Justus-Liebig-Universität Gießen

Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement

Institut für Phytopathologie und Angewandte Zoologie

Professur für Angewandte Entomologie

Isolierung und Strukturaufklärung peptidischer und niedermolekularer bioaktiver Naturstoffe mittels LC/ESI-MS und GC/MS

Habilitationsschrift

zur Erlangung der Lehrbefähigung für das Fach

Angewandte Mikrobiologie und Bioanalytik

im Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement der Justus-Liebig-Universität Gießen

vorgelegt von

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Gehe nicht,

wohin der Weg führen mag,

sondern dorthin, wo kein Weg ist,

und hinterlasse eine Spur.

(Jean Paul, 1763–1825)

In memoriam

Prof. Dr. Udo Gräfe (1941–2003),

meinem verehrten Doktorvater und Mentor

auf dem Gebiet der mikrobiellen Naturstoffchemie

Vorwort und Danksagung

In den vergangenen fünfundzwanzig Jahren hat die Bioanalytik als interdisziplinäres Bindeglied zwischen den Lebenswissenschaften und der klassischen analytischen Chemie eine überaus dynamische Entwicklung erfahren. Besonders hervorzuheben sind hierbei die Methoden der modernen massenspektrometrischen Hochleistungsanalytik, vor allem die Elektrosprayionisations-Massenspektrometrie (ESI-MS) und die Matrix-unterstützte Laser-Desorptionsionisations-Massenspektrometrie (MALDI). Als unverzichtbare klassische Methode hat sich die Elektronenstoßionisations-Massenspektrometrie (EI-MS) in den vergangenen 60 Jahren ihren festen Platz in der bioanalytischen Forschung etabliert. Die drei genannten Methoden leisteten und leisten entscheidende Beiträge dazu – selbst bei Vorliegen geringster Probemengen – die Suche nach Naturstoffen mit neuartiger chemischer Struktur und interessanten biologischen Aktivitäten wesentlich zu vereinfachen und zu beschleunigen.

Mit der vorliegenden kumulativen Habilitationsschrift werden eigene Original- und Übersichtsarbeiten zur Isolierung und Strukturaufklärung peptidischer und niedermolekularer bioaktiver Naturstoffe mittels HPLC-gekoppelter Elektrosprayionisations-Massenspektrometrie (LC/ESI-MS) und und Gaschromatographie/Elektronenstoßionisations-Massenspektrometrie (GC/MS) vorgestellt. Die Arbeit ist untergliedert in folgende drei Teile:

im ersten werden Arbeiten zur Isolierung und Sequenzierung neuer Peptaibiotika, nicht-ribosomal biosynthetisierter, α,α-dialkylaminosäurehaltiger Peptidantibiotika aus Pilzen sowie deren Bedeutung für den biologischen Pflanzenschutz und die moderne Pilztaxonomie dargestellt;

im zweiten wird der Einsatz moderner massenspektrometrischer Methoden im Rahmen des „Metabolite Profiling“ in der Pflanzenbiochemie erörtert;

und im dritten werden die Perspektiven der Peptaibiotikaforschung im nächsten Jahrzehnt sowie die Rolle und Bedeutung der Massenspektrometrie als Schlüsseltechnologie in den Lebenswissenschaften beleuchtet.

Der Großteil der dieser Habilitationsschrift zugrundeliegenden Original- und Übersichtsarbeiten ist im Zeitraum von März 2005 bis März 2008 an der Professur für

Lebensmittelwissenschaften des Instituts für Ernährungswissenschaft der Justus-Liebig-Universität Gießen entstanden.

Mein ganz besonderer Dank gilt daher dem damaligen Inhaber des Lehrstuhls, Herrn Professor Hans Brückner, der sich, verbunden durch das gemeinsame Interesse an Peptidantibiotika aus Pilzen, bereiterklärt hatte, mich in seinem Arbeitskreis aufzunehmen, mich stets förderte und forderte. Dadurch konnte ich dieses während meiner Tätigkeit in Jena unter der Betreuung meines viel zu früh verstorbenen Doktorvaters und Mentors, Herrn Professor Udo Gräfe, erschlossene Gebiet der Naturstoffchemie weiter bearbeiten. Ihm widme ich diese Arbeit in dankbarer Erinnerung an die – leider viel zu kurze – Zeit der gemeinsamen Arbeit am Hans-Knöll-Institut für Naturstoff-Forschung in Jena.

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Herrn Professor Andreas Vilcinskas bin ich für die Übernahme der Betreuung meiner Habilitation nach dem Ausscheiden von Herrn Professor Brückner aus dem aktiven Hochschuldienst zu großem Dank verpflichtet. Ohne seinen Einsatz wäre es mir nicht möglich gewesen, die Arbeit an meiner Habilitation fortzusetzen und meine Erfahrungen in der Lehre und bei der Betreuung von Bachelor- und Masterarbeiten in der Agrar- und Ernährungswissenschaften, insbesondere in den Bereichen der Lebensmittelsicherheit und des Vorratsschutzes sowie des Biologischen und Chemischen Pflanzenschutzes, zu sammeln.

Den Autoren der hier zusammengestellten Original- und Übersichtsarbeiten danke ich für die konstruktive Zusammenarbeit.

Herrn Dr. habil. Willibald Schliemann (ehemals Leibniz-Institut für Pflanzenbiochemie [IPB] Halle/Saale) danke ich für die gründliche Durchsicht des Manuskripts.

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Der Studienstiftung Mykologie (Köln) danke ich für die Förderung der Arbeiten in den Jahren 2005 bis 2006 und der Erwin-Stein-Stiftung (Gießen) für die ideelle und finanzielle Unterstützung, die mit der Vergabe eines Habilitationsstipendiums in den Jahren 2007 bis 2009 einherging.

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M von Roepenack-Lahaye, E.; Degenkolb, T.; Zerjeski, M.; Franz, M.; Roth, U.; Wessjohann, L.; Schmidt, J.; Scheel, D.; Clemens, S. (2004) Profiling of *Arabidopsis* secondary metabolites by capillary liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry. Plant Physiol., **134**: 548–559.

Folgende sieben der oben gelisteten Original- und Übersichtsarbeiten wurden außerdem als inhaltlich identische Buchbeiträge publiziert in:

Toniolo, C.; Brückner, H. (Eds.): Peptaibiotics – fungal peptides containing α-dialkyl α-amino acids. Verlag Helvetica Chimica Acta Zürich & Wiley-VCH Weinheim New York Chichester Brisbane Singapore Toronto. 702 p., 2009:

1. Degenkolb, T.; Brückner, H.: Peptaibiomics: towards a myriad of bioactive peptides containing C^{α}-dialkylamino acids? pp. 4–29.
2. Degenkolb, T.; Kirschbaum, J.; Brückner, H.: New sequences, constituents, and producers of peptaibiotics: an updated review. pp. 57–72.
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Zusammenfassung

Im Rahmen der vorliegenden Habilitationsschrift, die auf 13 Original- und Übersichtsarbeiten beruht, werden Beiträge zur Isolierung und Strukturaufklärung peptidischer und niedermolekularer bioaktiver Naturstoffe mittels LC/ESI-MS und GC/MS kumulativ vorgestellt:

Der erste Teil dieser Arbeit ist der Isolierung und Sequenzierung neuer und bereits bekannter Peptaibiotika – nicht-ribosomal biosynthetisierter, *α*,*α*-dialkylaminosäurehaltiger Peptidantibiotika aus Pilzen – gewidmet. Im Mittelpunkt standen hierbei die Suche nach neuen peptidischen Wirkstoffen für einen potentiellen Einsatz in der Medizin bzw. im biologischen Pflanzenschutz sowie die Bedeutung der gefundenen Sekundärmetaboliten für die moderne Pilztaxonomie. Unter Verwendung moderner massenspektrometrischer Verfahren wie der HPLC-gekoppelten ESI-Quadrupol/TOF-Hybrid-MS mit Pulsarfunktion (ESI-QqTOF-MS), der Kopplung der nicht-wäßrigen Kapillarelektrophorese (NACE) mit ESI-MS und der HPLC-gekoppelten ESI-Ionenfallen-MS (ion trap, IT) sowie einer peptaibiomischen Vorgehensweise als neuartigem methodisch-analytischem Ansatz wurden aus Pilzen der Gattung *Trichoderma*/*Hypocrea* etwa 100 größtenteils neue Sequenzen linearer Peptaibiotika ermittelt. Diese fielen entweder durch ihre neuroleptische Aktivität oder ihre antifungale Wirkung auf die Erreger latenter Rebholzkranheiten der Weinrebe auf. Erstmalig wurde über den Nachweis von *N*-terminalem Pyroglutamat und *C*-terminalem L-Alaninol als neue Strukturelemente von Peptaibiotika berichtet. Die Kombination von phylogenetisch-molekulartaxonomischer Methoden mit modernen massenspektrometrischen Verfahren führte zur Beschreibung des Brevicompactum-Cladus als neuem Zweig mit vier neuen Arten (*Trichoderma arundinaceum*, *T. protrudens*, *T. turrialbense* und *Hypocrea*/*Trichoderma rodmanii*) im Stammbaum der Gattung *Trichoderma*/*Hypocrea*. Außerdem wird auf neue bzw. ungewöhnliche Produzenten, Strukturelemente und Baupläne von Peptaibiotika, so z. B. die neue Unterfamilie der Cyclopeptaibiotika, eingegangen, die Geschichte und Perspektiven der Peptaibiotikaforschung werden beleuchtet sowie das Vorkommen von *α*,*α*-Dialkylaminosäuren in der belebten und unbelebten Natur thematisiert. Schließlich wurden die Trifluoracetolyse und die sich anschließende Analyse der Spaltprodukte mittels ESI-MS als geeignetes Verfahren zur Sequenzierung und Strukturaufklärung von Modellpeptiden und

Peptaibiotika untersucht und ein repetitiver Spaltmechanismus für homo-Aib-haltige Peptide und Peptaibiotika postuliert.

Der zweite Teil der Arbeit stellt die Kopplung von HPLC und hochauflösender ESI-Massenspektrometrie als eine unentbehrliche Komplementärtechnologie für die GC/MS beim so genannten „Metabolic Profiling“ am Beispiel von *Arabidopsis thaliana* als pflanzlichen Modellorganismus dar.

Der dritte Teil der Arbeit beleuchtet die Perspektiven der Peptaibiotikaforschung im nächsten Jahrzehnt sowie die Massenspektrometrie als analytische Hochleistungsmethode in den Lebenswissenschaften.

Zusammenfassend ist die Massenspektrometrie als derzeit vielseitigster und leistungsfähigster Zweig der Bioanalytik einzuschätzen, deren interdisziplinäre Rolle und Bedeutung für die Lebenswissenschaften in den kommenden Jahren noch weiter wachsen wird.

Summary

This cumulative habilitation thesis, which is divided into three chapters, comprises of 13 original research and review articles on the isolation and structural elucidation of bioactive peptidic and low-molecular weight natural products by LC/ESI-MS and GC/MS:

The first part of this work is devoted to the isolation and sequencing of new and recurrent peptaibiotics, *i. e.* fungal peptide antibiotics of non-ribosomal origin containing α-dialkyl α-amino acids. It is focussed on the search for novel peptidic components for potential use in medicine or as biocontrol agents (BCA) in agriculture. A second important aspect is the significance to modern fungal taxonomy of the secondary metabolites discovered. Using state-of-the-art mass spectrometric tools such as HPLC coupled to a pulsar ESI-QqTOF-MS, nonaqueous capillary electrophoresis coupled to ESI-MS, HPLC coupled to ESI-ion-trap-MS, as well as the recently established method of peptaibiomics, more than 100 novel and recurrent linear peptaibiotics produced by strains and species of the genus *Trichoderma*/*Hypocrea* were sequenced. Notably, some of them displayed neuroleptic activity, whereas others were highly effective against fungal diseases of grapevine trunks. Furthermore, *N*-terminal pyroglutamate and *C*-terminal L-Alaninol are reported as novel constituents of peptaibiotics for the first time. The combination of morphological analyses, molecular phylogenetics and state-of-the-art mass spectrometric tools culminated in the recognition of the Brevicompactum clade as a separate, novel lineage, including *T. brevicompactum* s. s. as well as four new species, *T. arundinaceum*, *T. protrudens*, *T. turrialbense*, and *H./T. rodmanii*. New and uncommon fungal producers, structural elements, and building schemes of peptaibiotics as well as the history of research in this field are reviewed, and a new subfamily of peptaibiotics, the so-called cyclopeptaibiotics, is introduced. Future prospects for peptaibiotic research are discussed, and the distribution of biotic and abiotic α-dialkyl α-amino acids is critically scrutinised. Finally, trifluoroacetolysis of peptaibiotics as well as peptides and subsequent analysis of its cleavage products by ESI-MS are presented as an effective tool for sequencing and structural elucidation of model peptides and peptaibiotics, and a repetitive cleavage mechanism for Aib-containing peptides is postulated.

The second part of this work introduces the benefits of HPLC coupled to electrospray ionisation quadrupole time-of-flight mass spectrometry as an indispensable, complementary

technique to GC/MS for profiling of secondary metabolites of *Arabidopsis thaliana* as a plant model organism.

The third part of this work discusses the future prospects for peptaibiotic research in the next decade and the role of mass spectrometry as an anaqlytical key technology in life sciences.

In conclusion, mass spectrometry can be characterised as the most versatile and powerful discipline in bioanalytics whose interdisciplinary role and importance in life sciences is predicted to grow further in the forthcoming years.

1. Isolierung und Strukturaufklärung neuer Peptaibiotika aus Pilzen der Gattung *Hypocrea*/*Trichoderma*

1.1. Allgemeine Bemerkungen zur Biosynthese nicht-ribosomaler Peptide

Die Knüpfung einer Peptidbindung kann *in vivo* durch drei grundlegende Mechanismen realisiert werden:

- die ribosomale Biosynthese linearer Polypeptide unter Verwendung der 20 proteinogenen Aminosäuren und eventuelle Prozessierung der gebildeten Proteine, so z. B. durch Proteolyse und/oder posttranslationale Modifikationen wie Glykosylierungen, Hydroxylierungen (Lys → Hyl, Pro → Hyp), Phosphorylierungen, Methylierungen, Acetylierungen u. a. Reaktionen;
- die enzymatische Synthese von Di- und Tripeptiden durch lösliche Enzyme;
- die nucleinsäure-unabhängige Biosynthese an modular organisierten Multienzymkomplexen, so genannten **n**icht-**r**ibosomalen **P**eptid**s**ynthetasen (**NRPS**), die – unter Verwendung ungewöhnlicher, nichtproteinogener Aminosäuren und gegebenenfalls nicht-peptidischer Bausteine – lineare und cyclische Peptide aus bis zu 21 Aminosäuren[1] generieren.

Durch die begrenzte Spezifität der beteiligten NRPS bedingt, werden nicht nur proteinogene L-Aminosäuren, sondern auch deren Isomere oder D-Enantiomere, aber auch *N*- oder *C*-Methylaminosäuren, *β*-Aminosäuren, Hydroxysäuren, Dehydroaminosäuren, schwefelhaltige Aminosäuren, Iminosäuren, Lipoaminosäuren, Fettsäuren, Aminoalkohole und andere ungewöhnliche Bausteine in nicht-ribosomal biosynthetisierte Peptidantibiotika eingebaut. Neben Bakterien wie dem Gramicidin-Bildner *Bacillus brevis* und dem Actinomycin-Bildner *Streptomyces antibioticus* sind auch Asco- und Basidiomyceten sowie vor allem anamorphe Pilze zur Biosynthese solcher Verbindungen befähigt [Gräfe, 1992]. Als Beispiele für praktisch bedeutsame, nicht-ribosomale Peptide aus Pilzen sind zu nennen:

[1] Das aus 21 Resten bestehende SCH 643432 [Hegde et al., 2003] gilt als das derzeit größte Peptaibiotikum.

- die insektizid wirksamen cyclischen Depsipeptide aus entomopathogenen, im Rahmen der biologischen Schädlingsbekämpfung verwendeten Pilzarten, wie z. B. die Destruxine aus *Metarhizium anisopliae* und *Aschersonia* sp., das Beauvericin aus *Beauveria bassiana* und *Isaria fumosorosea* (syn. *Paecilomyces fumosoroseus*), die Beauveriolide aus *Beauveria* sp., *Isaria* sp. und *Lecanicillium* (syn. *Verticillium*) *lecanii* sowie die Enniatine aus *Fusarium* spp. [Anke & Sterner, 2002; Anke & Antelo, 2009].

- die insektizid und immunsuppressiv wirksamen Cyclosporine, cyclische Undecapeptide [Kleinkauf & von Döhren, 1999; Kürnsteiner et al., 2002], welche vor allem aus *Tolypocladium*-Arten, besonders *T. inflatum* (syn. *T. niveum* u. a., siehe www.cbs.knaw.nl) und dessen Teleomorphe *Elaphocordyceps* [Sung et al., 2007] *subsessilis* (syn. *Cordyceps subsessilis* [Hodge et al., 1996]), isoliert werden.

Neben der Hauptkomponente, dem Cyclosporin A, weisen weitere 14 der bislang 32 aus natürlichen Quellen isolierten oder durch gerichtete Biosynthese (precursor-directed biosynthesis) erzeugten Cyclosporine mindestens einen L-α-Aminobuttersäure-Rest (Abu) als strukturelles Charakteristikum in ihrem Ring auf. Diese Aminosäure ist wegen ihres auf wenige natürliche Quellen beschränkten Vorkommens bemerkenswert: exemplarisch seien hier folgende von Bakterien ribosomal produzierten Lantibiotika genannt:

Nisin (*Streptococcus lactis*), Subtilin (*Bacillus subtilis*), Gallidermin (*Staphylococcus gallinarum*) [Gräfe, 1992] und die Lantibiotika des Duramycins-Typs, welche aus Actinomyceten der Gattungen *Streptomyces* und *Streptoverticillium* isoliert worden sind [Fredenhagen et al., 1991]. Außer in den Lantibiotika ist Abu bislang auch in Cyclosporin-produzierenden Pilzen nachgewiesen worden. [Übersicht der Bildner in: Kürnsteiner et al., 2002].

1.2. Peptaibiotika – eine Klasse ungewöhnlicher nicht-ribosomaler Peptid-Antibiotika aus Pilzen

Ein Isomer der oben genannten L-α-Aminobuttersäure ist die achirale α-Aminoisobuttersäure (Aib), der namensgebende Bestandteil einer Klasse linearer und cyclischer, ebenfalls durch nicht-ribosomale Biosynthese gebildeter Peptid-Antibiotika, der **Peptaibiotika** [Brückner et

al., 1991(a) und (b)]. Seit etwa 15 Jahren erlebt diese Klasse von Peptidantibiotika eine Renaissance in der mikrobiologischen und naturstoffchemischen Forschung. Dies ist vor allem bedingt durch ihre einzigartigen Konformationen [Toniolo & Benedetti, 1991] und Bioaktivitäten, wie beispielsweise die neuroleptische Wirkung der Ampullosporine [Ritzau et al., 1997; Kronen et al., 2001; Krügel et al., 2006; Berek et al., 2009] und Trichofumine (siehe Abschnitt 1.5. bzw. ***Anhang B***), die Inhibition der HIV-1-Integrase [Singh et al., 2002; de Zotti et al., 2009; de Zotti et al., 2010(a)], die Hemmung der Bildung von Amyloid-*β*-Peptiden [Hosotani et al., 2007] sowie die Ionenkanalbildung in Biomembranen [Boheim et al., 1983; Leitgeb et al., 2007]. Letztere trägt mit großer Wahrscheinlichkeit zur Wirksamkeit der im biologischen Pflanzenschutz eingesetzten Präparate auf Basis von *Trichoderma*-Konidien (siehe Abschnitt 1.7. bzw. ***Anhang D***) bei.

Peptaibiotika sind durch folgende charakteristische Merkmale gekennzeichnet (siehe auch ***Anhang J***):

- Es handelt sich um lineare oder cyclische, antibiotisch wirksame Polypeptide mit einem Molekulargewicht zwischen 500 und 2.200 Da, die aus 4 bis 21 monomeren Einzelbausteinen bestehen und durch NRPS biosynthetisiert werden [Reiber et al., 2003].

- In den meisten (> 98%) dieser Verbindungen ist *α*-Aminoisobuttersäure (Aib) als Marker-Aminosäure enthalten[2]. In manchen Sequenzen, z. B. dem Adenopeptin [Hayakawa et al., 1998], einigen Stilboflavinen [Jaworski & Brückner, 2001] oder dem Peptaibiotikum SCH 643432 [Hegde et al., 2003], liegt der Anteil der Aib-Reste bei 40 bis 50%. Andere Peptaibiotika, z. B. das Trichobrachin III A a, besitzen hingegen nur einen einzigen Aib-Rest [Brückner et al., 1993].

- Neben Aib können noch weitere nicht-proteinogene *α*,*α*-Dialkylaminosäuren im Molekül enthalten sein. Dabei handelt es sich um vor allem um das chirale Homologe der Aib, das Isovalin (Iva), welches entweder in der L- bzw. *(S)*-, oder in der D- bzw. *(R)*-Form vorliegen kann. Außerdem ist L- bzw. *(S)*-Ethylnorvalin (*α*-Amino-*α*-ethyl-

[2] Auf Ausnahmen wird im Text explizit hingewiesen. Die Massendifferenzen am *N*-Terminus der Trichobrachine III A b und III B b lassen den Schluß zu, daß in diesen Verbindungen kein Aib- bzw. Iva-Rest mehr enthalten sein kann. Die *N*-terminale Sequenz beider Peptide erwies sich letztendlich als nicht bestimmbar; denn bei diesen beiden Partialsequenzen handelt es sich lediglich um Minorkomponenten in einem hochkomplexen, mikroheterogenen Gemisch homologer und positionsisomerer Peptide [Brückner et al., 1993].

n-valeriansäure, EtNva) als Bestandteil von Peptaibiotika nachgewiesen worden [Tsantrizos et al., 1996] sowie die achirale 1-Aminocyclopropan-1-carbonsäure (Acc) [Fredenhagen et al., 2006; siehe auch Abschnitt 1.9. bzw. ***Anhang F***]. In einzelnen Sequenzen ist die Aib vollständig durch Iva- oder Acc-Reste substituiert, so z. B. in den Cyclotetrapeptaibiotika Phoenistatin [Masuoka et al., 2001], FR 235222 [Mori et al., 2003(a), (b)] und AS1387392 [Sasamura et al., 2010(a)] sowie den Acc-haltigen Cycloheptapeptaibiotika Serinocyclin A und B [Krasnoff et al., 2007].

- Neben den bereits genannten nicht-proteinogenen Aminosäuren können auch weitere ungewöhnliche Bestandteile, wie *β*-Aminosäuren oder *N*-Methylaminosäuren, vorhanden sein.

- Der *N*-Terminus linearer Peptaibiotika ist stets acyliert, während ihr *C*-Terminus meist aus einem freien oder acetylierten, amidartig gebundenen 2-Aminoalkohol besteht. Alternative *C*-terminale Strukturvarianten umfassen die Substitution durch ein Amin, Amid, eine freie Aminosäure oder ein 2,5-Diketopiperazin als Kondensationsprodukt zweier Aminosäuren.

Die überwiegende Mehrheit der bislang bekannten Peptaibiotika, die **Peptaibole** [Benedetti et al., 1982; Brückner & Graf, 1983], besitzt einen acetylierten *N*-Terminus, während ihr *C*-Terminus durch einen 2-Aminoalkohol gebildet wird. Stärker lipophile Peptaibiotika, deren *N*-Terminus durch einen *n*-Octan-, *n*-Decan- oder *cis*-Dec-4-ensäurerest substituiert ist, sind die **Lipopeptaibole**. Eine dritte Unterfamilie stellen die **Lipoaminopeptide** dar, die gelegentlich auch als **Aminolipopeptide** bezeichnet werden. Ihr *N*-Terminus ist durch unverzweigte, *α*- oder *γ*-methylverzweigte gesättigte oder ungesättigte Fettsäuren mit einer Kettenlänge zwischen vier und 15 *C*-Atomen substituiert. In Position 1 der Peptidkette findet sich ein L-Prolin-, *trans*-4-Hydroxy-L-prolin- oder *cis*-4-Methyl-L-prolin-Rest. Auf diesen folgt in Position 2 zumeist der namensgebende Lipoaminosäure-Rest. Bei diesem handelt es sich überwiegend um 2-Amino-6-hydroxy-4-methyl-8-oxodecansäure (AHMOD), es können statt dessen jedoch Desmethyl-AHMOD (siehe Abschnitt 1.8. bzw. ***Anhang E***] oder 2-Amino-4-methyl-8-oxodec-6-ensäure (AMOD) [Pruksakorn et al., 2010] vorhanden sein. Außerdem kann sich eine zweite Lipoaminosäure in der Kette befinden, so z. B. die 2-Amino-4-methyldecansäure (AMD) in den Culicininen A–D [He et al., 2006]. Alle linearen Peptaibiotika, die nicht in eine der drei vorgenannten eingeordnet werden können, werden in

einer vierten Unterfamilie gruppiert. Die fünfte und zugleich kleinste Unterfamilie bilden die **Cyclopeptaibiotika**, die durch das Vorhandensein von *α,α*-Dialkylaminosäuren in Ringstrukturen gekennzeichnet sind[3].

Die bislang beschriebenen rund 900 Einzelsequenzen von Peptaibiotika werden ausschließlich von Pilzen biosynthetisiert[4]. Die meisten Bildner sind in Anamorphen-Gattungen zu finden, deren Teleomorphen zur Ordnung Hypocreales zählen. Dabei ist die Gattung *Trichoderma* mit Teleomorphen in *Hypocrea* als weitaus wichtigste Quelle für Peptaibiotika zu benennen, gefolgt von den Gattungen *Acremonium, Emericellopsis* (Incertae sedis) und *Sepedonium* (Hypocreaceae; Teleomorphe in *Hypomyes*) sowie *Tolypocladium* (mit *Elaphocordyceps*-Teleomorphen in den Ophiocordycipitaceae: Sung et al., 2007). Weitere peptaibiotika-bildende Arten und Stämmen finden sich in folgenden Gattungen der Hypocreales: *Stilbella*, *Myrothecium*, *Culicinomyces* (Incertae sedis), *Mycogone* (Hypocreaceae), *Mariannaea* (Nectriaceae), *Clonostachys* (Bionectriaceae), *Dendrodochium*[5] und *Pochonia* (Clavicipitaceae; Teleomorphe in *Metacordyceps*: Sung et al., 2007). Als Bildner außerhalb der Hypocreales sind folgende Arten zu erwähnen:

- *Paecillium lilacinum* (Thom) Luangsa-ard, Hywell-Jones & Samson (syn. *Paecilomyces lilacinus*) und *Paranomuraea marquandii* (Massee) Luangsa-ard, Hywell-Jones & Samson[6] (syn. *Paecilomyces marquandii*) gelten als klassische Quellen der Leucinostatine (Literaturübersicht in ***Anhang J***).

- *Paecilomyces variotii*, die Typusart der Gattung, bildet zwei antifungale Peptaibiotika [Hegde et al., 2003]. Die drei vorgenannten Species gehören zur Familie Trichocomaceae der Ordnung Eurotiales.

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[3] Auf diese neu eingeführte Unterfamilie wird bei der Besprechung der entsprechenden Übersichtsarbeit (siehe Abschnitt 1.12. bzw. ***Anhang I***) näher eingegangen

[4] In diesem Zusammenhang ist bemerkenswert, daß biotisches Aib, Iva und EtNva bisher nur aus pilzlichen Organismen isoliert worden sind, abiotisches Aib/Iva und andere *α*-Dialkyl-*α*-aminosäuen auch in Meteoriten vorkommen können. Darauf wird im Abschnitt 1.14. dieser Arbeit bzw. in ***Anhang K*** näher eingegangen.

[5] Die Nennung der Gattung *Dendrodochium* folgt lediglich den Angaben in Singh et al. [2002]. Die Identität des Integramid-Bildners ist als zweifelhaft werten, da sie nicht durch Daten zu Isolierung und Identifizierung des Stammes verifiziert worden ist.

[6] Die Nomenklatur folgt hier Domsch et al. [2007]. Dabei ist jedoch zu berücksichtigen, daß eine gültige Beschreibung der Gattungen *Paecillium* und *Paranomuraea* bislang noch nicht publiziert worden ist.

- Eine taxonomisch nicht näher bestimmte *Chrysosporium* sp. (Onygenaceae, Onygenales) ist als Bildner des Adenopeptins angegeben worden [Hayakawa et al., 1998].

- Aus *Geotrichum candidum* (Teleomorphe: *Galactomyces candidus*; Dipodascaceae, Saccharomycetales) wurden von Fredenhagen et al. [2006] die Neoefrapeptine isoliert.

- *Peniophora* cf. *nuda* (Peniophoraceae, Russulales), aus dessen Kulturen Chlamydocin und zwei seiner Analoga isoliert wurden, gilt als bislang einziger verifizierter peptaibiotika-produzierender Basidiomycet [Tani et al., 2001].[7]

1.3. Massenspektrometrische Methoden als unentbehrliche Werkzeuge bei der Sequenzierung und Strukturaufklärung von Peptaibiotika

Die Sequenzierung und Strukturaufklärung der „klassischen" Peptaibole wurde zu Beginn der systematischen Beschäftigung mit diesen Verbindungen durch mehrere Faktoren erschwert:

- Aufgrund der negativen Ninhydrin-Reaktion wurde irrtümlich davon ausgegangen, daß Alamethicin (ALM) [Meyer & Reusser, 1967], Antiamoebin (AAM) [Vaidya et al., 1968] und Trichotoxin A (TXT-A) [Hou et al., 1972] eine cyclische Struktur besitzen. Daher wurden zunächst weder die Acetylierung des *N*-Terminus, noch die *C*-terminale Position des 2-Aminoalkohols erkannt.

- Obwohl die Aminosäurezusammensetzung der jeweiligen mikrokristallin vorliegenden Hauptkomponenten ermittelt worden war, ergab die Analyse bei einigen Aminosäuren keine ganzzahlige Stöchiometrie, so z. B. im Falle des TXT [Irmscher et al., 1978].

[7] Berichte über die Bildung von Peptaibiotika in Fruchtkörpern von Basidiomyceten [Aretz et al., 1997; Lee et al., 1999(a) und (b)] wurden bereits von Kiet et al. [2002] mit einer nicht erkannten Infektion der betreffenden Fruchtkörper durch einen peptaibolbildenden Mykoparasiten der Gattung *Sepedonium* in Verbindung gebracht. Dieses Phänomen wird in den ***Anhängen A***, ***F*** und ***J*** diskutiert und gilt seit dem Nachweis der Tylopeptin-Bildung durch Kulturen von *Sepedonium chalcipori* [Neuhof et al., 2007(a)] als widerlegt.

Letzteres Phänomen wurde folgerichtig auf die so genannte **Mikroheterogenität** zurückgeführt, d. h. das Vorliegen chromatographisch nur schwer oder gar nicht trennbarer Gemische homologer und positionsisomerer Peptide. Diese ergibt sich aus der nicht-ribosomalen Biosynthese der Peptaibiotika, die bereits 1967 von Reusser für ALM postuliert wurde. Später wurde gezeigt, daß dieser bereits in Bakterien beschriebene, an Multienzymkomplexen mit reduzierter Spezifität ablaufende Prozeß ebenfalls in Pilzen stattfindet [Rindfleisch & Kleinkauf, 1976].

Zur Sequenzierung und Strukturaufklärung von Peptaibolen wurden Methoden wie Edman-Abbau und hochauflösende Felddesorptions-Massenspektrometrie (FD-HR-MS), Kopplung aus Gaschromatographie und hochauflösender Elektronenstoß-Massenspektrometrie (GC/HR-EI-MS) bzw. Feldionisations-Massenspektrometrie (FI-HR-MS) angewendet. Von besonderem Wert erwies sich dabei, beruhend auf der Erkenntnis einer relativen Labilität der Bindung zwischen Aib und Pro, die Verwendung wasserfreier Trifluoressigsäure (TFA) als Reagenz zur selektiven Spaltung von Peptaibolen und die nachfolgende Analyse der entsprechend derivatisierten Spaltprodukte mittels GC/MS [Brückner et al., 1979; Brückner & Jung, 1980; Brückner et al., 1980; siehe auch ***Anhang L***]. Ein weiterer Fortschritt ergab sich aus der Einführung der Fast-Atom-Bombardment-Massenspektrometrie (FAB-MS) [Rinehart, 1983], die allein oder in Kopplung mit der Hochdruckflüssigchromatographie (HPLC) eine Analytik intakter, nicht-derivatisierter Peptaibiotika, so z. B. der Leucinostatine, ermöglichte [Isogai et al., 1992 und darin zitierte Referenzen]. Mit Hilfe dieser beiden hochenergetischen, so genannten „harten“, Ionisierungsmethoden EI- und FAB-MS wurden bis 1988 etwa 60 Peptaibiotika sequenziert.

In diesem Zusammenhang sei abschließend noch auf den relativ hohen Probenbedarf, den erheblichen apparativen sowie den vergleichsweise großen Zeit- und Arbeitsaufwand verwiesen, den eine Peptid-Sequenzierung mittels dieser beiden klassischen Methoden der Massenspektrometrie nach sich zog.

1.4. Die Elektrosprayionisations-Massenspektrometrie revolutioniert die Sequenzierung und Strukturaufklärung von Peptaibiotika

Selbst bei Verwendung von FAB-MS können oftmals weitere sekundäre oder tertiäre Serien mehr oder minder unspezifischer Fragment-Ionen auftreten, welche die Sequenzierung zusätzlich erschweren. Mit der Einführung der Elektrosprayionisations-Massenspektrometrie (ESI-MS) steht seit etwa 20 Jahren ein niederenergetisches, so genanntes „weiches" Ionisierungsverfahren zur Verfügung, das hervorragend zur Sequenzierung von Peptaibiotika geeignet ist. Dies wird durch die Tatsache illustriert, daß bis Februar 2003 bereits etwa 300 Einzelsequenzen von Peptaibiotika beschrieben worden sind.

Bei der ESI-MS werden nicht primär Fragment-Ionen gebildet, sondern es kommt – durch Abstraktion ($[M–H]^-$) oder Anlagerung von Protonen, Na^+ oder K^+ – zur Bildung von Addukten, so genannten Quasi-Molekülionen. Diese geben Auskunft über das Molekulargewicht der betreffenden Analyten, während Fragmentierungsreaktionen größtenteils unterbleiben. Diagnostische Fragment-Ionen, die meist den *a*-, *b*- oder *c*- bzw. *x*-, *y*- oder *z*-Serien, entsprechend der Roepstorff/Fohlman/Biemann-Nomenklatur [Roespstorff et al., 1984, 1985; Biemann, 1992], zuzuordnen sind, werden erst durch Kollision ausgewählter Quasi-Molekülionen mit einem inerten Stoßgas in einer so genannten Kollisionzelle erzeugt.

Das vorrangige Ziel dieser Übersichtarbeit besteht darin, die verschiedenen massenspektrometrischen Methoden zur Sequenzierung von Peptaibiotika kurz darzustellen, relevante Strukturdaten (diagnostische Fragment-Ionen, Molekulargewichte), einschließlich möglicher Modifikationen des *N*- bzw. *C*-Terminus der bislang bekannten Peptaibiotika, in Tabellenform zu vermitteln, und proteinogene[8] und ungewöhnliche nicht-proteinogene Aminosäure-Bausteine vorzustellen. Außerdem werden Aspekte der Nomenklatur bei der Klassifizierung von Peptaibiotika sowie der Ökophysiologie und der Einordnung der Bildner ins System der Pilze diskutiert.

[8] Schwefelhaltige (Cys, Met) sowie L-Lys und L-His als basische proteinogene Aminosäuren sind bisher noch nicht als Bausteine von Peptaibiotika beschrieben worden. Auch L-Orn (*Bacillus* sp.: Gramicidin S, Tyrocidin) bzw. D-Orn (Bacitracin A: *Bacillus licheniformis*) und die bisweilen in Peptid-Antibiotika (Polymyxin-Gruppe: *Bacillus polymyxa*) vorkommende 2,4-L-Diaminobuttersäure (Dab) [Gräfe, 1992] wurden in Peptaibiotika noch nicht nachgewiesen. D-Lys wurde bislang lediglich einmal – als Baustein des Cycloheptapeptaibiotikums Serinocyclin B – beschrieben [Krasnoff et al., 2007].

1.5. Die Trichofumine – vier neuroleptisch wirksame, neue Peptaibole aus *Trichoderma* sp. HKI 0276

Aus Oberflächenkulturen des taxonomisch nicht näher bestimmten Stammes *Trichoderma* sp. HKI 0276 wurden vier Peptaibole, die Trichofumine A, B, C und D, präparativ isoliert. Trichofumin A und B bestehen aus elf, Trichofumin C und D aus dreizehn Resten. Sie sind in der Lage, die Morphogenese der Hyphen des Coelomyceten *Phoma destructiva* (Pleosporales) zu beeinflussen. Dieser Effekt macht sich, wie bei der Referenzsubstanz Cyclosporin A, durch die zeitlich beschleunigte Bildung eines bräunlich pigmentierten Hofes um das Testloch auf einer Agardiffusionsplatte bemerkbar [Dornberger et al., 1995] und ist als Vortest für eine neuroleptische (antipsychotische) Wirkung geeignet. Diese wird schließlich durch die Auslösung von Hypothermie bei Mäusen bestätigt, wobei sich letztere in der Abnahme spontaner lokomotorischer Aktivität widerspiegelt [Bansinath & Ramabadran, 1990]. Die neuroleptische Aktivität der vier Trichofumine war der der Ampullosporine A, B und D vergleichbar [Ritzau et al. 1997; Kronen et al., 2001].

Die massenspektrometrische Sequenzierung der Trichofumine konnte durch den Einsatz eines ESI-Qq-TOF-Geräts mit Pulsar-Funktion wesentlich erleichtert und beschleunigt werden. Mit Hilfe des so genannten „Pulsars“ können Ionen über einen bestimmten *m/z*-Bereich in der Stoßzelle zuerst gesammelt und nachfolgend – gewissermaßen „gebündelt“ – ins Flugrohr des MS weitergeleitet („gepulst“) werden. Dies führte zu einer erheblichen Verbesserung der Sensitivität des Gerätes. Dadurch war es möglich, die Trichofumine mit Hilfe der gebildeten *b*-Typ-Fragmente komplett zu sequenzieren.

1.6. Detektion neuer, modifizierter Alamethicine F30 durch Kopplung von nicht-wäßriger Kapillarelektrophorese und ESI-Massenspektrometrie

In Abhängigkeit von den Fermentationsbedingungen produziert *Trichoderma arundinaceum* CBS 123793[9] ein Gemisch mehrerer, als Alamethicin (ALM) bezeichneter Polypeptide, die aufgrund ihres unterschiedlichen dünnschichtchromatographischen Laufverhaltens als ALM F30 (ca. 85%) und ALM F50 (ca. 12%) bezeichnet werden; daneben wurden ALM F20 (2%) sowie ALM F40, F60 und F70 (insgesamt 1%) nachgewiesen [Melling & McMullen, 1975]. Mittels Direkteinlaß sowie Kopplung aus HPLC und Ionenfallen-Massenspektrometrie (ESI-IT-MS^n) und GC/MS-Analyse der derivatisierten Aminosäuren in den salzsauren Hydrolysaten wurden die einzelnen Peptaibole im ALM F30 bzw. ALM F50 sequenziert. Insgesamt wurden zehn saure ALM F30 (Glu^{18}–Gln^{19}) und 13 neutrale ALM F50 (Gln^{18}–Gln^{19}) beschrieben [Kirschbaum et al., 2003].

Das Ziel dieser Arbeit bestand darin, das Potential einer Kopplung aus nicht-wäßriger Kapillarelektrophorese (NACE) und ESI-IT-MS bzw. ESI-TOF-MS für die Analyse und Sequenzierung von Peptaibolen zu untersuchen. Mittels beider Methoden konnten zehn ALM F30-Sequenzen identifiziert werden. Unter diesen befanden sich drei, die bereits von Kirschbaum et al. [2003] beschrieben worden sind:

die beiden Hauptkomponenten ALM F30/3 (ca. 46%) und F30/7 (ca. 40%) sowie ALM F30/6 (ca. 2%), das durch einen Austausch Gln→Glu in Position 6 charakterisiert ist. Die restlichen von Kirschbaum et al. [2003] gefundenen Minorkomponenten konnten mittels NACE nicht nachgewiesen werden. Dies wird auf die zu geringe Massendifferenz zwischen den in ohnehin geringen Konzentrationen vorliegenden Homologen von ± 14 Da zurückgeführt, die für eine kapillarelektrophoretische Trennung unzureichend war.

Das Potential der Methode wird jedoch durch die Tatsache verdeutlicht, daß mittels NACE/MS-Kopplung die Sequenzen von sieben neuen Alamethicinen aus der F30-Reihe ermittelt werden konnten, die mit Hilfe der HPLC/MS-Kopplung nicht identifiziert werden

[9] Dieser Stamm wurde ursprünglich als *Trichoderma viride* NRRL 3199 hinterlegt und ist unter dieser Bezeichnung als Alamethicin-Produzent patentiert worden [Coats et al., 1974]. In den meisten Publikationen zum Thema „Alamethicin“ wird diese mittlerweile veraltete Nomenklatur verwendet. Zur Revision der systematischen Einordnung des Stammes siehe Abschnitt 1.11. und die Originalarbeit H des ***Anhangs***.

konnten. Diese Minorkomponenten zeichnen sich durch eine zusätzliche Carboxyl-Gruppe aus, die durch einen Gln→Glu-Austausch bzw. den Verlust des *C*-terminalen Aminoalkohols generiert wird. Diese strukturellen Veränderungen sind offensichtlich der Grund dafür, daß diese Substanzen mittels CE detektiert werden konnten, während sie durch die HPLC nicht erfaßt wurden. Im Hinblick auf eine Erhöhung der strukturellen Vielfalt der Peptaibiotika ist der Nachweis zweier drastisch verkürzter Sequenzen, die einen *N*-terminalen Pyroglutaminsäure-Rest (Pyr) tragen, von besonderer Bedeutung. Diese repräsentieren die jeweiligen Hauptkomponente ALM F30/3 bzw. ALM F50/5, denen das *N*-terminale Hexapeptid fehlt. Somit wurde der erstmalige Nachweis von Pyr als ungewöhnlicher Bestanteil von Peptaibiotika erbracht. Nachdem Spuren dieser Peptide auch im Ausgangsmaterial detektiert werden konnten, wurde ALM F30 verschiedenen Erhitzungsversuchen unterworfen, um die Bedingungen der Aufarbeitung zu simulieren. Diese Experimente erbrachten keine Erhöhung des Gehalts an Pyr-Peptiden im Ausgangsmaterial. Dies erhöht die Wahrscheinlichkeit, daß diese Verbindungen tatsächlich nativ in der Probe vorliegen könnten. Pyr ist als Bestandteil von Peptiden bei Bakterien, Pflanzen, Invertebraten und Vertebraten bereits mehrfach beschrieben worden, was anhand von Beispielen aus der Literatur diskutiert wird.

1.7. Peptaibiomics – das zielgerichtete Screening nach Peptaibiotika in phytoprotektiven *Trichoderma*-Arten

Die von Krause et al. [2006(a)] eingeführte Methode der **„Peptaibiomics“** zielt darauf ab, die Gesamtheit der von einem Pilzstamm unter definierten Kulturbedingungen gebildeten Peptaibiotika, das so genannte **„Peptaibiom“**, zu erfassen. Mit dieser Vorgehensweise ist es u. a. möglich, die zeitabhängige Dynamik der Biosynthese und des Abbaus von Peptaibiotika zu verfolgen, wie am Beispiel der extrem mikroheterogenen Trichobrachine aus *Trichoderma ghanense* CBS 936.69 demonstriert wurde [Krause et al., 2007]. Methodisch wird wie folgt vorgegangen:

An die Extraktion der Pilzkultur mit organischen Lösungsmitteln schließt sich eine Festphasenextraktion (SPE) über C_{18}-Kartuschen an. Das organische Eluat wird danach mittels ESI-MS analysiert. Als besonders geeignet hierfür hat sich die Ionenfallen (ion trap)-Massenspektrometrie (IT-MS) erwiesen; denn diese ermöglicht es, wie in der ***Publikation A***

des ***Anhangs*** dargestellt, die Genealogie von Produkt-Ionen zu verfolgen. In einem ersten Schritt wird die Probe durch Applikation verschiedener Spannungsdifferenzen (typischerweise 0, 45 und 65%) in der Skimmer-Region des IT-MS der so genannten „Quellenfragmentierung“ (**c**ollision-**i**nduced **d**ecomposition, **CID**-MS) unterworfen. Somit können ausgewählten Quasimolekül-Ionen eine Reihe von zugehörigen Fragment-Ionen zugeordnet werden. Von besonderer Bedeutung ist dabei das Auftreten der Differenz Δ (*m/z*) 85, da diese das Vorhandensein eines Aib-Restes im Molekül anzeigt. Eine weitere diagnostische Differenz ist Δ (*m/z*) 99, die Hinweise auf das Vorliegen eines Vxx-Restes (D- oder L-Iva bzw. L-Val[10]) gibt. Die durch CID-MS ermittelten Partialsequenzen werden in einem nächsten Schritt durch Stoßexperimente (MS^2, MS^3, MS^4, MS^n) nach Auswahl geeigneter Vorläufer-Ionen bestätigt.

Acht Stämme aus sieben *Trichoderma*-Arten (*T. erinaceus*, *T. pubescens*, *T. spirale*, *T. strigosum* und *T. stromaticum* sowie *T.* cf. *pubescens* und *T.* cf. *strigosum*) wurden mittels der oben vorgestellten Methode auf die Bildung von Peptaibiotika untersucht. Diese Isolate wurden im Rahmen eines Screenings von mehr als 400 Stämmen aus etwa 30 *Trichoderma*-Arten aufgrund ihrer antimikrobiellen Wirkung auf die Erreger der Eutypiose, *Eutypa lata*, und der Esca-Krankheit, *Phaeomoniella chlamydospora* und *Phaeoacremonium aleophilum*, für eine vertiefende Bearbeitung ausgewählt. Beide traten in Deutschland in den vergangenen Jahren verstärkt als Erkrankung von Stamm und mehrjährigem Holz der Weinrebe (*Vitis vinifera*) auf [Gräfenhan, 2006]. In allen untersuchten Stämmen wurden Peptaibiotika nachgewiesen, die zur antifungalen Aktivität der Bildner beitragen könnten:

Neben bekannten Sequenzen wurden neue, aus sieben, zehn und elf Resten bestehende Lipopeptaibole mit *N*-terminalen Alkanoyl- und *C*-terminalen Leucinol- (Leuol) oder Isoleucinol-Resten (Ileol) nachgewiesen[11]. Diese werden als Lipostrigocine (LSG) bzw. Lipopubescine (LPB) bezeichnet. Zwei neue, aus 19 Resten bestehende Peptaibole, die Trichostrigocine (TSG) A und B, werden von *T. strigosum* und *T. erinaceus* gebildet. In

[10] Über den Nachweis von D-Val in Peptaibiotika ist bislang noch nicht berichtet worden.

[11] Alle bislang in linearen Peptaibiotika nachgewiesenen *C*-terminalen Aminoalkohole sind L-konfiguriert. Da eine Konfigurationsanalyse mittels GC/MS nicht durchgeführt werden konnte, sind die isobaren Aminosäuren bzw. -alkohole der in den ***Anhängen D***, ***E*** und ***H*** gefundenen Peptaibiotika mit Vxx und Lxx bzw. Vxxol und Lxxol angegeben.

Kulturen von *T. stromaticum* [Samuels et al., 2000] wurden fünf neue Peptaibole, die Trichstromaticine (TSM) A–E, detektiert, die aus 18 Resten bestehen und zum Erfolg dieser Art bei der biologischen Bekämpfung des Erregers der Hexenbesenkrankheit des Kakaos (*Theobroma cacao*), *Moniliophthora* (syn. *Crinipellis*) *perniciosa,* beitragen könnten.

Schließlich wird die chemotaxonomische Relevanz der gefundenen Peptaibiotika diskutiert. Für die Systematik besitzen diese Peptid-Antibiotika bei isolierter Betrachtung keine Bedeutung, wohl aber im Kontext mit anderen, für die Einordnung der Bildner ins System der Pilze wichtigen Merkmalen, wie klassisch-morphologischen Daten und molekularbiologischen Markern, aber auch der Bildung von Mykotoxinen und anderen niedermolekularen Sekundärmetaboliten.

1.8. Der *Trichoderma brevicompactum*-Komplex als reiche Fundgrube bekannter und neuer Sequenzen von Peptaibiotika

Im Rahmen des im vorherigen Abschnitt besprochenen Screenings nach Trichodermen, die zur biologischen Bekämpfung von Eutypiose und Esca-Krankheit im Weinbau geeignet sind, wurden drei Isolate von *T. brevicompactum* sowie vier mit dieser Art nahe verwandte Stämme (vorläufig als *Trichoderma* cf. *brevicompactum* klassifiziert)[12] eingehend auf die Bildung von Peptaibiotika untersucht.

Die Auswahl der sieben oben genannten Stämme erfolgte aufgrund ihrer besonders stark ausgeprägten antibiotischen Aktivität gegen die Erreger der beiden Rebholz-Krankheiten. Methodisch wurde in dieser Arbeit, wie bereits im Abschnitt 1.7. beschrieben, vorgegangen. Alle untersuchten Stämme produzierten die aus 20 Resten bestehenden Alamethicine F30.[13] Insgesamt wurden folgende Peptaibole sequenziert: 14 aus zwölf Resten bestehende Trichocryptine B (TCT-B), zwölf aus elf Resten bestehende Trichocryptine A (TCT-A), 19

[12] Zur endgültigen taxonomischen Einordnung und Hinterlegung der betreffenden Stämme siehe ***Anhang H***. Zu einer dieser Arten gehört auch der ALM-Patentstamm *Trichoderma „viride*“ NRRL 3199, der als „klassische“ Quelle dieser aus 20 Resten bestehenden Peptaibole bekannt ist.

[13] ALM gilt allgemein als ***d a s*** klassische Beispiel für membranaktive Peptaibole [vgl. Übersichtsarbeit: Leitgeb et al., 2007]. Deren antibiotische Aktivität beruht auf der Bildung von spannungsabhängigen Ionenkanälen nach dem „Flip-Flop-Modell“ [Boheim et al., 1983], das aus dem „barrel stave“-Modell [Boheim, 1974] hervorgegangen ist.

aus elf Resten bestehende Trichobrevine (TBV) A und B. Außerdem bildeten die Isolate sechs aus zehn Resten bestehende Lipoaminopeptide, die Trichoferine (TFR), und 17 aus sieben Resten bestehende Lipopeptaibole, die Trichocompactine (TCT). Somit konnten – allein aus diesen sieben Stämmen – fast 70 neue Sequenzen von Peptaibiotika ermittelt werden.

Die gefundenen Peptaibiotika, insbesondere die als Hauptkomponenten auftretenden ALM F30[9], könnten – wegen ihrer ionenkanalbildenden Eigenschaften – als ***d a s*** maßgebliche antibiotische Prinzip der sieben untersuchten Stämme interpretiert werden. Aufgrund der distinkten HPLC-Elutionsprofile der einzelnen Stämme und des in zwei Gruppen aufspaltenden Musters der gebildeten, auf Warmblüter cytotoxisch wirkenden Mykotoxine[14] Trichodermin [Godtfredsen & Vangedal, 1964; 1965] und Harzianum A [Corley et al., 1994] ergab sich die Notwendigkeit einer taxonomischen Revision der Sammelart *Trichoderma brevicompactum*, d. h. ihre Neubeschreibung und die Schaffung zusätzlicher Arten. Diese Studie verdeutlicht zwei Tatsachen:

Einerseits illustriert sie eindrucksvoll das Potential einer peptaibiomischen Vorgehensweise bei der Suche nach neuen Peptabiotika mit interessanten Bioaktivitäten. Andererseits bestätigt sie bereits im ***Anhang D*** niedergelegten Aussagen zur Relevanz von Peptaibiotika in der Pilzsystematik, da die Ergebnisse der morphologischen und molekulartaxonomischen Untersuchung dieser Isolate im Einklang mit chemotaxonomischen Befunden stehen.

[14] Auf die Sicherheitsrisiken, die sich aus einem Einsatz mykotoxinbildender Trichodermen in der landwirtschaftlichen Praxis ergäben, wurde bereits von Nielsen et al. [2005] verwiesen.

1.9. Neue Sequenzen, Bausteine und Produzenten von Peptaibiotika

In den vier Jahren seit dem Erscheinen von ***Anhang A*** sind etwa 300 neue Peptaibiotika vollständig oder zumindest partiell sequenziert worden; darüber hinaus wurden neue Bildner und weitere ungewöhnliche, nicht-proteinogene Bausteine beschrieben. Hieraus ergab sich im Jahre 2007 die Notwendigkeit, diese neuen Befunde in einer Übersichtarbeit, welche im ***Anhang F*** aufgelistet ist, darzustellen:

Neue Arten von Peptaibiotikabildnern werden vorgestellt und deren Position im System der Pilze diskutiert. Außerdem wird auf weitere, potentielle Quellen von Peptaibiotika eingegangen. Dabei handelt es sich um solche Arten und Stämme, für die zwar bereits der Nachweis von peptidisch gebundenem Aib, D- oder L-Iva mittels GC/MS erbracht wurde, in denen aber bislang noch keine Peptaibiotika sequenziert worden sind[15].

Repräsentative Sequenzen von Peptaibiotika und ungewöhnliche monomere Bausteine werden im Formelbild illustriert. Auf die erfolgreiche Anwendung der Peptaibiomics bei der Untersuchung neuer Bildner aus der Gattung *Trichoderma*/*Hypocrea* sowie bei der Sequenzierung der Suzukacilline [Krause et al., 2006(b)] und der extrem mikroheterogenen Trichobrachine [Krause et al., 2007] wird eingegangen. Als besonders interessanter Befund wird die erstmalige Isolierung von Antiamoebinen (AAM) aus dem natürlichen, von *Stilbella fimetaria* (syn. *S. erythrocephala*) besiedelten Substrat ausführlich diskutiert, d. h. der Nachweis der Peptaibiotikabildung *in vivo*. Hierbei ist hervorzuheben, daß die gefundenen Mengen an Antiamoebin im deutlich inhibitorischen Bereich lagen und somit für den koprophilen Bildner einen bemerkenswerten Vorteil bei der Besiedlung von Herbivoren-Dung als ökologische Nische darstellen.

Als Gründe für die erwähnte Verdopplung der in der Literatur beschriebenen Peptaibiotika innerhalb von nur vier Jahren werden die Einführung neuer analytischer Methoden, die zielorientierte Auswahl und Optimierung von Trennmethoden und der Einsatz neuester massenspektrometrischer Verfahren diskutiert. In diesem Zusammenhang werden analytische Aspekte der Trennung stereoisomerer Aminosäuren an zwei für die Sequenzierung von Peptaibiotika relevanten Beispielen (DL-Iva sowie 3- und 4-Hyp) erörtert.

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[15] Auf diese bislang nur in Ansätzen bearbeiteten Bildner nicht-proteinogener α,α-Dialkylaminosäuren wird im ***Anhang K*** eingegangen.

Als ein weiterer Grund für die rapide Zunahme der in der Literatur beschriebenen Peptaibiotika wird die Erforschung von Bildnern aus hochkompetitiven bzw. ungewöhnlichen Substraten besprochen. Der Bearbeitung von Pilzen aus ökologischen Nischen, so z. B. aus koprophilen (AAM aus *Stilbella*-Arten), entomopathogenen (Cicadapeptine aus *Cordyceps heteropoda*), und fungicolen Assoziationen (Acretocin aus *Acremonium crotocinigenum*; *Trichoderma stromaticum* als Hyperparasit des Erregers der Hexenbesenkrankheit des Kakaos, *Moniliophthora* [syn. *Crinipellis*] *perniciosa*), erhöht die Wahrscheinlichkeit des Auffindens von neuartigen Sequenzen und interessanten Bioaktivitäten.

1.10. Die Bedeutung und künftige Perspektiven aktueller Ergebnisse auf den Gebieten der Peptaibiotika-, Hydrophobin- und Mykotoxinforschung für die Chemotaxonomie der Gattung *Trichoderma*/*Hypocrea*

Auf die überragende Bedeutung der Anamorphen-Gattung *Trichoderma* und ihrer *Hypocrea*-Teleomorphen als Bildner von Peptaibiotika wurde im Rahmen dieser Ausführungen schon mehrfach verwiesen. Ungeachtet dessen ist es, wie in ***Anhang G*** ausgeführt, stets notwendig, Aussagen zur Bildung von Sekundärmetaboliten durch bestimmte Arten der Gattung *Trichoderma*/*Hypocrea* kritisch zu hinterfragen. Dies beruht vor allem auf der Tatsache, daß deren Systematik seit etwa Mitte der 1990er Jahre, bedingt durch die Einführung molekulartaxonomischer und phylogenetischer Methoden, bedeutsamen Veränderungen unterworfen ist. Diese finden jedoch erst seit etwa zehn Jahren ihren Niederschlag in der naturstoffchemischen und bioanalytischen Natur. Von wenigen Ausnahmen abgesehen, wurde die Bildung von Sekundärmetaboliten, einschließlich der Peptaibiotika, bis zu diesem Zeitpunkt meist den vier verbreitetesten *Trichoderma*-Arten, *T. viride, T. koningii, T. harzianum* und *T. longibrachiatum*, zugeschrieben. Diese Zuordnung kann nach heutigem Kenntnisstand nicht mehr aufrecht erhalten werden, da die ursprüngliche Identifizierung dieser Produzenten in der Zwischenzeit entweder bereits revidiert wurde oder fortwährend Gegenstand berechtigter Zweifel ist[16]. Derzeit sind etwa 140 *Trichoderma*-Arten gültig

[16] Es sei an dieser Stelle auf die nach wie vor bestehende, da nicht verbindlich gelöste Problematik verwiesen, daß etliche Peptaibiotikabildner nicht in einer anerkannten, öffentlich zugänglichen Stammsammlung (**I**nternational **D**epositary **A**uthority; **IDA**) hinterlegt sind. Diese Tatsache erschwert nicht nur die Nachbestimmung eines peptaibiotika-bildenden Stammes, dessen publizierte Identität zweifelhaft erscheint, sondern hat in der Vergangenheit bereits zum wahrscheinlich endgültigen Verlust von Isolaten geführt.

beschrieben. Daher gelten moderne Methoden der DNA-Sequenzierung – neben klassisch-morphologischen Bestimmungsmethoden – als derzeitiger Standard in der Pilztaxonomie. Als weitere vielversprechende Methode hat sich in diesem Zusammenhang die **„I**ntact **C**ell **MALDI-TOF MS“ (IC-MS)** [Neuhof et al., 2007(a), (b), (c)] erwiesen. Im Falle intakter pilzlicher Zellen werden die im Solvens löslichen Komponenten der Zellwand, vor allem Proteine, sowie niedermolekulare Metaboliten, einschließlich der Peptaibiotika, detektiert. Dabei gelten die **H**ydro**ph**o**b**ine **(HFB)** als exzellente Biomarker, die eine Differenzierung von Isolaten unterhalb der Artebene ermöglichen und somit für die Aufspaltung von Sammelarten, wie *Trichoderma brevicompactum*[17], besonders gut geeignet sind.

Wann immer möglich, sollten molekulargenetische und chemotaxonomische Methoden, wie z. B. die Bestimmung der HFB mittels IC-MS, ein LC/MS-basierenden Mykotoxin-Screening [Nielsen et al., 2005] und ein peptaibiomischen Ansatz (siehe Abschnitte 1.7., 1.8. und 1.11.), miteinander kombiniert werden. Eine solche interdisziplinäre Vorgehensweise, die der genomischen, proteomischen und metabolomischen Komplexität der Pilze Rechnung trägt, wird bei der Besprechung des *Trichoderma brevicompactum*-Cladus im folgenden Abschnitt ausführlich erörtert.

Die praktische Bedeutung von Sekundärmetaboliten der Gattung *Trichoderma*/*Hypocrea* wird einerseits dadurch verdeutlicht, daß nicht nur stetig neue Sequenzen und Bioaktivitäten von Peptaibiotika, wie die Hemmung der Amyloid-*β*-Peptide [Hosotani et al., 2007], beschrieben werden, sondern mittlerweile auch bekannte phytoprotektive Wirkungen von so genannten pflanzenstärkenden oder im biologischen Pflanzenschutz eingesetzten Präparaten auf *Trichoderma*-Basis durch die Präsenz von Peptaibiotika erklärbar sind. Andererseits ist zu berücksichtigen, daß solche Präparate, die potentiell in die Nahrungskette gelangen können, frei von Metaboliten unterschiedlichster Strukturklassen, die als Mykotoxine **i**m **e**ngeren **S**inne [**i. e**. **S**.: Frisvad et al., 2006(b)] klassifiziert worden sind, sein müssen. Anhand repräsentativer Beispiele wird erläutert, warum manche Peptaibiotika – allein aufgrund ihres irreführenden Namens (vgl. Trichotoxin!) oder einer toxischen Wirkung gegenüber Invertebraten – nicht pauschal als Mykotoxine (i. e. S.) eingestuft werden sollten.

[17] Weitere Beispiele aus der neuesten Literatur werden im ***Kapitel 3*** diskutiert.

1.11. Der *Trichoderma brevicompactum*-Cladus: ein separater Zweig im Stammbaum mit neuen peptaibiotika- und mykotoxinbildenden Arten

Ausgehend von den im Abschnitt 1.8. dargestellten Diskrepanzen, die sich aus der Untersuchung des Peptaibioms und des Mykotoxin-Musters ausgewählter Isolate des Brevicompactum-Komplexes ergaben, resultierte die Notwendigkeit einer taxonomischen Revision des nunmehr als Sammelart zu bezeichnenden *Trichoderma brevicompactum*. Mit Hilfe des interdisziplinären im ***Anhang H*** beschriebenen Ansatzes, der

- die morphologische Analyse und deren nachfolgender statistische Auswertung (MDS);
- die phylogenetische Analyse (*tef1*, *rpb2*, ITS);
- die Peptaibiom-Analyse (durch HPLC/ESI-IT-MS^n, MALDI-TOF-MS und GC/MS);
- die Hydrophobin-Analyse der sporulierenden Mycelien (durch IC-MALDI-TOF-MS) und
- die Mykotoxin-Analyse (durch Kopplung von DAD-HPLC und hochauflösender ESI-TOF-MS)

umfaßte, konnte dieses Ziel erreicht werden. Die in ***Anhang E*** temporär als *Trichoderma* cf. *brevicompactum* klassifizierte Stämme und weitere Isolate gehören den folgenden drei neuen Arten an und zwar

- *Trichoderma arundinaceum* sp. nov. Zafari, Gräfenhan & Samuels;
- *Trichoderma protrudens* sp. nov. Samuels & Chaverri sowie
- *Trichoderma turrialbense* sp. nov. Samuels, Degenkolb, K. F. Nielsen & Gräfenhan.

Gemeinsam mit *Trichoderma brevicompactum*, dessen Artbeschreibung revidiert wurde, und *Hypocrea rodmanii* sp. nov. Samuels & Chaverri (Anamorphe: *Trichoderma rodmanii*) bilden sie den neuen Brevicompactum-Cladus, der systematisch dem Lutea-Cladus am nächsten steht. Dabei unterscheidet sich die neue *Hypocrea*-Art durch das Fehlen von einfachen, nicht-makrocyclischen Trichothecenen von den vier übrigen Arten, die entweder Trichodermin (*T.*

brevicompactum)[18] oder Harzianum A (*T. arundinaceum*[19], *T. protrudens* und *T. turrialbense*) bilden. Außerdem ist das Peptaibiotika-Muster von *H. rodmanii* völlig verschieden von dem der übrigen Arten des Cladus – neben sieben neuen, aus sieben Resten bestehenden Lipopeptaibolen, den Hypocompactinen (HCP), und fünf neuen, aus 14 Resten bestehenden Peptaibolen, den Hyporodicinen (HRC), bildet diese Art aus 19 Resten bestehende Trichokonine (TKO). Im Gegensatz dazu bilden die sieben untersuchten Isolate von *Trichoderma brevicompactum* hauptsächlich ALM[20] sowie die TCT der A- und B-Reihe. Als Minorkomponenten wurden Lipoaminopeptide der TFR-Gruppe gefunden. In Abhängigkeit vom untersuchten Isolat können Spuren von TBV vorhanden sein. *T. turrialbense* unterscheidet sich durch die Bildung von Harzianum A von *T. brevicompactum*. Das Muster der produzierten TCT ist vom Isolat abhängig; besonders bemerkenswert ist in diesem Zusammenhang der erstmalige Nachweis von L-Alaninol (L-Alaol) als *C*-terminaler 2-Aminoalkohol von Peptaibolen mittels GC/MS. Ferner wurden TBV und TFR als Minorkomponenten nachgewiesen. Somit ist *T. turrialbense* als Bindeglied zwischen *T. brevicompactum* und *T. arundinaceum* einzuordnen. Das Peptaibiom der beiden Harzianum A-bildenden Species *T. arundinaceum* und *T. protrudens* ist identisch; die Abgrenzung der letzteren erfolgt hier anhand ihres einzigartigen Hydrophobin-Musters und morphologisch-physiologischer Parameter.

[18] Der ursprünglich als *T. viride* beschriebene Trichodermin-Bildner [Godtfredsen & Vangedal, 1964; 1965] gehört zu *T. brevicompactum*.

[19] Zu *T. arundinaceum* gehören u. a. der von Corley et al., [1994] als *T. harzianum* ATCC 90237 klassifizierte Harzianum A-Bildner sowie „*Hypocrea* sp." F000527, die von Lee et al. [2005] und Jin et al. [2007] als Bildner von Harzianum A bzw. B publiziert wurde. Auch der ALM-Patentstamm *T.* „*viride*" NRRL 3199 ist nunmehr als *T. arundinaceum* eingeordnet.

[20] Das Verhältnis zwischen ALM F30 und F50 hängt vom jeweiligen Isolat und von den Fermentationsbedingungen ab. Die verwendeten Abkürzungen werden im Abschnitt 1.6. erläutert.

1.12. Natürlich vorkommende Cyclopeptaibiotika und verwandte cyclische Tetrapeptide: ihre strukturelle Vielfalt und biologischen Aktivitäten

Wird deren Linearität nicht mehr als ein notwendiges Kriterium zur Einordnung von bioaktiven Peptiden als Peptaibiotika betrachtet, so bietet dies die Möglichkeit, die klassische Definition eines Peptaibiotikums [Brückner et al., 1991(a) und (b); ***Anhang A*** und ***Anhang F***] auf cyclische, *α,α*-dialkylaminosäure-haltige Peptide zu erweitern. Diese schließt nunmehr eine kleine Gruppe von Verbindungen ein, die – wie in ***Anhang I*** dargestellt – als fünfte Unterfamilie der Peptaibiotika, die **Cyclopeptaibiotika**, in die Literatur eingeführt wurden. Dabei werden auch taxonomische und ökophysiologische Aspekte der Bildner diskutiert. Strukturell lassen sich die bisher bekannten Verbindungen in dieser Unterfamilie in die **Cyclotetrapeptaibiotika** und die **Cycloheptapeptaibiotika** unterteilen. Cyclotetrapeptaibiotika sind durch einen vergleichsweise einheitlichen Bauplan gekennzeichnet: in ihrem Ring befinden sich ein substituierter L-2-Aminodecansäure-, ein D-Pro- oder *trans*-4-D-MePro-[21], ein Phenylalanyl-[22] und ein *α,α*-Dialkylaminosäure-Rest. Letzterer wird in sieben von neun bislang beschriebenen Cyclotetrapeptaibiotika durch Aib gebildet, welche im Phoenistatin [Masuoka et al., 2001][23] durch Iva[24] sowie im FR235222 und im AS1387392[25] durch L-Iva substituiert ist. In den drei Cycloheptapeptaibiotika fehlt hingegen der Lipoaminosäure-Rest; stattdessen sind in den Syctalidamiden A und B als ungewöhnliche Bausteine neben Aib *N*-Methylaminosäuren vorhanden sowie Pro bzw. *(2S,3S)*-3-MePro. In den Serinocyclinen ist Acc als *α,α*-Dialkylaminosäure enthalten;

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[21] Bislang nur im FR235222 nachgewiesen; alle übrigen Cyclotetrapeptaibiotika besitzen einen D-Pro-Rest.

[22] Als eine Ausnahme hiervon ist AS1429716 zu nennen, das statt des Phe-Restes einen Tyr-Rest trägt. Es wird aus AS1387392, cyclo(Aoh-D-Pro-L-Phe-L-Iva) [Sasamura et al., 2010(a)], durch Biokonversion m. H. verschiedener Actinomyceten erzeugt [Sasamura et al., 2010(b)]. Weitere Derivate des AS1387392 sind cyclo(Aoh-D-Pro-L-*m*-Tyr-L-Iva), cyclo(Add-D-Pro-L-Phe-L-Iva), cyclo(Add-D-Pro-L-Tyr-L-Iva) und cyclo(Add-D-Pro-L-*m*-Tyr-L-Iva). Sie werden durch Biokonversion mit *Amycolatopsis azurea* JCM 3275 gewonnen [Sasamura et al., 2010(c)]. **Aoh** = 2-**A**mino-8-**o**xo-9-**h**ydroxydecansäure; **Add** = 2-**A**mino-8,9-**d**ihydroxy**d**ecansäure.

[23] Das von *Acremonium fusigerum* produczierte Phoenistatin [Masuoka et al., 2001] und die Serinocycline A und B aus *Metarhizium anisopliae* [Krasnoff et al., 2007] werden im ***Anhang I*** nicht abgehandelt.

[24] Die Konfiguration des Iva-Rests im Phoenistatin ist bisher noch nicht bestimmt worden.

[25] AS1387392 [Sasamura et al., 2010(a)] und FR235222 wurden aus *Acremonium* sp. No. 27082 isoliert.

weiterhin ein D-Ser-, zwei L-Ser-, ein *(2S,4R)*-Hyp, ein *β*-Ala- und ein 2-*(R,4S)*-*γ*-Hylys- bzw. D-Lys-Rest [Krasnoff et al., 2007].

Alle bisher beschriebenen Cyclotetrapeptaibiotika und natürlich vorkommende, strukturell verwandte *α,α*-dialkylaminosäurefreie Cyclotetrapeptide[26] werden von Pilzen gebildet. Sie besitzen eine stark inhibierende Wirkung auf Histon-Deacetylasen (HDAC), durch die das breite Spektrum der biologischen Aktivitäten dieser Verbindungen, auf die in ***Anhang I*** ausführlich eingegangen wird, im allgemeinen erklärt werden kann. Als Beispiele mit möglicher praktischer Relevanz sind zu nennen die immunsuppressive Wirkung des FR235222 oder die TGF-*β*-artige Aktivität[27] des Diheteropeptins.

Im Falle der beiden Cycloheptapeptaibiotika Scytalidamid A und B wurde eine *in vitro*-Hemmung von Krebszell-Linien beobachtet, während das von *Metarhizium anisopliae* var. *anisopliae* und *M. anisopliae* var. *acridum*[28] biosynthetisierte Serinocyclin A subletale lokomotorische Defekte bei *Aedes aegypti*-Larven hervorruft [Krasnoff et al., 2007].

Eine durch das Vorkommen nicht-proteinogener Aminosäuren wahrscheinliche nicht-ribosomale Biosynthese dieser Verbindungen schlägt sich im Falle der Apicidine, Azumamide und der Analoga der HC-Toxine [Kim et al., 1985; Tanis et al., 1986; Rasmussen & Scheffer, 1988] in einer Mikroheterogenität nieder:

Die im letzteren Falle beschriebenen Substitutionen von D-Ala gegen Gly (HC-Toxin II) bzw. von D-Pro gegen *trans*-3-Hyp (HC-Toxin III) geben Anlaß zu der Hypothese eines möglichen Austauschs von Ala gegen einen *α,α*-Dialkylaminosäure-Rest. Die bislang noch ausstehende systematische Untersuchung dieser Mikroheterogenität, z. B. durch Applikation eines peptaibiomischen Ansatzes, erscheint als interessante Alternative zur potentiellen

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[26] Hierzu zählt auch JM47, cyclo(Aoh-D-Pro-Ala-D-Ala), das von einem marinen *Fusarium* sp. [Jiang et al., 2002] gebildet wird. Die beiden Ala-Reste erscheinen ebenfalls als für einen hypothetischen Austausch gegen Aib geeignet.

[27] Hierbei handelt es sich um den Transformierenden Wachstumsfaktor Beta, ein Cytokin, das u. a. Hirnzellen vor Zerstörungen schützt, wie sie nach ischämischen Verletzungen oder infolge der *Alzheimer*-Krankheit auftreten können.

[28] Beide Subspecies werden bei der biologischen Bekämpfung von Schadinsekten kommerziell eingesetzt. Der verwendete Stamm von *M. anisopliae* var. *acridum* wurde aus dem Präparat „Green Muscle“® isoliert, das in Westafrika sehr effektiv zur biologischen Bekämpfung von Wanderheuschrecken verwendet wird.

Auffindung von Analoga bzw. Minorkomponenten mit neuen monomeren Bausteinen und interessanten Bioaktivitäten.

1.13. Peptaibiomics: auf dem Wege zu einer Myriade von bioaktiven, *α,α*-dialkylaminosäure-haltigen Peptiden?

Bereits aus den bisherigen Ausführungen ist erkennbar, daß Pilze eine buchstäblich unerschöpfliche Quelle bioaktiver Peptide darstellen. Daher wird in der als ***Anhang J*** aufgelisteten Übersichtsarbeit eingangs die **Definition des Terminus „Peptaibiotikum“** aktualisiert und präzisiert. Chronologisch wird die Geschichte der Peptaibiotika-Forschung von den Anfängen im Jahre 1958 bis 2008 vorgestellt und die dabei wichtigsten Literaturreferenzen besprochen. Diese Arbeit fokussiert einerseits auf die Darstellung der strukturellen Diversität und Mikroheterogenität der bis dahin etwa 850 beschriebenen Sequenzen von Peptaibiotika, andererseits auf die Biodiversität der sie produzierenden Pilze.

Ausgehend von der Entdeckung von peptidisch gebundener Aib im Hydrolysat des nicht-kristallinen Antibiotikums ICI 13959 werden die ersten zwei Dekaden der Peptaibiotika-Forschung geschildert. Daran schließen sich Ausführungen über Entwicklung und Relevanz von hochenergetischen Ionisierungstechniken, wie EI-MS und FAB-MS, für die Sequenzierung von Peptaibiotika an; und es wird auf die Bedeutung von GC/MS-, GC-FID, FD- und CI-MS sowie HPLC-Methoden zur Aminosäure-Analytik, einschließlich der Bestimmung des *N*-terminalen Aminosäure-Rests eingegangen. Danach wird die Einführung von Spray-Ionisationstechniken als entscheidender, die Sequenzierung von Peptaibiotika revolutionierender Schritt geschildert. Einen breiten Raum nimmt die Darstellung der strukturellen Diversifizierung in den Jahren 1996 bis 2008 ein. Es werden Beispiele für Verbindungen mit ungewöhnlichen Bausteinen[29] und Bioaktivitäten (vgl. Abschnitt 1.2.) vorgestellt. Dabei kommen auch taxonomisch-systematische Aspekte und die Ökophysiologie der Bildner zur Sprache. Beispielsweise wird die Frage der Peptaibiotika-Bildung in Fruchtkörpern von Basidiomyceten abschließend diskutiert; und es wird auf die erstmalige

[29] In diesem Zusammenhang sei auf eine Problematik verwiesen, die sich im Rahmen von Literaturrecherchen nach neuen Sequenzen, Strukturelementen, Bildnern oder biologischen Aktivitäten von Peptaibiotika gelegentlich ergibt: Ungeachtet des Nachweises von *α,α*-Dialkylaminosäure-Resten, vor allem von Aib, werden die gefundenen bioaktiven Peptide nicht von allen Autoren als Peptaibiotika erkannt und entsprechend klassifiziert. Dies führt vereinzelt zu dazu, daß relevante Literatur nicht zeitnah erfaßt wird.

Isolierung von Peptaibolen aus dem natürlichen Substrat verwiesen. Weiterhin werden anhand von Beispielen aus der neuesten Literatur peptaibiomische und auf die Verwendung der IC-MS beruhende methodische Ansätze vorgestellt, die ein beschleunigtes Screening potentieller Bildner und eine rasche Sequenzierung von Peptaibiotika ermöglichen. Schließlich werden künftige Perspektiven der Peptaibiotika-Forschung auf dem Wege zu einer sprichwörtlichen Myriade einzelner Sequenzen diskutiert, so z. B. Möglichkeiten zur weiteren strukturellen Diversifizierung durch Untersuchung von aus ökologischen Nischen isolierten Bildnern und die Erweiterung des auf bislang kaum bzw. noch nicht bearbeitete systematische Gruppen von Pilzen. Auf letzteren Aspekt wird im folgenden Abschnitt näher eingegangen.

1.14. Aib und Iva in der Biosphäre – weder selten noch zwangsläufig extraterrestrischen Ursprungs

Gegenwärtig wird von einer geschätzten Gesamtzahl von nahezu anderthalb Millionen rezenten Pilzarten ausgegangen, wovon rund 98.000 gültig beschrieben sind [Kirk et al., 2008]. Demgegenüber stehen bisher nur einige Tausend Arten, aus denen bioaktive Sekundärmetaboliten isoliert worden sind. Noch deutlicher wird dieses bislang unerforschte Potential im Falle der Peptaibiotika: als Quellen der annähernd 900 publizierten Einzelsequenzen kommen annähernd 100 Pilzarten in ca. 25 Gattungen[30] in Frage, wobei die weitaus meisten Sequenzen von *Trichoderma*/*Hypocrea* (siehe auch Abschnitt 1.2.) biosynthetisiert werden. Die überwiegende Mehrheit der Pilze wurde noch nicht auf die Bildung von Peptaibiotika untersucht.

Aus diesem Grunde wurden organische Extrakte aus 49 Stämmen filamentöser Pilze hydrolysiert und nach entsprechender Derivatisierung auf das Vorhandensein von peptidgebundenem Aib, D- und/oder L-Iva mittels GC/SIM-MS untersucht. In 37 der untersuchten Isolate konnte mindestens eine der drei oben genannten α,α-Dialkylaminosäuren nachgewiesen werden. Eine Übersicht der geprüften Stämme gibt Tabelle 1 der als ***Anhang K*** bezeichneten Originalarbeit. Von besonderem Interesse sind hierbei die positiv getesteten Arten und Stämme aus den Gattungen *Lecythophora* (Teleomorphe: *Coniochaeta*, Coniochaetaceae, Coniochaetales), *Nectriopsis* (Bionectriaceae, Hypocreales), *Monocillium*,

[30] Die hier genannten Zahlenangaben ergeben sich aus einer Schätzung zum Zeitpunkt des Einreichens dieser Habilitationsarbeit im November 2010.

Niesslia (Niessliaceae, Hypocreales) und *Wardomyces* (Microascaceae, Microascales); denn über Peptaibiotika-Bildung in den drei genannten Familien ist bisher kaum bzw. noch nicht berichtet worden. Weitere Arten, wie *Bionectria pityrodes*, *Clonostachys rosea* und *Clonostachys candelabrum* (Bionectriaceae, Hypocreales), sind ebenfalls für eine vertiefende Bearbeitung prädestiniert, da in deren Hydrolysaten sowohl Aib als auch beide Iva-Enantiomere nachgewiesen wurden.

Die Ergebnisse dieses GC/MS-Screenings widerlegen langjährige Postulate von Astrochemikern und Meteoritenforschern, daß Aib und Iva ausschließlich extraterrestrischen Ursprungs sind und in der Biosphäre extrem selten vorkommen.

Hinzu kommt die an sich ubiquitäre Verbindung möglicher Produzenten, welche anhand von Beispielen illustriert wird. *Trichoderma*/*Hypocrea* ist als Pilzgattung mit vielen bodenbürtigen Arten weltweit verbreitet. In diesem Zusammenhang spielt die mögliche Verbreitung von Pilzsporen als Bestandteile von Bioaerosolen eine nicht zu unterschätzende Rolle; außerdem können sie Sand- und Staubpartikeln anhaften und so über Hunderte oder gar Tausende von Kilometern verbreitet werden. Daher kann eine möglicherweise biotische Herkunft von Aib und Iva, die in marinen und nicht-marinen Sedimenten nahe (aber nicht in!) der sog. Kreide-Tertiar (K/T)-Grenze gefunden wurden, nicht ausgeschlossen werden[31]. Selbst über eine nachträgliche mikrobielle Kontamination von aminosäurehaltigen Meteoriten, wie dem *Murchinson* oder dem *Murray*, wurde berichtet, da diese nicht unter streng aseptischen Bedingungen gelagert werden. Seit einigen Jahren wird eine stetig wachsende Anzahl von Sekundärmetaboliten aus psychrotoleranten, kälteadaptierten Pilzspecies [Frisvad et al., 2006(a); Frisvad 2008] publiziert. Selbst aus Meteoriten oder Eisbohrkernen antarktischen Ursprungs werden regelmäßig lebensfähige Mikroorganismen, einschließlich psychrophiler Pilze, isoliert[32]. Darunter befinden sich auch bereits als Peptaibiotika-Bildner beschriebene Pilzgattungen, wie *Acremonium*, *Paecilomyces* und *Trichoderma*[33].

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[31] Beispiele für die Sequenzierung und Isolierung von Peptaibiotika aus Pilzstämmen mariner Herkunft und aus marinen Sedimenten wurden bereits im ***Anhang J*** vorgestellt.

[32] Die Pilzarten des antarktischen Festlands werden eingehend von Onofri et al. [2007] abgehandelt.

[33] Auf die erstmalige Isolierung von Peptaibolen aus einem marinen *Trichoderma asperellum* antarktischer Herkunft wird im ***Kapitel 3*** eingegangen.

Zusammenfassend ist festzustellen, daß ein Screening von bislang noch nicht oder wenig bearbeiteten Pilzarten, die aus ökologischen Nischen und Extremstandorten, so z. B. Eisbohrkernen, Boden-, oder marinen Sedimentproben aus arktischen oder antarktischen Habitaten, die Wahrscheinlichkeit des Auffindens neuer Bildner, Bausteine und Sequenzen von Peptaibiotika beträchtlich erhöht. Eine vertiefende Bearbeitung der in Tabelle 1 des ***Anhangs K*** aufgelisteten, positiv auf peptidisches Aib, D- und/oder L-Iva getesteten Stämme erscheint daher folgerichtig und naheliegend.

1.15. Untersuchungen zur selektiven trifluoracetolytischen Spaltung von nativen Peptaibolen und Modellpeptiden mittels HPLC und ESI-CID-MS

Unerwartete, obgleich selektive Spaltreaktionen von Peptidbindungen, insbesondere bei Anwesenheit nicht-proteinogener Aminosäuren im Molekül, gelten allgemein als Herausforderung für die Planung manueller oder automatisierter Peptidsynthesen bzw. bei der Aufarbeitung, Aufreinigung oder Sequenzierung von Peptiden.

Demgegenüber steht der zielgerichtete Einsatz acidolytischer Spaltungen der von speziellen Aminosäuren gebildeten Amidbindungen, beispielsweise bei der Strukturaufklärung von Peptaibiotika, z. B. Chlamydocin [Closse & Huguenin, 1974], AAM [Pandey et al., 1977(a), 1978], ALM [Pandey et al., 1977(b); Brückner & Jung, 1980], TXT-A40 [Brückner et al., 1979] und TXT-A50 [Przybylski et al., 1984].

Untersucht wurde, wie in der Originalarbeit im ***Anhang L*** geschildert, das Spaltverhalten folgender Peptaibole: der nativen, mikroheterogenen AAM- und ALM-F30-Mischungen, des aufgereinigten nativen Paracelsins A (PC-A) und des synthetischen TXT-A50/E. Als Vergleichssubstanzen wurden drei Homo-Aib-Peptide analysiert, und zwar Z- und Ac-$(Aib)_{10}$-O^{t}Bu sowie Ac-$(Aib)_7$-O^{t}Bu. Alle untersuchten Peptide wurden bei 37 °C für 0,5 bis 26 h mit wasserfreier Trifluoressigsäure (TFA) inkubiert, die acidolytisch erzeugten Fragmente mittels HPLC aufgetrennt und schließlich durch ESI-CID-MS analysiert.

Im Falle der nativen Peptaibole war bereits nach 30-minütiger Inkubation eine selektive Spaltung der Bindung zwischen Aib (bzw. Iva) und Pro (bzw. Hyp) festzustellen. Außerdem wurde eine rasche Veresterung der Hydroxylgruppe des *C*-terminalen Aminoalkohols durch TFA beobachtet. In Abhängigkeit von der Inkubationszeit kam es zur

Freisetzung *C*-terminaler Tri- bzw. Tetrapeptide sowie zur Abspaltung einzelner Aminosäure-Reste aus den dominanten, trifluoracetolytisch erzeugten Fragmenten.

Bei der Trifluoracetolyse des Z^{34}-$(Aib)_{10}$-O^{t}Bu wurde schon nach 30-minütiger Spaltung eine reguläre Fragment-Serie von Z-$(Aib)_{10}$-OH bis Z-$(Aib)_5$-OH erzeugt. Außerdem wurden gleichzeitig Fragment-Serien H-$(Aib)_{10}$-OH bis H-$(Aib)_3$-OH generiert.

Nach dreistündiger Inkubation des Z-$(Aib)_7$-O^{t}Bu kam es zur Freisetzung einer regulären Fragment-Serie von Z-$(Aib)_7$-OH bis Z-$(Aib)_3$-OH.

Von diesen experimentellen Daten ausgehend, wurde ein repetitiver Spaltmechanismus für Homo-$(Aib)_n$-Peptide vorgeschlagen, der über die sich wiederholende Bildung intermediärer Oxazolinolium/Oxazolinium-Ionen verläuft:

Ein interner nucleophiler Angriff des Sauerstoff-Atoms der Carbonylgruppe in der Carboxamid-Bindung wird dabei wahrscheinlich durch den so genannten *geminalen* Dimethyl- oder *Thorpe-Ingold*-Effekt begünstigt. Dabei kommt es zur schnellen Bildung instabiler vierbündiger Zwischenstufen, die nachfolgend durch einen Protonentransfer und die Abspaltung des ursprünglich *C*-terminalen Aib-Rests stabilisiert werden. Die somit entstandenen Oxazolonium-Peptide werden bereits durch die überall – selbst in wasserfreier TFA – vorhandenen Wasserspuren leicht hydrolysiert. Dieser Mechanismus ist auf die Spaltung der Aib(Iva)–Pro(Hyp)-Bindung in Peptaibiotika übertragbar.

Die gezielte Anwendung der Trifluoracetolyse ist von größtem Wert für die Sequenzierung und Strukturaufklärung von Peptaibiotika, da diese aufgrund ihrer nicht-ribosomalen Biosynthese einem enzymatischen Abbau nicht oder nur in sehr begrenztem Umfange [Fredenhagen et al., 2006] zugänglich sind. In solchen Fällen ist eine zielgerichtete acidolytische Spaltung von besonderer Bedeutung. Sie ermöglicht die Erzeugung von Fragmenten, welche für die Positions- und Konfigurationsanalyse von Aminosäuren, beispielsweise der Iva-Enantiomere, geeignet sind.

[34] Die Abkürzung „Z“ steht hierbei für die Benzoyloxycarbonyl-, die Abkürzung „^{t}Bu“ für die *tert*-Butyl-Schutzgruppe.

2. Profiling von Sekundärmetaboliten aus *Arabidopsis thaliana* durch Kopplung von Kapillar-HPLC und ESI-QqTOF-Massenspektrometrie

Im ersten Teil dieser Habilitationsschrift wurde anhand ausgewählter Beispiele ein peptaibiomischer Ansatz vorgestellt. Dieser zielt darauf ab, die Gesamtheit der von einem Pilzstamm unter definierten Kulturbedingungen gebildeten Peptaibiotika, das Peptaibiom, mittels Kopplung aus HPLC und ESI-MS zu erfassen. In Analogie zu dieser peptaibiomischen Vorgehensweise werden – insbesondere in der Pflanzenbiochemie – metabolomische Ansätze dazu verwendet, die Gesamtheit der von einer pflanzlichen Zelle, einem Gewebe, Organ oder Organismus unter definierten Bedingungen gebildeten Primär- und Sekundärmetaboliten, das Metabolom, vergleichend zu analysieren. Metabolomische Ansätze stellen somit – neben der Analyse von Transkriptom (mRNA-Profiling) und Proteom – die dritte Säule der funktionellen Genomforschung dar. Der überwiegende Teil der vor 2004 publizierten Arbeiten zum Profiling pflanzlicher Metaboliten fußt auf der Verwendung von auf GC/MS-Methoden basierenden analytischen Strategien. Diese sind jedoch vorwiegend zur Analyse sehr polarer sowie unpolarer Metaboliten geeignet, während Substanzen mittlerer Polarität kaum erfaßt werden.

In der als ***Anhang M*** aufgeführten Originalarbeit wurde daher untersucht, ob die Verwendung einer Kopplung aus Kapillar-HPLC und ESI-QqTOF-MS geeignet ist, die Lücke im Spektrum der für das Profiling von Sekundärmetaboliten bisher verwendeten analytischen Methoden schließen zu können. Durch den Einsatz einer aseptischen hydroponischen Kultivierungsmethode war die Anzucht der Pflanzen unter hochstandardisierten Kultivierungsbedingungen möglich; zur Schonung empfindlicher Metaboliten wurde das Pflanzenmaterial in flüssigem Stickstoff homogenisiert und mit Methanol/Wasser (8:2) extrahiert.

Insgesamt konnten, ungeachtet der hohen Komplexität der untersuchten Proben, durch Kombination von Retentionszeit und hochaufgelöster Masse in Wurzelextrakten rund 800 Peaks im Bereich zwischen [*m/z*] 106 und 1000 reproduzierbar detektiert werden, während in Blättern etwa 1400 Peaks nachgewiesen wurden. Die technische Varianz lag

hierbei unter 30%, die biologische bei etwa 40%. Nach umfangreicher Optimierung der geräteeigenen Software wurde schließlich eine automatische Dekonvolution und Prozessierung der generierten Daten ermöglicht, die somit der Strukturaufklärung und Quantifizierung zur Verfügung standen. Durch die hochauflösende, auf der QqTOF-Technologie basierenden Arbeitsweise des Massenspektrometers kann prinzipiell jedem einzelnen detektierten Ion eine Präzisionsmasse (Δ m zwischen 5 und 15 ppm) zugeordnet werden, die nach Abgleich des berechneten gegen das gemessene Isotopenmuster zur Ermittlung einer möglichen Summenformel führten. Nach entsprechenden Tandem-MS-Experimenten (CID-MS, MS/MS) und darauffolgenden Literaturrecherchen konnte eine Anzahl von Sekundärmetaboliten des *Arabidopsis*-Wildtyps (Columbia, Col-O) in ihrer Struktur aufgeklärt sowie Unterschiede im Metabolom des Ökotyps Landsberg erecta (Ler) und seiner *tt4*-Mutante analysiert werden.

Zusammenfassend ist festzustellen, daß die Kopplung von HPLC und hochauflösender ESI-Massenspektrometrie eine unentbehrliche Komplementärtechnologie für die GC/MS beim so genannten „Metabolic Profiling“ darstellt – erfordert doch die hohe strukturelle Diversität pflanzlicher Sekundärmetaboliten die Kombination unterschiedlicher analytischer Technologien zur Erfassung von Naturstoffen mit unterschiedlichsten Polaritäten.

3. Ausblick

3.1. Quo vadis? – Perspektiven der Peptaibiotika-Forschung im nächsten Jahrzehnt

Die im ersten Teil dieser Arbeit diskutierten ungewöhnlichen Bioaktivitäten ausgewählter Peptaibiotika, beispielsweise Neurolepsie, Inhibition der Histon-Decacetylase, Hemmung der Bildung von Amyloid-*β*-Peptiden sowie die phytoprotektive und antimikrobielle Wirkung der Pflanzenstärkungsmittel und Biofungizide auf *Trichoderma*-Basis, sprechen für eine weiterhin intensive Bearbeitung dieser hochinteressanten Gruppe nicht-ribosomal biosynthetisierter Peptidantibiotika.

Im Abschnitt 1.13. und ***Anhang J*** ist auf die Perspektiven einer weiteren strukturellen Diversifizierung der Peptaibiotika durch zielgerichtete Untersuchung potentieller Bildner aus ökologischen Nischen verwiesen worden. Dieser Aspekt wurde in Abschnitt 1.14. und ***Anhang K*** u. a. durch Beispiele für die Isolierung von Sekundärmetaboliten aus kältetoleranten und psychrophilen Pilzen aus arktischen und antarktischen Habitaten präzisiert Schließlich wurde die Hypothese einer möglichen Peptaibiotika-Bildung an allen Standorten, an denen potentiell zur Produktion dieser Metaboliten befähigte Pilzstämme vorkommen könnten, aufgestellt. Die Bildung von Peptaibiotika unter *in vivo*-Bedingungen wurde erstmals von Lehr et al. [2006] als Standortvorteil bei der Besiedlung ungewöhnlicher Habitate beschrieben und in Abschnitt 1.9. bzw. ***Anhang F*** diskutiert.

Bereits fünf Monate nach dem Erscheinen von ***Anhang K*** berichteten Ren et al. [2009] über die Isolierung und Strukturaufklärung der Aspereline A–F aus Kulturen von *Trichoderma asperellum* Y19-07, eines aus marinen Sedimenten vor Penguin Island (Südliche Shetlandinseln) isolierten Stammes. Diese sechs, aus zehn Resten bestehenden Peptaibole zeichnen sich durch den erstmaligen Nachweis von *(S)*-Prolinol (= L-Prolinol) als *C*-terminalen Bestandteil[35] von Peptaibiotika aus[36].

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[35] *N*-terminales AcPro wurde bislang nur in dem aus 14 Resten bestehenden Peptaibiotikum Adenopeptin nachgewiesen [Hayakawa et al., 1998].

[36] Die Chiralität der Aminosäuren wurde nach Komplexbildung zwischen den im Hydrolysat befindlichen Aminosäuren und dem chiralen Reagenz Rutheniumporphyrin, Ru((+)D_4-Por*)CO, mittels ^{1}H-NMR bestimmt.

Von Maddau et al. wurde ebenfalls 2009 über die Isolierung eines aus homologen und positionsisomeren Sequenzen bestehenden Peptaibol-Gemischs berichtet, das von *Trichoderma citrinoviride* S25 gebildet wird. Dieser endophytische Stamm wurde aus gesundem Gewebe einer sardischen Korkeiche (*Quercus suber* L.) isoliert und ist in der Lage, das Wachstum von sieben korkeichenpathogenen Pilzen zu hemmen. In dem bioaktiven Gemisch fanden sich mindestens 28, aus 20 Resten bestehende Peptaibole vom Paracelsin-Typ, von denen sieben als neu eingeordnet wurden. Dies verdeutlicht, wie bereits im Abschnitt 1.7. und ***Anhang D*** ausgeführt, das Potential ausgewählter *Trichoderma*-Arten für einen möglichen Einsatz als Biofungizide. Auf einen möglichen Einsatz endophytischer Trichodermen bei der biologischen Bekämpfung pilzlicher Kakao-Pathogene in den Anbaugebieten Südamerikas und Westafrikas ist bereits von Holmes et al. [2004], Tondje et al. [2007], Mejía et al. [2008] und Bailey et al. [2008; 2009] verwiesen worden. Eine Übersicht über in jüngster Zeit isolierte endophytische *Trichoderma*-Species, wie *T. evansii*, *T. lieckfeldtiae* [Samuels & Ismaiel, 2009] und *T. amazonicum* u. a., geben Chaverri et al. [2010]. Eine vertiefende chemisch-analytische Bearbeitung dieser ausnahmslos neuen Arten, einschließlich eines Peptaibiotika-Screenings, ist als vordringlich zu bewerten, da beispielsweise *T. paucisporum* und *T. theobromicola in vitro* und in Inokulationsexperimenten hemmend auf den Erreger der Monilia-Fruchtfäule des Kakaos (*Moniliophthora roreri*) wirkten [Samuels et al., 2006(a)], während *T. martiale* im Rahmen von *in situ*-Feldversuchen Symptomatik und Ausbreitung der Braunfäule des Kakaos (*Phytophthora palmivora*) reduzierte [Hanada et al., 2008; 2009].

Ein weiteres Beispiel für die Bedeutung mariner endophytischer Pilze als Quelle neuer Naturstoffe gaben kürzlich Pruksakorn et al. [2010] mit ihrem Bericht über die Isolierung neuer Lipoaminopeptide vom Helioferin/Roseoferin-Typus [Gräfe et al., 1995; Degenkolb et al., 2000, ***Anhänge E*** und ***H***]. Diese drei als Trichoderine A, A1 und B bezeichneten, aus neun Resten bestehenden Peptaibiotika wurden aus dem taxonomisch nicht näher bestimmten, schwammassoziierten *Trichoderma* sp. 05FI48 isoliert. Als für Peptaibiotika neues Strukturelement im Trichoderin A1 wird erstmals 2-Amino-4-methyl-8-oxodec-6-ensäure (AMOD) angegeben. Besonders bemerkenswert ist die potente inhibitorische *in vitro*-Aktivität der Trichoderine gegen wachsende und dormante Mykobakterien, einschließlich *M. tuberculosis* H37Rv [Pruksakorn et al., 2010].

Aus dem bodenbürtigen *Trichoderma polysporum* FKI-4452 wurden unlängst fünf bereits beschriebene und zwei neue, antitrypanosomal wirksame Trichosporin-Analoga, TSP B-VIIa und B-VIIb, isoliert [Iwatsuki et al., 2010].

Die bereits erwähnten extremophilen und endophytischen Isolate illustrieren das überaus reiche Potential der Gattung *Trichoderma*/*Hypocrea* als nach wie vor wichtigste Quelle von Peptaibiotika. Während der vergangenen sieben Jahre sind eine Reihe weiterer *Hypocrea*/*Trichoderma*-Arten erstbeschrieben worden, deren eingehende chemische Bearbeitung noch aussteht. Viele dieser Species kommen in ökologischen Nischen vor [Chaverri et al., 2003; Bissett et al., 2003; Lu et al., 2004; Jaklitsch et al., 2005; 2006(a) und (b), 2008(a) und (b), 2009; Overton et al., 2006(a) und (b); Samuels et al., 2006(a) und (b); Yu et al., 2007; Zhang et al., 2007] – ein Faktum, welches das Auffinden von Peptaibiotika mit neuen Bioaktivitäten, Strukturelementen, oder Bauplänen überaus wahrscheinlich macht.

Weitere Pilzstämme, die aufgrund des bereits erfolgten Nachweises peptidisch gebundener *α*,*α*-Dialkylaminosäuren als Quelle neuer Peptaibiotika in Frage kommen, sind in Tabelle 1 des ***Anhangs K*** aufgelistet. Als eine sprichwörtliche Fundgrube empfiehlt sich die Gattung *Acremonium*. Dies wird u. a. durch die zwei Publikationen belegt, in denen über die Isolierung und Bioaktivitäten der Acrebole A und B berichtet wurde. Hierbei handelt es sich um zwei lediglich partiell sequenzierte Peptaibiotika mit freiem Serin am *C*-Terminus, die von *A. exuviarum* DSM 21752 gebildet werden [Andersson et al., 2009; Kruglov et al., 2009][37].

Besondere Aufmerksamkeit ist in diesem Jahrzehnt den Integramiden A und B zuteilgeworden. Dabei handelt es sich um zwei aus 16 Resten bestehende Peptaibiotika aus *Dendrodochium* sp.[38], die als Inhibitoren der HIV-1-Integrase fungieren [Singh et al., 2002].

[37] Die Einordnung von Peptaibiotika als Mykotoxine wird in der Literatur teilweise kontrovers diskutiert, siehe dazu auch Abschnitt 1.10. und ***Anhang G***. Zwar weisen die längerkettigen Peptaibole (Alamethicin, Suzukacillin und Trichotoxin) amphiphile, detergensähnliche Eigenschaften auf, so daß eine lytische Wirkung auf die Membran isolierter Human-Erythrocyten beobachtet werden kann [Irmscher & Jung, 1977]. Weder eine solche Hämolyse, noch die *in vitro* beobachtete Hemmung der Motilität von Eberspermatozoen, oder die Hemmung des Komplexes III der Atmungskette isolierter Rattenleber-Mitochondrien rechtfertigt die von Andersson et al. [2009] und Kruglov et al. [2009] vorgenommene Einordnung der Acrebole als Mykotoxine. Es ist vielmehr zu vermuten, daß der Bildner neben den Acrebolen nicht detektierte Mykotoxine i. e. S. [Frisvad et al., 2006(b)] produzieren könnte, welche die von den Autoren nicht näher spezifizierten Krankheitssymptome bei den Bewohnern eines durch Wasserschaden zerstörten Hauses auslösten.

[38] Siehe Kommentar zur Identifizierung des Integramid-Bildners in Fußnote 3.

Die Strukturaufklärung der Integramide erwies sich als analytische Herausforderung, da drei der fünf in der Kette befindlichen Iva-Reste L-konfiguriert sind, während die restlichen zwei die D-Konfiguration aufwiesen. Zur Ermittlung der Chiralität der beiden Iva-Reste in Position 14 und 15 wurden die LD- und DL-Diasteromeren synthetisiert und ihre physikochemischen, analytischen und biologischen Eigenschaften mit denen der isolierten Naturstoffe verglichen. Dies führte letztendlich zur Zuordnung der korrekten Stereochemie –L-Iva^{14}–D-Iva^{15}. Ungeachtet dessen wirkte sich die Inversion der Sequenz zu –D-Iva^{14}–L-Iva^{15}– nicht nachteilig auf die Hemmung der HIV-Integrase aus [de Zotti et al., 2009 und 2010(a)]. In diesem Zusammenhang wurde kürzlich eine nicht-destruktive, auf der Verwendung von 2D-NMR basierende Methode zur Ermittlung der Konfiguration von D- und L-Iva-Resten in Peptiden ausgearbeitet, die derzeit auf ihre Eignung zur Sequenzierung anderer Iva-haltiger Peptaibiotika untersucht wird [de Zotti et al., 2010(b)].

Mit Blick auf das System der Pilze ist festzustellen, daß bisher nur etwa 100 der derzeit rund 2.700 Arten der Ordnung Hypocreales [Kirk et al., 2008] auf die Produktion von Peptaibiotika geprüft worden sind. Noch deutlicher wird dieses bisher wenig erforschte Potential bei Hochrechnung auf die über 64.000 Arten der Ascomycota [Kirk et al., 2008]; finden sich doch einige Peptaibiotika-Bildner in den Ordnungen Eurotiales, Onygenales und Saccharomycetales. Ein umfangreiches, MS-basiertes Screening peptaibiotika-bildender Pilze außerhalb der Ascomyceten und ihrer Anamorphen ist bislang noch nicht Gegenstand der Forschung gewesen; lediglich Tani et al. berichteten 2001 über einen einzigen Cyclopeptaibiotika-Bildner unter den Basidiomycota (siehe Abschnitt 1.2.). Infolge der systematischen Erforschung neuer ökologischer Nischen werden jedoch, wie vorab dargestellt, fortwährend neue Pilzspecies beschrieben, die als potentielle Produzenten von Peptaibiotika in Frage kommen könnten.

In den vergangenen zehn Jahren haben moderne Methoden der Molekularbiologie, so z. B. die Multilocus-Sequenzierung repräsentativer, voneinander unabhängiger Genregionen, Einzug in die Pilztaxonomie gehalten. Von besonderer Bedeutung sind dabei die Sequenzierung der RNA-Polymerase II-Untereinheit (*rpb2*), des Translationselongationsfaktors 1 α (*tef1*) und der „**i**nternal **t**ranscribed **s**pacer"-Regionen (**ITS**1; 5,8 S und **ITS**2); häufig werden jedoch auch andere Genabschnitte, z. B. Actin (*act*) oder GH18-Chitinase (*chi18-5*), sequenziert. Dadurch

ist die Systematik der Pilze gegenwärtig einem tiefgreifenden Wandel hin zu einer phylogenetischen Klassifizierung unterworfen; so wurde u. a. eine Auflösung der Zygomycota als poly- oder paraphyletisches Taxon vorgeschlagen [Hibbett et al., 2007]. Diese Entwicklung schlägt sich selbstverständlich auch in der Systematik peptaibiotika-bildender Gattungen nieder. Besonders deutlich wird dies im Falle der Gattung *Hypocrea* und ihrer *Trichoderma*-Anamorphen; gibt es doch Species, die morphologisch nicht oder nur kaum voneinander zu unterscheiden sind, wohl aber molekulargenetisch. Ein Beispiel hierfür stellt *T. reesei s. l.* dar, welches eine große ökonomische Bedeutung als industrieller Produzent von Cellulasen und Hemicellulasen [Druzhinina et al., 2010(a)] und somit für die Herstellung von Bioethanol [Xu et al., 2009] besitzt. Eine genetisch isolierte, sympatrische Schwesterart, *T. parareesei* sp. nov. ist erst kürzlich beschrieben worden. Diese wird als Vorfahr von *T. reesei* und seiner Teleomorphe *H. jecorina* interpretiert; und es wurde gezeigt, daß die Mehrheit der bislang als *T. reesei* identifizierten anamorphen Stämme zu dieser neuen Art gehört [Atanasova et al., 2010]. Weiterhin ist die Sammelart *Trichoderma asperellum s. l.* [Lieckfeldt et al., 1999; Samuels et al., 1999] zu nennen, die erst kürzlich in zwei Species, *T. asperellum s. s.* und *T. asperelloides* sp. nov., aufgespalten wurde [Samuels et al., 2010]. Moderne massenspektrometrische Methoden, z. B. Peptaibiomics und IC-MS, zeigen eindrucksvoll die Komplementarität molekulargenetischer und chemotaxonomischer Ansätze in der Pilzsystematik. So ist beispielsweise das einzigartige Hydrophobin-Muster Art das sicherste Unterscheidungsmerkmal von *T. arundinaceum* und *T. protrudens* (siehe Abschnitt 1.11. bzw. ***Anhang H***). Desweiteren gelten *T. stromaticum s. l.* [Samuels et al., 2000] und *T. harzianum s. l.* als Sammelarten, [de Respinis et al., 2010; Druzhinina et al., 2010(b)], bei denen Unterschiede in Hydrophobin-Muster und Peptaibiom für die ausstehende, notwendige Artabgrenzung verwendet werden könnten. Eine detaillierte chemische Untersuchung der genannten Sammelarten unter Verwendung moderner massenspektrometrischer Methoden wäre bereits aufgrund der praktischen Relevanz dieser Pilzspecies als Enzymproduzenten bzw. aktive Bestandteile von Präparaten im biologischen Pflanzenschutz von erheblicher Bedeutung Darüber hinaus kann die Chemotaxonomie, wie in ***Anhang H*** dargestellt, wichtigen Beiträge zur Weiterentwicklung der modernen phylogenetischen Pilzsystematik des 21. Jahrhunderts leisten.

Zusammenfassend ist festzustellen, daß sich in neuester Zeit neue Perspektiven für eine mögliche praktische Anwendung von Peptaibiotika – sowohl in der Medizin als auch in Land- und Forstwirtschaft – ergeben:

Beispielsweise wird das auf Reis mit Konidien von *T. stromaticum* formulierte Präparat „Tricovab“ seit einigen Jahren mit Erfolg zur biologischen Bekämpfung im Südosten des Bundesstaates Bahia, dem Zentrum des brasilianischen Kakaoanbaus, eingesetzt [Pomella et al., 2007], wobei das Mikroklima in der Plantage entscheidend für die Sporulation und den Antagonismus gegenüber *Moniliophthora perniciosa* ist [Loguercio et al., 2009]. Erste Formulierungen zur biologischen Bekämpfung der Braunfäule des Kakaos (*Phytophthora palmivora*) auf Basis von *T. martiale* ALF 247 haben sich als unter Freilandbedingungen effizient erwiesen [Hanada et al., 2009].

Ferner läßt der aktuelle Stand der Forschung auf dem Gebiet der Ampullosporine begründeten Bedarf für eine weitere intensive Bearbeitung im Hinblick auf die mögliche Entwicklung eines potentiellen Neuroleptikums erkennen [Berek et al., 2009]. Nicht zuletzt besitzen Peptaibiotika wie die Integramide, das SPF-5506-A_4, die Tricoderine und Trichosporine wegen ihrer – im Rahmen dieser Arbeit erörterten – anti-HIV-, antituberkulotischen bzw. antiprotozoischen Aktivitäten eine große Bedeutung für die human- und veterinärmedizinische Forschung, sind sie doch als Modellsequenzen geeignet zur Entwicklung neuer pharmakologisch aktiver Leitstrukturen.

3.2. Massenspektromtrie als analytische Schlüsseltechnologie in den Lebenswissenschaften

Gegenüber anderen instrumentell-analytischen Methoden bieten massensprektrometrische Verfahren eine Reihe von Vorteilen:

Erstens sind die Anforderungen an die Reinheit der zu analysierenden Proben nicht so hoch, z. B. im Falle der NMR-Spektroskopie oder Röntgenstrukturanalyse. Somit kann die ESI-MS beispielsweise für bioaktivitätsbasierende Screening-Ansätze partiell aufgereingter Proben verwendet werden, während die MALDI-TOF-MS zur Analyse intakter Zellen (IC-MS) prädestiniert ist. Eine – zumindest partielle – Aufreinigung wird vielfach durch Vortrennung der Probe bei Kopplung von HPLC und ESI-MS erreicht. Zudem wird durch die

Verwendung von Streulicht-Detektoren („**D**iodena**r**ray-**D**etektoren“, **DAD**) während der HPLC-Trennung zusätzlich ein UV/VIS-Spektrum jedes einzelnen Peaks erzeugt, das im Idealfalle Rückschlüsse auf bestimmte Strukturelemente des jeweiligen Analyten erlaubt. Hinzu kommen der relativ geringe Probenbedarf moderner Massenspektrometer und ihre hohe Empfindlichkeit, die bei Triple-Quadrupol-Geräten im unteren Femtogramm-Bereich liegen kann.

Zweitens sind beide Methoden prinzipiell automatisierbar; somit besteht die Möglichkeit, in vergleichsweise kurzer Zeit Hochdurchsatz-Screenings durchzuführen, die selbst industriellen Anforderungen genügen. Dies eröffnet neue Perspektiven zur schnellen Auffindung biologisch aktiver Substanzen, die als Leitstrukturen für die Entwicklung neuer Arznei- und Pflanzenschutzmittel dienen können.

Drittens kann der Informationsgehalt einer massenspektrometrischen Analyse vergleichsweise hoch sein. Im Allgemeinen gestatten niederauflösende Spray- oder Laserdesorptions-Verfahren die Ermittlung der Nominalmasse ausgewählter Substanzen durch die Bildung von Quasi-Molekülionen. Mit Hilfe hochauflösender Verfahren ist es prinzipiell möglich, die Elementarzusammensetzung und das Isotopenmuster jedes einzelnen Peaks in der Probe zu ermitteln und dadurch Rückschlüsse auf mögliche Summenformeln zu ziehen. Die Verwendung von Tandem-Massenspektrometern ermöglicht darüber hinaus die Fragmentierung ausgewählter Ionen durch Stoßexperimente mit Inertgasen in einer so genannten Kollisionszelle. Die Fragmentierung des Analyten erfolgt überwiegend an so genannten Sollbruchstellen, so daß durch die Masse der entstehenden Produkt-Ionen und die Massendifferenzen zwischen ihnen Aussagen zu möglichen Strukturen der einzelnen Fragmente, Abgangsgruppen bzw. Vorläufer-Ionen ermöglicht werden. Darauf beruht nicht zuletzt die *de novo*-Sequenzierung von Proteinen und Peptiden, einschließlich der Peptaibiotika. Als für die massenspektrometrische Naturstoffanalytik besonders wertvoll haben sich Ionenfallen erwiesen, gestatten diese doch, die Genealogie von Produkt-Ionen nachzuverfolgen und somit positionsisomere bzw. homologe Substanzen zu untersuchen. Die letztere Option ist von unschätzbarem Wert bei der Analyse chromatographisch nur schwer oder nicht trennbarer hochkomplexer Gemische, wie die von Peptaibiotika.

Viertens sind die analytischen Möglichkeiten der modernen GC/MS-Kopplung zur Analyse flüchtiger, aber auch hochpolarer und unpolarer Metaboliten den im Rahmen dieser

Arbeit dargestellten LC/MS-Ansätzen komplementär. So wurde u. a., in Analogie zum in ***Kapitel 2*** diskutierten LC/MS-Profiling von *Arabidopsis*-Sekundärmetaboliten, die hochauflösende GC/TOF-MS-Kopplung zur Analyse löslicher Primär- und Sekundärmetaboliten sowie von zellwandgebundenen Phenolen von *Medicago truncatula* (Fabaceae, Fabales) verwendet, um das Metabolom nicht-mykorrhizierter Wurzeln mit den metabolischen Veränderungen nach Besiedlung mit dem arbuskulären Mykorrhizapilz *Glomus intraradices* (Glomeraceae, Glomerales, Glomeromycota) zu vergleichen [Schliemann et al., 2008]. Ferner bietet auch die Kopplung aus GC und Ionenfalle eine Möglichkeit zur massenspektrometrischen Strukturaufklärung flüchtiger Naturstoffe [de Pinho et al., 2009; Fernandes et al., 2009].

Somit ist die Massenspektrometrie als derzeit vielseitigster und leistungsfähigster Zweig der Bioanalytik einzuschätzen, deren interdisziplinäre Rolle und Bedeutung für die Lebenswissenschaften in den kommenden Jahren ständig weiter wachsen wird.

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Review

The Occurrence of Peptaibols and Structurally Related Peptaibiotics in Fungi and their Mass Spectrometric Identification via Diagnostic Fragment Ions‡

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Abstract: Peptaibols and related peptide antibiotics (peptaibiotics) display diagnostically useful fragmentation patterns during mass spectrometry (FAB-MS, ESI-CID-MS/MS and CID-MS^n). The paper compiles fragmentation data of pseudo-molecular ions reported in the literature as a guide to the rational identification of recurrently isolated and new peptaibols and peptaibiotics. Taxonomic and ecological aspects of microorganisms producing peptaibols and peptaibiotics are discussed. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptaibols; lipopeptaibols; lipoaminopeptides; peptaibiotics; fungicolous fungi; ESI-CID-MS/MS; CID-MS^n; b-type fragmentation

INTRODUCTION

Peptaibols and peptaibiotics (lipopeptaibols, lipoaminopeptaibols, lipoaminopeptides) constitute a constantly growing family of peptide antibiotics of fungal origin.

They show interesting physico-chemical and biological activities depending on particular structural properties, such as the formation of pores in bilayer lipid membranes as well as antibacterial, antifungal, occasionally antiviral [1] and antiparasitic activities [2]. Inhibition of mitochondrial ATPase, uncoupling of oxidative phosphorylation, immunosuppression [3–7], inhibition of platelet aggregation [8], induction of fungal morphogenesis and neuroleptic effects [9–12] have been reported.

The broad-spectrum antibiotic and membrane-disrupting activities of peptaibiotics could, to some extent, account for their action against plant and fungal hosts of the producing microorganisms.

Peptaibols and related peptaibiotics are composed of 5–20 amino acids, and amongst them there are several α-aminoisobutyric acid (Aib) moieties as a characteristic of these structures. Their structural diversity is caused by the varying amount and nature of the constituting amino acids, and different substitutions of the *N*- and *C*-terminus (see below). The name peptaibol was originally proposed by Benedetti *et al.* [13], but also by Brückner and co-workers [14] — independently of each other.

We recommend that the name should be used for linear peptide antibiotics that exhibit the following characteristics, thus expanding the original definition [15 and tables 3–4]:

(i) have a molecular weight between 500 and 2200 Dalton; (ii) show a high content of α-aminoisobutyric acid; (iii) are characterized by the presence of non-proteinogenic amino acids and (iv) possess an acetylated *N*-terminus, whereas the *C*-terminus is reduced to an amino alcohol.

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In 1991, Brückner *et al.* [16,17] introduced the comprehensive term peptaibiotic to include such components lacking the amino alcohol. To further expand their definition, considering recent findings in this area of research, we suggest the term peptaibiotic for linear peptide antibiotics that

(i) have a molecular weight between 500 and 2200 Dalton; (ii) show a high content of α-aminoisobutyric acid; (iii) are characterized the presence of non-proteinogenic amino acids and/or lipoamino acids and (iv) possess an acylated *N*-terminus, whereas the *C*-terminus may consist of a free or methoxy-substituted 2-amino alcohol, amine, amide, free amino acid, diketopiperazine or sugar alcohol [see tables 3–4].

A common feature of this heterogeneous class of substances is their biosynthesis via non-ribosomal pathways following the so-called 'thio-template mechanism'. This mode of biosynthesis enables the presence of unusual, non-proteinogenic amino acids, amino alcohols, lipoamino acids, amines and fatty acids in these structures [18,19].

Presently, the family of peptaibols and related peptaibiotics amounts to more than 300 members. For information on special compounds 'The Peptaibol Database' can be recommended which is available on the World Wide Web at: http://www.cryst.bbk.ac.uk/peptaibol/welcome.html [20,21]. However, searching for structures and producers of peptaibols and peptaibiotics is enabled, too, by other databases, such as the Chapman & Hall, Dictionary of Natural Products on CD ROM, and Antibase 2.0 [22]. An excellent review, summarizing related sub-families of peptaibols, has recently been presented by Chugh and Wallace [23]. Lipopeptaibol antibiotics were reviewed by Toniolo *et al.* [24]; and detailed information on mass spectrometry of leucinostatin antibiotics was given by Isogai *et al.* [25]. Due to the still growing number of peptaibiotics, recurrent isolations may occur frequently. Therefore, assistance in preventing re-isolations or disclosure of new structures may be welcome.

Mass spectrometric methods such as fast atom bombardment ionization (FAB-MS) including linked B/E-MIKES scan approaches, ESI-CID-MS/MS, ESI-CID-MS^n and MALDI-TOF-PSD-MS have been indispensable tools for the determination of molecular weights via the pseudo-molecular ions such as $[M+H]^+$ and $[M+Na]^+$ as well as sequence analysis via the generation of diagnostic fragment ions. Such ions can be generated by **c**ollision-**i**nduced **d**issociation (**CID**, synonymous: **c**one-**v**oltage **f**ragmentation, **CVF**) of the selected parent ion(s) using triple quadrupole or ion-trap mass spectrometers.

The present paper compiles data on the mass spectrometric behaviour of peptaibols and peptaibiotics including pseudo-molecular ions and the formation of diagnostic fragment ions. This compilation is intended to serve as a guide to a more rational identification of recurrent as well as novel representatives of these fungal peptides.

MICROORGANISMS PRODUCING PEPTAIBIOTICS

Peptaibols and related peptides (peptaibiotics) are biosynthesized exclusively by fungi, mainly soil-borne and plant-pathogenic, as well as fungicolous (also known as mycophilic) taxa. The latter occur on, as well as within, the fruit-bodies of asco- and basidiomycetes. Genera containing fungicolous species or fungicolous species themselves mentioned in this paper are marked with a superscript #.

More detailed information on modern taxonomic aspects of the producers of peptide antibiotics has been given [26–29].

Most of the structures reviewed here were isolated from *fungi imperfecti* belonging to the genera *Trichoderma*#, *Acremonium*#, *Paecilomyces*# and *Emericellopsis*#. Less commonly reported producers are listed in Table 1.

The occurrence of peptaibols such as boletusin [30] as well as the tylopeptins A and B [31] has been reported for a few basidiomycete fruit-bodies such as *Boletus* ssp. and *Tylopilus neofelleus*.

There are doubts, however, about the isolation of a peptaibiotic from the fruit-bodies of basidiomycetes. The authors making this claim pointed out the obvious structural homologies with the even co-isolated chrysospermins A–D [32], but they did not mention whether the fresh (in the case of boletusin) or dried material (in the case of the tylopeptins), used for extraction, showed any sign of decay or infection by mycophilic fungi.

In some cases, an infection of an asco- or basidiomycete fruiting-body by the genus *Sepedonium*# is easily recognized by the presence of large quantities of yellow aleurioconidia (synonym: chlamydospores), the colour of which is mainly due to the presence of sepedonin and its derivatives as well as skyrin [33]. However, the colour starts to appear relatively late, when masses of chlamydospores become mature. For that reason, it cannot be excluded that accidentally younger stages of infection were

Table 1 A Survey of the Less Frequently Reported Fungal Producers of Peptaibiotics

Name of peptaibiotic	Isolated from	Reference(s)
Chrysospermins	*Sepedonium chrysospermum*[#] (Teleomorph: *Hypomyces chrysospermus* (Bull.) Tul. Synonym: *Apiocrea chrysosperma*)	[32]
Peptaibolin	*Sepedonium* sp.[#]	[89]
Ampullosporins	*Sepedonium ampullosporum*[#]	[9,10]
Hypomurocins A and B	*Hypocrea muroiana*[#] Hino & Katsumoto	[90]
Some of the hypelcins	*Hypocrea peltata*[#] (Jungh.) Sacc.	[91]
Lipohexin, Texenomycin A	*Acremonium lindtneri* (Kirschstein) Samuels & Rogerson (Synonyms: *Moeszia lindtneri* (Kirschstein) G. Arnold *Cylindrocarpon lindtneri*); Teleomorph: *Sporophagomyces chrysostomus* [92] (Synonym: *Hypomyces chrysostomus* Berk. & Broome [93])	[67,68]
Clonostachin	*Clonostachys* sp.	[8]
Aibellin	*Verticimonosporium ellipticum* Matsushima	[63,64]
LP237-F5, -F7, -F8	*Tolypocladium geodes* W. Gams	[42,43]
Some of the Antiamoebins	*Stilbella fimentaria* [94] (*Synonym*: Stilbella erythrocephala) *Gliocladium catenulatum*[#] *Gilman & Abbott*	[94–96]
Stilboflavins	*Stilbella flavipes* (Peck) Seifert	[96]
Antiamoebin I	*Verticillium epiphytum* Hansford [97] (Synonym: *Cephalosporium pimprina*[#])	[76]
Antiamoebin I	*Clonostachys rosea* f. *catenulata* Gilman and Abbott [98] (Synonym: *Gliocladium catenulatum*[#])	[77]
Gliodeliquescin	*Gliocladium deliquescens*	[99]
Efrapeptins	*Tolypocladium inflatum* W. Gams (*Synonym: Tolypocladium niveum* (Rostrupp) Bisset) and other *Tolypocladium* species	[100]

not recognized macroscopically, thus leading to the extraction of infected material.

Moreover, it is well known from the literature [34] that a primary infection by *Sepedonium chrysospermum*[#], attacking its preferred hosts in the order *Boletales*, e.g. *Boletus, Paxillus, Tylopilus* and *Scleroderma* species, [for a review see: 33,35] normally causes total necrosis of the infected host cells. However, if 'weak pathogens', such as *Botrytis cinerea*[#] or *Trichothecium roseum*[#], invade fruit-bodies, previously colonized by *Sepedonium chrysospermum*, the latter can grow biotrophically within the mycelium of these so-called 'secondary parasites' [36].

NOMENCLATURE OF PEPTAIBOLS AND PEPTAIBIOTICS

The name peptaibol was introduced originally for small fungal peptides containing a high portion of the non-proteinogenic amino acid Aib (α-aminoisobutyric acid or α-methyl alanine; Figure 1). The Aib residues may number two, as in the pentameric peptaibolin from *Sepedonium* strains, or up to nine in some of the stilboflavins.

The term 'peptaibol' should be applied only to peptides having an acetylated *N*-terminus, and an 2-amino alcohol as *C*-terminus (see above).

X-Aaa-$(Aaa)_n$-AaaY

Figure 1 A general, simplified building scheme of the peptaibiotics. n = in the range of 3 [89] to 19 [106] residues, containing a high proportion of Aib as well as other non-proteinogenic amino acids; Xaa = *N*-terminal amino acid substituted with unbranched, or 2- or 4-methyl branched, saturated or unsaturated fatty acid; Yaa = substituent covalently linked to the *C*-terminal amino acid: free or methoxy-substituted amino alcohol, amine, amide, free amino acid, diketopiperazine, or sugar alcohol. A peptaibol synthetase from *Trichoderma virens* has recently been cloned [107].

Structurally varying compounds such as lipopeptaibols have been named 'peptaibiotics' [16,17]. Variations of *N*-acetylated peptaibol-type substances concern a heterologous *C*-terminal moiety, such as a mannitol residue [clonostachin: 8], a free carboxy group formed by glycine [XR 586: 37], glutamine, valine [16,17] or serine [cephaibols P and Q: 2], a methoxylated Aib [NA VII: 38], L-prolineamide, or a diketopiperazine such as *cyclo*-L-Pro-Aib [pseudokonins KL III and KL VI: 39].

In the so-called lipopeptaibols, the *N*-terminus is substituted by a fatty acid with more than four carbons [38] and the *C*-terminus is substituted by an amino alcohol. This group comprises trichogin GA IV [38], the trikoningins KB I and KB [40], trichodecenins TD-I and TD-II [41] as well as LP237_F7 and LP237_F8 [42,43].

In the subfamily of lipoaminopeptides (also reported as aminolipopeptides) the *N*-terminus is substituted by long-chain, α-methyl-branched fatty acids (Figure 1). An L- proline-, L-4-hydroxy-proline or *cis*-4-methyl-L-proline residue is found in position 2 of the peptide chain, and in most cases it is followed by a lipoamino acid residue in position 3. To our present knowledge, this compound, 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid (AHMOD), has only been recorded from this subfamily.

In some of the leucinostatins the AHMOD moiety is replaced by L-Leu or L-Val [25,44–46]. The *C*-terminus of leucinostatin is substituted, too, by an amino alcohol (cf Table 2).

It should be mentioned that the primary structures of some leucinostatins remain uncertain, because the assignment of structures by LC-Frit-FAB-MS/MS was not entirely conclusive [25].

Up to now, the following lipoaminopeptides have been published in the literature:

- 22 leucinostatins from different strains of *Paecilomyces marquandii*[#] (Massee) Hughes and *Paecilomyces lilacinus*[#] (Thom) Samson [5,25,47–53]

Table 2 Reference Masses of the Amino Acid Residues Occurring in Peptaibiotics ($[M + H - H_2O]^+$)

Amino acid	Abbreviation		Monoisotopic molecular weight	Molecular formula of the residue
	3-letter code[a]	1-letter code[b]		
Alanine	Ala	A	71.0371	C_3H_5NO
α-Aminoisobutyric acid	Aib	U	85.0528	C_4H_7NO
2-Amino-4-methyl-6-hydroxy-8-oxo-decanoic acid	AHMOD	—	213.1365	$C_{11}H_{19}NO_3$
Asparagine	Asn	N	114.0429	$C_4H_6N_2O_2$
Aspartic acid	Asp	D	115.0269	$C_4H_5NO_3$
α-Ethyl-norvaline	EtNor	Z	127.0997	$C_7H_{13}NO$
Glutamine	Gln	Q	128.0586	$C_5H_8N_2O_2$
Glutamic acid	Glu	E	129.0426	$C_5H_7NO_3$
Glycine	Gly	G	57.0215	C_2H_3NO
β-Hydroxyleucine	Hyleu	—	129.0790	$C_6H_{11}NO_2$
4-Hydroxyproline	Hyp	O	113.0477	$C_5H_7NO_2$
Isoleucine	Ile	I	113.0841	$C_6H_{11}NO$
Isovaline	Iva	J	99.0684	C_5H_9NO
Leucine	Leu	L	113.0841	$C_6H_{11}NO$
4-Methylproline	MePro	—	111.0684	C_6H_9NO
Phenylalanine	Phe	F	147.0684	C_9H_9NO
Pipecolic acid	Pip	—	111.0684	C_6H_9NO
Proline	Pro	P	97.0528	C_5H_7NO
Serine	Ser	S	87.0320	$C_3H_5NO_2$
Tryptophan	Trp	W	186.0793	$C_{11}H_{10}N_2O$
Tyrosine	Tyr	Y	163.0633	$C_9H_9NO_2$
Valine	Val	V	99.0684	C_5H_9NO

[a] In some cases, the abbreviations commonly used for non-proteinogenic amino acids do not match the 3-letter code.
[b] Note, that there is no 1-letter code for some of the non-proteinogenic amino acids.

Table 3 Modifications of the *N*- and *C*-terminus in Peptaibol-type Antibiotics

N-terminus			*C*-terminus		Name of compound (selected examples)	MW of compound $(M)^+$
Fatty acid moiety (abbr., MW)	Modified amino acid	MW (m/z)	Structure (abbr.)	MW[a] (m/z)		
Acetyl- (Ac; 43)	Ala	114	Phenylalaninol (Pheol)	150	Trichoaureocin 1a [101]	
	Aib	128	Phenylalaninol (Pheol)	150	Longibrachins LGA, LGB; atroviridins	
			Valinol (Valol)	102	Harzianin Pc_u4	1401
			Tryptophanol (Trpol)	190	Trichorzins PA	
			Esterified mannitol	165	Clonostachin	1667
			Leucinol (Leuol)	117	Trikoningin KA V, other hypelcins	
			Isoleucinol (Ileol)	117	Hypelcins A-V, -VI; Stilboflavins SF B 9, 10; TrichovirinTV_XIIa [102]	1927; 1913
			Prolineamide (Pro-NH_2)	114	Pseudokonin KL III	1074
			cyclo-Aib-L-Prolinal	183	Pseudokonin KL VI	1045
			Aib-OCH_3	99	NA VII	
			2-[(2-Amino-3-phenylpropyl)amino]ethanol	183	Aibellin	2000
			Glutamine (Gln)	128	Trichobrachin TB I A, B, C and D; Trichobrachin TB IIa C and D [109][a]	
			Valine (Val)	99	Trichobrachin TB IIa A and B [109][a]	
	Val	142	Phenylalaninol (Pheol)	150	Bergofungins; antiamoebins XII, XIV	
	Iva	142	Leucinol (Leuol)	117	Hypomurocin HM A-2	1174
	Leu	156	Phenylalaninol (Pheol)	150	Peptaibolin; antiamoebins XV, XVI	589; 1622; 1636
	Phe	190	Tryptophanol (Trpol)	190	Chrysospermins; boletusin; peptavirin B	
			Phenylalaninol (Pheol)	150	Other cephaibols; other antiamoebins	
			Serine (Ser)	104	Cephaibols P, Q	1872; 1856
	Trp	230	Leucinol (Leuol)	117	Ampullosporins; tylopeptins	
			Phenylalaninol (Pheol)	150	Zervamicins	
			Glycine	71	XR586	1878
	Pip	154	Pipecolic acid (Pip)	Different	Efrapeptins [108]	

(*continued overleaf*)

Table 3 (*Continued*)

N-terminus			*C*-terminus		Name of compound (selected examples)	MW of compound $(M)^+$
Fatty acid moiety (abbr., MW)	Modified amino acid	MW (m/z)	Structure (abbr.)	MW[a] (m/z)		
n-Octanoyl- (Oc, 127)	Aib	212	Leucinol (Leuol)	117	Trichogin GA IV; trikoningin KB I; LP237_F5, LP237_F8	1065; 1037; 1341; 1325
	Iva	226	Leucinol (Leuol)	117	Trikoningin KB II	1051
n-Decanoyl- (Dec, 155)	Aib	240	Leucinol (Leuol)	117	LP237_F7	1311
cis-4-Decenoyl- (155)	Gly	210	Leucinol (Leuol)	117	Trichodecenins-TD_I and—TD_II	751
(4S,2E)-4-Methylhex-2-enyl- (MeHA, 111)	Pro	208	MPD, DPD or DPD-N-oxide[b]	87; 101 or 117	Leucinostatins L; T	1189; 1089
	cis-4-methyl-L-Pro	222	MPD, DPD or DPD-N-oxide[b]	87; 101 or 117	Most of the other leucinostatins	
2-Methyloctanoyl- (MOA, 141)	Pro	238	AAE or AMAE[b]	119/133	Helioferins; Roseoferins D_2, D_3, E, F	1121; 1135
2-Methyldecanoyl- (MDA, 169)	Pro	266	AMAE (trichopolyns); AAE or AMAE (roseoferins)	119/133	Trichopolyns I (or A), II (or B); Roseoferins A, B, C, D_1, G[a]	1206; 1192
	L-4-Hyp	282	AAE or AMAE	119/133	Roseoferins H; I; K	1179; 1165;1151
	cis-4-methyl-L-Pro	280	MPD, DPD or DPD-N-oxide		Acremostatins A; B; C	1262; 1276; 1292
3-Hydroxy-2-methyldecanoyl- (HMDA, 185)	Pro	282	AMAE (trichodiaminol)	133	Trichopolyns III; IV; V	1192; 1178; 1222
β-Keto-2-methyl-tetradecanoyl- (MOTDA, 239)	Pro	336	Free β-alanine (β-Ala)	89	Lipohexin	765
	Pro	336	Arginol (Argol)	160	Texenomycins A, B	2028

[a] The molecular weight is calculated from the $[M + H]^+$ ion (m/z). All *C*-terminal residues tabulated were demonstrated to possess the *S*-configuration (L-isomers).
[b] Trichobrachins TB III A and TB III B are hexapeptides, the *N*-terminus of which has not been assigned yet. Remarkably, they no longer contain Aib.
[c] MPD: N^1-methyl-propane-1,2-diamine; DPD: N^1,N^1-dimethyl-propane-1,2-diamine; DPD-NO: (2S)-N^1,N^1-dimethyl-propane-1,2-diamine-N-oxide;
AAE: 2-(2′-aminopropyl)-aminoethanol; AMAE: 2-(2′-aminopropyl)-N-methylamino-ethanol. For detailed information on structure assignment of selected compounds see [19,53–55]. In such cases where no $[M]^+$ peak is tabulated the reader is directly referred to the original reference(s).

Table 4 Non-proteinogenic amino acids[a] found in peptaibiotics

Non-proteinogenic amino acid (Abbr.)	Name of the peptaibiotic
α-Aminoisobutyric acid (Aib)	Characteristic feature of all peptaibiotics
L-Isovaline (Iva)	Bergofungins A, B and D; peptavirins A and B; clonostachin in position 7 and 10; some efrapeptins
D-Isovaline (Iva)	Harzianins HC III, HC VIII, HC IX, HC XII, HC XIII, HC XV; atroviridins; tylopeptin A; boletusin; XR586; trichokindins I–VII [91], clonostachin in position 4 and 13, antiamoebins in different positions (except for antiamoebin VI, which does not contain Iva)
cis-4-L-Hydroxyproline (Hyp)	Bergofungins [103,104], clonostachin; XR586; zervamicins; Roseoferins H, I and K
trans-4-L-Hydroxyproline (Hyp)	Heptaibin [105], antiamoebins, emerimicins
cis-4-L-Methylproline (MePro)	Most of the leucinostatins;[b] acremostatins[c]
β-Hydroxy-L-leucine (Hyleu)	Most of the leucinostatins; acremostatins
β-Alanine (β-Ala)	Efrapeptins [109], leucinostatins, acremostatins, lipohexin, texenomycins
L-Pipecolic acid (Pip)	Efrapeptins A–H
α-Ethyl-norvaline (Etnor)	LP237_F5 and LP237_F8
2-Amino-4-methyl-6-hydroxy-8-oxo-decanoic acid (AHMOD)	All lipoaminopeptides, exceptions are mentioned in the text (see above)

[a] Unless otherwise indicated amino acids possess *S*-configuration (L-isomers).
[b] The structure of a number of leucinostatins has not been completely assigned, plausible sequences were tabulated by Isogai *et al.* [25].
[c] The optical configuration of the amino acids of the acremostatins could not be analyzed.

as well as the conjugate structure leucinostatin-β-O-di-glucoside [54] isolated from the endophyte *Acremonium* sp. Tbp-5 [55].

- five trichopolyns from *Trichoderma polysporum*[#] (Link ex Pers.) Rifai TMI 60146 [56–58];
- two helioferins from *Mycogone rosea*[#] Link DSM 8822 [59].
- and 16 roseoferins from *Mycogone rosea*[#] Link DSM 12973 [60]. Another nine novel roseoferins (H_1–H_4; I_1,I_2; K_1–K_3) were obtained by replacing the L-Pro moiety of the corresponding MDA-containing roseoferins by L-Hyp via 'precursor-directed biosynthesis' [61].
- and three acremostatins formed by co-cultivation of *Mycogone rosea* DSM 12973 and *Acremonium* sp. Tbp-5 [62].

There are five compounds that build a bridge between peptaibols and lipoaminopeptides at present.

Aibellin [63,64], one of these compounds, carries an *N*-terminal Aib, which is acetylated, whereas the *C*-terminus is an amino alcohol, 2-[(2-amino-3-phenylpropyl)amino]ethanol. Lipohexin [65,66], and texenomycins A and B [67,68], are *N*-terminally substituted by a 2-methyl-3-oxo-tetradecanoyl moiety (MOTDA). The *C*-terminus of lipohexin representing a partial structure of texenomycins is formed by β-alanine. The 20meric texenomycins A and B carry an L-arginol residue as the *C*-terminus. Both lipohexin and texenomycin were co-isolated from cultures of *Acremonium* (*Moeszia*) *lindtneri* DSM 11119; but texenomycin A and its C-2-epimer texenomycin B (which is rapidly converted into A under basic conditions) by contrast, were originally claimed to be obtained from *Scleroderma texense* [62]. This puffball, which was recently classified as a member of the *Boletales* [69–71], is occasionally infected by species of *Sepedonium* (W. Gams, personal observation).

The occurrence of a free serine residue as the *C*-terminus was reported for cephaibols P and Q [2].

The basic amino acids histidine and lysine as well as the sulphur-containing amino acids cysteine and methionine, have not been found as yet in the peptaibiotics.

L-Serine occurs in a few peptaibols and related structures. Examples are harzianin HC I, III, XI, XII, and XIII [72]; trichorzin PA II, IV, V, VI, VII, VII and XI; PA_U4 and PC_U4 [73,74]; trichokindins I–VII [75]; chrysospermins [32] and tylopeptins [30,31].

L-Threonine has to date only been described as a constituent of some emerimicins and zervamicins, and as occurring in the cephaibols P and Q [2; 76,

77]. However, to date, L-tyrosine has been found only in LP237_F5 [41].

The chirality of amino acids in the peptaibols and peptaibiotics was determined to be L in most cases. A common method for making this determination is derivatization of amino acids in the hydrolysate with N_α-(2,4-dinitro-5-fluoro-phenyl)-1-L-alanine-amide (FDAA, Marfey's reagent), followed by HPLC separation and comparison of the retention times with those of derivatized standard amino acids [78]. However, classical GC/MS approaches have also been used in amino acid analysis [37,39,72].

MASS-SPECTROMETRIC METHODS FOR STRUCTURAL CHARACTERIZATION

Sequencing of peptaibols and peptaibiotics with a molecular weight up to 2000 Dalton can be furnished by classical (FAB-MS) and modern methods of tandem mass spectrometry (ESI-CID-MS/MS, ESI-CID-MS^n, MALDI-TOF-PSD-MS).

In the case of high-energy-ionization (FAB-MS), a remarkable number of rather non-specific fragment ions are observed in addition to $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$. It appears important to mention that different CID methods will generate different types of fragments. Hence, the simultaneous use of different MS methods is recommended.

According to the original Roepstorff nomenclature [80] which was revised by Biemann [81], six series of fragment ions can be generated during cleavage of a peptide bond, corresponding to *C*-heteroatom- and α-bond cleavages. The resulting positive fragment ions are classified as *a*-, *b*-, and *c*-type if the positive charge remains on the *N*-terminal fragment. However, if it remains on the *C*-terminus, the fragments generated are characterized as *x*, *y* and *z*. Even under high energy ionization conditions, *b*-type fragments are the most commonly produced while the others are weak or even not observable.

The interpretation of spectra generated by high-energy ionization (EI, FAB) is often hampered by the presence of an additional series of secondary or tertiary fragment ions, thus complicating the subsequent structural assignment.

Soft ionization techniques such as electrospray ionization (ESI or MALDI) will usually not generate fragments. Such spectra almost exclusively show pseudo-molecular ions such as $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, $[2M + H]^+$ and $[2M + Na]^+$. These single ions can be activated subsequently by collision with argon or helium gas in collision cells. Some of the modern HR-ESI-Qq-TOF instruments use highly purified nitrogen. In the case of FT-ICR/MS instruments the collision gas is sometimes substituted by soft-laser pulses inducing fragmentation. This method causes a low-energy fragmentation, which is referred to as CID-MS/MS and CID-MS^n.

Fragments of the B_n-type- ($[M - H_2O]^+$) are thereby produced with a bias toward the positive ion mode, i.e the charge remains on the *N*-terminus after cleavage of the peptide bond. The presence of leucine or isoleucine will lead, for example, to a *b*-type fragment of m/z 113, whereas the occurrence of valine or isovaline in the peptide chain will be signalled by a corresponding fragment of m/z 99. Thus, in comparison with FAB-MS, CID-MS/MS enables a more precise assignment of fragments arising from a defined pseudo-molecular ion. However, there is an obvious disadvantage of ESI-CID-MS/MS of larger peptides in comparison with FAB-MS: In most cases, not all of the possible *b*-type fragments will be visible.

The possibility of generating further generations of daughter ions (MS^n) using electric or magnetic ion trap (IT) analysers enables both the detection and the subsequent sequencing of positional isomers (see below). In contrast to what occurs in linear quadrupole analysers, but much like that seen in ICR, the ions within an ion trap rotate for microseconds on stable orbits in a high-frequency field of alternating current, adjacent to a ring electrode. By varying the amplitude of the current a selective measurement of the mass of ions can be achieved, which thus are forced to leave their orbits in a stepwise manner. The real advantage of ion trap mass spectrometers is the generation of up to ten generations of daughter-ions. For instance, every MS^2 daughter ion generated by collision (mostly with helium) can be fragmented separately, thus generating MS^3 grand-daughter ions. The further fragmentation of these ions will result in MS^4 great-grand-daughter ions and so on [82–86].

DISCUSSION

Tandem-MS methods, especially spray ionization or MALDI coupled to quadrupole, ion trap or TOF analysers have been proven useful for assignment of structure in studies of peptaibols and peptaibiotics. In spite of the remarkable structural diversity of these substances a series of general and repeating

structural elements will often be present enabling the identification of a given structure as a member of the peptaibiotics.

The analysis of saturated fatty acyl moieties occurring in lipopeptaibols and lipoaminopeptides often requires additional ESI-'In-Source'-fragmentation experiments.

1D- and 2D-NMR studies are also needed to distinguish between isoleucine or leucine as a part of a given molecule. For fatty acid analysis, GC/MS-, EI/MS and NMR investigations of the etheric or $CHCl_3$ extracts of the hydrochloric acid hydrolysate are indispensable.

In some cases, *b*-type fragmentation is rather weak. For instance, the presence of an Aib-Pro bond often suppresses formation of b_n-acylium ions. According to Rebuffat *et al.* [72], this tertiary amide bond undergoes a preferential cleavage, leading to an *N*-terminal acylium ion N^+, and a di-protonated *C*-terminal ion. This pattern of cleavage results in the absence of fragment ions at higher masses and the superimposition of two independent b_n-type series at lower masses, starting from the N^+ and also from the di-protonated *C*-terminal ions. To overcome this problem, the use of the so-called 'pulsar function' was proven to be beneficial. This function was recently developed for ESI-QqTOF-MS/MS instruments to collect, firstly, ions of a defined m/z range or even a single mass and to subsequently release the 'ion package', thus remarkably enhancing the sensitivity of the instrument. The superiority of this technique was recently shown for the peptaibols of the trichofumin family [87]. Also, the presence of a saturated *N*-terminal fatty acid moiety next to a proline or proline-derived residue will generally induce the formation of the corresponding a-type fragment while suppressing the b_1-fragment ion [59–62]. The presence of a β-hydroxy amino acid within the peptide chain may favour the formation of rather intense $[b_n\text{-}H_2O]^+$-ions.

No cleavage between the β-alanyl residue and the *C*-terminal propane-amine moieties was observed in the case of leucinostatins (reviewed by Isogai *et al.*, [25]) and acremostatins [62]. This could be ascribed to the presence of a non-α-peptide linkage.

Last but not least, the mass spectrometric behaviour of 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid (AHMOD) [49,88] as a constituent of leucino- and acremostatins, trichopolyns, helioferins and roseoferins must be discussed briefly. ESI-MS/MS and ESI-MS^n of these lipoaminopeptides are characterized by the loss of a side chain from the AHMOD moiety (m/z 72) as a result of an α-cleavage. This diagnostic feature leads to the formation of a rather intense fragment ion $[M + H\text{-}C_3H_5O]^+$. Acid hydrolysis of lipoaminopeptides with HCl or H_2SO_4 and subsequent analysis of the hydrolysate by ESI-MS produces 4-methyl-6-(2-oxobutyl)-2-piperidine carboxylic acid (MOBPA) from AHMOD by dehydration followed by a Michael-addition and subsequent cyclization. Thus, a characteristic fragment with m/z 214 is visible in the positive ESI mass spectrum [52,56–58,59–62,88].

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Isolation, Structure Elucidation and Biological Activities of Trichofumins A, B, C and D, new 11 and 13mer Peptaibols from *Trichoderma* sp. HKI 0276‡

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Abstract: Trichofumins A–D were isolated from cultures of *Trichoderma* sp. HKI 0276 as new 11 and 13mer peptaibols. Similar to 15mer peptaibols they promote morphogenesis of the fungus *Phoma destructiva* and cause hypothermia in mice as a characteristic of neuroleptic activity. Membrane measurements using a synthetic BLM model showed that A, B, C and D increased membrane permeability for cations in a similar manner as was shown for larger peptaibols but with comparably lower efficiency. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: trichofumins; 11 and 13mer peptaibols; structure elucidation; effects on fungal morphogenesis; pigment formation; membrane activity

INTRODUCTION

Peptaibols are helical peptides forming ion-penetrable channels in artificial bilayer membranes [1,2]. They owe this unique property to the presence of hydrophobic amino acids such as α-aminoisobutyric acid (Aib), an acetylated nitrogen terminus and an alcoholic instead of a carboxylic acid group at the *C*-terminus. As an explanation for their biological activities membrane channel formation was established for the 15–20mer peptaibols [1–6]. Peptaibols with >18 amino acids were subjected to various studies of their molecular conformation, ion channel formation and biological activities [3]. However, little information is available on the 'smaller' peptaibols such as 10–14mer representatives (c.f. the trichorozins [4]).

In the course of our continuing search for new peptaibols, *Trichoderma* sp. HKI 0276 was recently disclosed as a producer of the new peptaibols trichofumins A–D (Figure 1). The structures of A–D were elucidated by mass spectrometry (HRESI-MS, ESI-CID-MS/MS and ESI-QqTOF-MS/MS) as will be shown below. Moreover, the biological activities of A–D and their interactions of A–D with an artificial bilayer membrane are reported.

MATERIAL AND METHODS

Compounds A–D were isolated from cultures of *Trichoderma* sp. HKI 0276. The strain was cultivated

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A Acetyl-Val-Gln-Leu-Val-Aib-Pro-Leu-Leu-Aib-Pro-Leuol

B Acetyl-Val-Gln-Leu-Leu-Aib-Pro-Leu-Leu-Aib-Pro-Leuol

C Acetyl-Val-Gln-Val-Aib-Gln-Gln-Leu-Leu-Pro-Leu-Aib-Pro-Leuol

D Acetyl-Val-Gln-Leu-Aib-Gln-Gln-Leu-Leu-Pro-Leu-Aib-Pro-Leuol

Figure 1 Amino acid sequences of trichofumins A, B, C and D.

as a surface culture (60 l) at 26 °C in 500 ml Erlenmeyer flasks containing 100 ml malt medium [5,6] composed as follows (g/l): malt extract 20, glucose 10, yeast extract 1, $(NH_4)_2SO_4$ 5, pH 6.0.

After 20 days of cultivation at 26 °C, the culture broth was harvested and separated by filtration. Thereafter, the culture filtrate and the mycelium were extracted twice with ethyl acetate. The combined extracts were dried and evaporated. The residue (3 g) was subjected to silica gel chromatography (silica gel 60, Merck, 0.063–0.1 mm, column 600 × 40 mm, $CHCl_3$–MeOH, 9 : 1 v/v), and 20 ml portions were collected. Fractions containing trichofumins A–D were detected by ESI-MS. Final purification was done by isocratic preparative HPLC (Spherisorb ODS-2, 5 µm, RP_{18}, Promochem, 250 × 25 mm, acetonitrile H_2O, 83 : 17 v/v; 12 ml/min, 210 nm).

Antimicrobial Activities

The antimicrobial activity of the new peptaibols A–D was determined against several bacteria and fungi by the agar plate diffusion assay [7]. The inducing effect on pigment formation by *Phoma destructiva* was investigated as reported earlier [8,10].

Assay of Hypothermia in Mice (Neuroleptic Effect)

The neuroleptic activity of the new peptaibols was investigated according to Thompson [9,10]. Solutions of peptaibols were administered intraperitoneally in male mice (strain NMR) obtained from the Central Breeding Laboratory for Animals, Beutenberg Campus Jena, in a dosage of 20 mg/kg body weight. The decrease of body temperature (less 2–6 °C) was recorded 30 min after administration. The effect was in the same order of magnitude as was described for chlorpromazine, a known neuroleptic agent [10]. The basal body temperature was measured by a thermistor probe placed in the rectum of mice until a stable temperature was indicated on the thermometer. The colon temperature was recorded 0.5, 1, 3, 5, 7 and 24 h after administration of the trichofumins A–D.

Measurements Employing Lipid Bilayer Membranes

Planar bilayer lipid membranes (BLM) were prepared from soya bean phosphatidylcholine (Sigma, P5638) 20 mg/ml in *n*-heptane [11].

The measuring glass cell (25 ml of total volume) was equipped with a teflon cylinder (1 cm diameter), which contained a hole of 0.5 mm diameter to harbour the BLM. The membranes were formed by painted method on the hole of the teflon cylinder. Formation of the BLM was controlled by the use of a binocular microscope. Both the measuring cell (10 ml outside (*cis*)-volume) and the inner side of Teflon cylinder (*trans*-volume; 1 ml) were filled with a solution of potassium chloride ranging from 100 to 1000 mM depending on the type of experiment.

The membrane current was measured by the voltage-clamp method [12]. The current measuring device consisted of an operational amplifier model Keithly.301 (USA). The amplitude current noise of the amplifier was less than 10^{-13} A in the frequency range of 0.1–20 Hz. Then 1–10 µl of the stock solutions of the peptaibols (0.1–1 mg/ml in methanol) was added into the glass chamber containing the teflon cylinder with a bilayer membrane. The solution was mixed for 5 min by a magnetic stirrer at 500 rotations/min. The progress of the bilayer formation and estimation of its area was monitored on the screen of the PC by the amplitude of the membrane capacitance currents in response to the rectangular shape voltage pulses applied to the membrane. Measurements of the membrane currents corresponding to the applied peptaibol concentration were done 5 min after termination of the process of black membrane formation marked by the constant amplitude of the membrane capacitive current.

RESULTS AND DISCUSSION

The physicochemical properties of the new peptaibols, trichofumins A, B, C and D are shown in Table 1. The structures of A–D (Figure 1) were elucidated by HRESI-MS (Finnigan MAT 95 XL, Finnigan, Bremen, Germany; showing for trichofumin A: m/z 1189.7918 ($([M+H]^+$), calcd. 1189.7929 for $C_{59}H_{105}N_{12}O_{13}$); trichofumin B: m/z 1203.8096 ([M + H], calcd. 1203.8081 for $C_{60}H_{107}N_{12}O_{13}$); trichofumin C: m/z 1444.9026

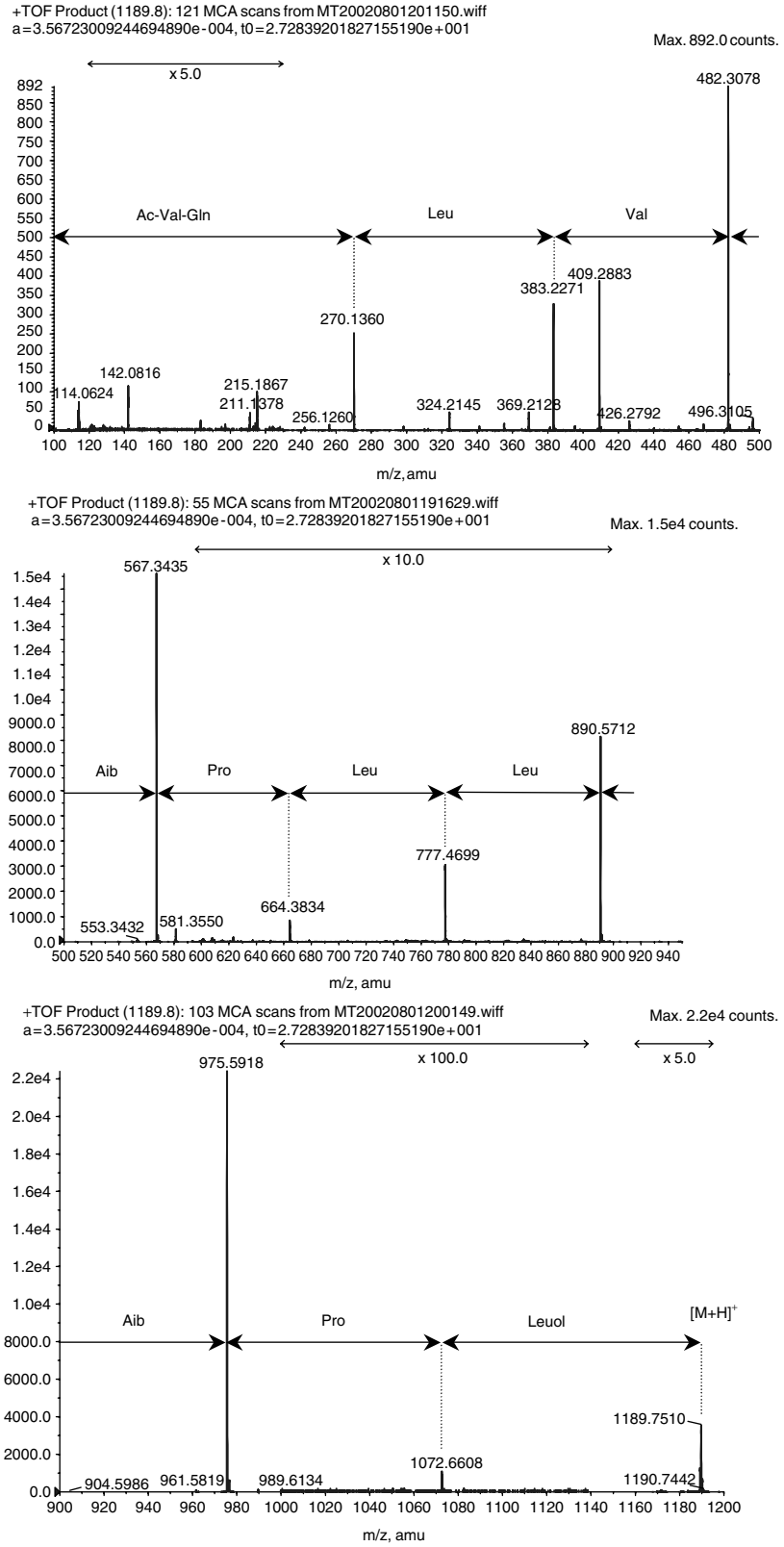

Figure 2 Diagnostic fragment formation by trichofumin A during ESI-QqTOF-MS/MS.

Table 1 Physico-chemical properties of trichofumins A–D

Trichofumin	A	B	C	D
Appearance	White solid	White solid	White solid	White solid
Melting point	157–160 °C	162–164 °C	167–169 °C	178–180 °C
HRESI-MS (m/z)	1189.7918	1203.8096	1444.9026	1458.9163
$[M+H]^+$	calcd. 1189.7929	calcd. 1203.8081	calcd. 1444.9017	calcd. 1458.9174
Molecular formula	$C_{59}H_{105}N_{12}O_{13}$	$C_{60}H_{107}N_{12}O_{13}$	$C_{69}H_{120}N_{16}O_{17}$	$C_{70}H_{122}N_{16}O_{17}$
$[\alpha]_D^{25}$ (MeOH, 3 mg/ml)[a]	—	−1.6°	−3.7°	−4.2°
R_t on HPLC (min)[b]	15.1	15.6	14.7	14.5

[a] Propol polarimeter (Dr Kernchen, Seelze, Germany).
[b] Nucleosil 100-5, RP18, 250 × 4.6 mm, 1 ml/min, 210 nm gradient acetonitrile/H_2O trifluoroacetic acid.

($[M+H]^+$, calcd. 1444.9017 for $C_{69}H_{120}N_{16}O_{17}$); and trichofumin D: m/z 1458.9163 ($[M+H]^+$, calcd. 1458.9174 for $C_{70}H_{122}N_{16}O_{17}$). The sequence of amino acids in A–D was deduced from ESI-CID-MS/MS (Triple Quadrupole Mass Spectrometer Quattro (VG Biotech, Altrincham, England) and ESI-Qq-TOF-MS/MS (API QStar Pulsar, Hybrid Quadrupole-TOF mass spectrometer, Applied Biosystems, MDS Sciex, USA) due to the diagnostic B-type fragments arising from cleavage of the amide bonds. An example for structure elucidation of A–D is depicted in Figure 2.

Hydrolysis of A–D, derivatization of the amino acids by Marfey's reagent [18] and HPLC-analysis

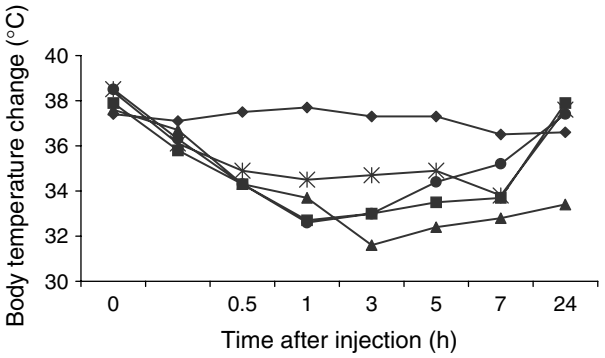

Figure 4 Induction of hypothermia in mice by A–D (♦ control; ■ trichofumin A; ▲ trichofumin B; ● trichofumin C; ∗ trichofumin D).

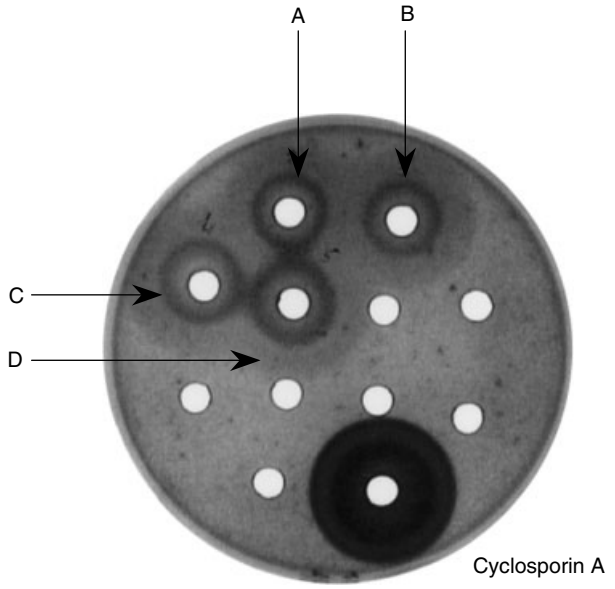

Figure 3 Effect of trichofumins A–D on morphogenesis of *Phoma destructiva* (70 h incubation, 1 mg/ml (MeOH), 23 °C). Cyclosporin A was used as standard.

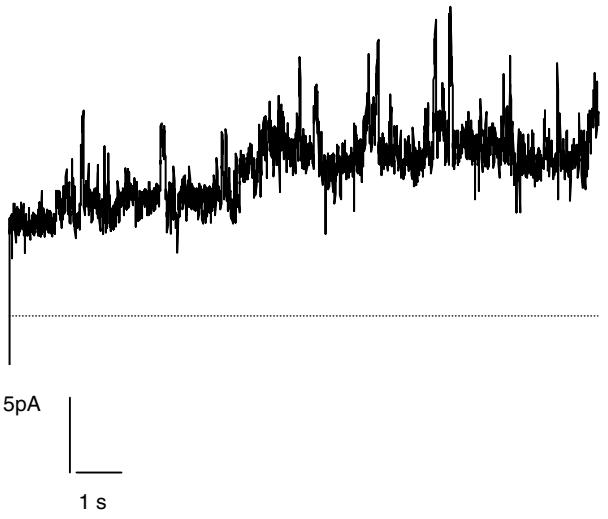

Figure 5 Trace of the membrane current in time in presence of trichofumin A (0.3 μM). Conditions: 500 mM KCl, membrane voltage 170 mV. Note characteristic pulse shape of the current corresponding to the opening and closing of the discrete ion conducting pores.

of the derivatives showed the presence of L-valine, L-leucine, L-glutamine, L-proline, L-leucinol and α-aminoisobutyric acid (Aib).

Compounds A–D thus represent new members of the 'smaller' peptaibols containing 11–14 amino acids, c.f. trichorozins from *Trichoderma harzianum*, trichogin-A-IV from *Trichoderma longibrachiatum* and trichorovins from *Trichoderma viride* [4]. Trichofumins A–D displayed moderate antibacterial activity against Gram-positive bacteria such as *Bacillus subtilis* ATCC 6633 in a concentration of >50 μg/ml during the agar well diffusion assay [17]. No activity was found against fungi and yeasts in the same concentration.

Administration of A–D to surface cultures of the phytopathogenic fungus *Phoma destructiva* caused an accelerated morphogenesis of hyphae as was indicated by earlier onset of brownish pigmentation. The effect of A–D on this fungus depending on the incubation time is shown in Figure 3. The pigment formation was comparable to the standard inducer cyclosporin A after 70 h.

Up to now little information has been available on the biological properties of the smaller peptaibols. Compounds A–D caused hypothermia in mice after intraperitoneal application in the same range of concentration and order of magnitude as the 15mer peptaibols ampullosporin A, B and D [8]. This effect can be taken as a measure of neuroleptic activity [9,10]. The vital functions (shock, climbing, refusal) were altered strongly after application of A, B, C or D. The decrease of body temperature after

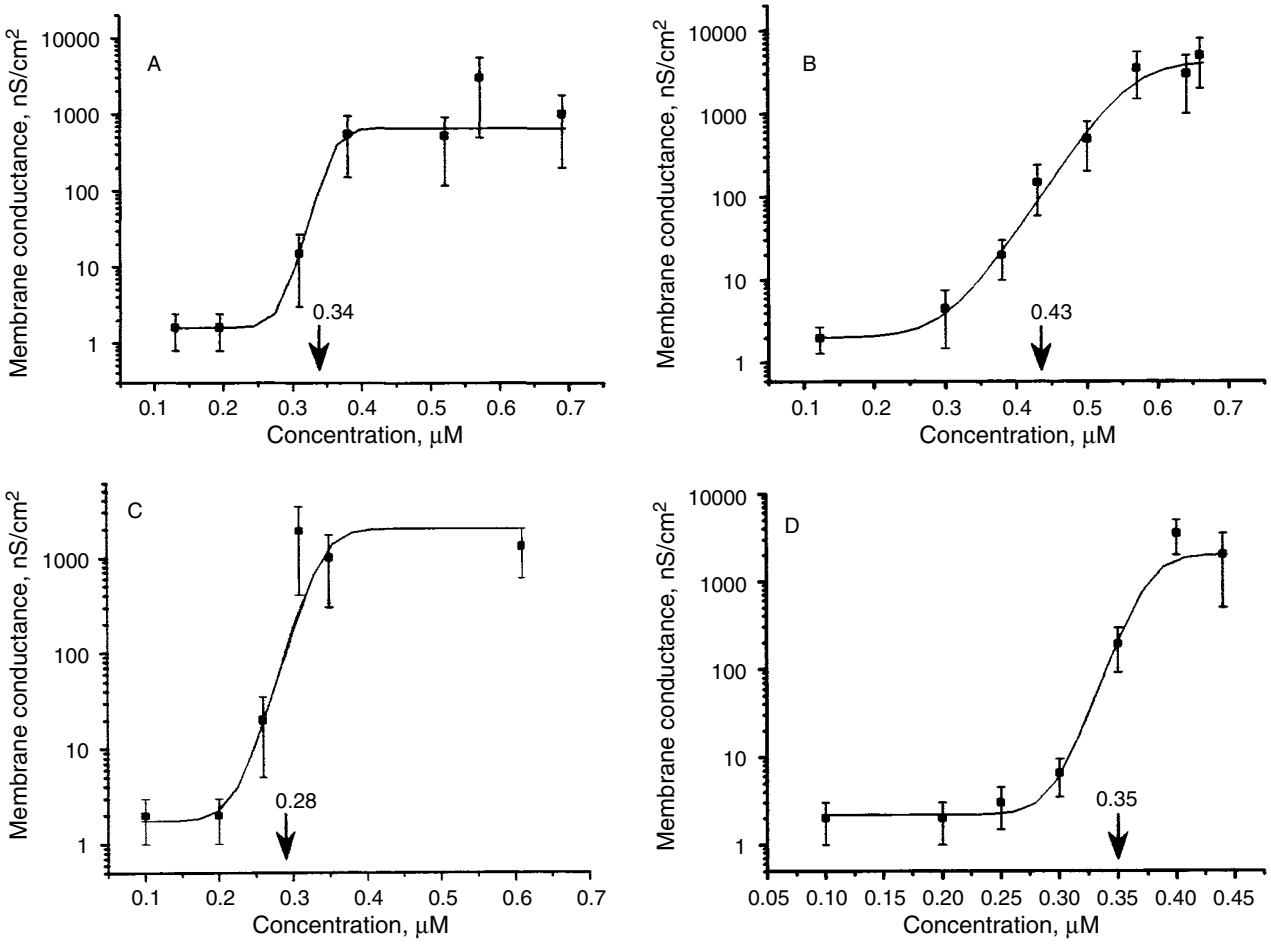

Figure 6 Membrane conductance as a function of the peptaibol concentration: (a) for trichofumin A, (b) for trichofumin B, (c) for trichofumin C and (d) for trichofumin D. The functions are saturated at about the same level of the membrane conductance 1000–2000 nS/cm^2, and the midpoints between two levels of the sigmoids corresponding to the relative effectiveness of the peptaibols are: 0.34 μM for trichofumin A, 0.43 μM for trichofumin B, 0.28 μM for trichofumin C and 0.35 μM for trichofumin D.

intraperitoneal dosage of A–D (20 mg/kg) is depicted in Figure 4.

Compounds A–D exerted these effects on mice and the fungus *Phoma destructiva* although the sequences of amino acids are different from those of the ampullosporins A, B and D. There are visible variations in the activities of A, B, C and D indicating that changes of the constituting amino acids caused alterations of biological activity.

Trichofumins A–D were shown to interact with artificial bilayer lipid membranes suggesting the formation of unstable pores as was reported for other peptaibols [13]. Figure 5 shows traces of membrane current measured in the presence of compound A suggesting that pores with a short life-time were formed. Almost identical pictures were observed with compounds B, C and D. The increase of electric conductivity of the bilayer membrane was dependent on the concentrations of peptaibols A, B, C or D which were given to the *cis*-side of the membrane (Figure 6).

Thereby, the 13mer peptaibols C and D displayed membrane effects comparable to the 11mer peptaibols A and B. The results are compatible with the view that even 11–13mer peptaibols are capable of disturbing the integrity of synthetic bilayer membranes by forming pores or channel-like aggregates. However, their efficiency and channel stability is lower than observed with the 15–20mer representatives of the peptaibol family [4,13]. No membrane activity was observed with peptaibolin [14] as a 5mer representative of the peptaibol structure. It can be suggested that the capacity of channel formation decreases in parallel with the decreasing number of the constituting amino acids. Probably a peptide chain of less than 10 amino acids would not enable membrane activity.

Ampullosporins A, B and D, on the one side, and trichofumins A–D on the other thus show comparable biological activities and effects on conductivity of artificial bilayer membranes. Hence, limited permeabilization of cellular membranes could be responsible, at least partly, for the observed effects of 11–15mer peptaibols on fungal morphogenesis and induction of hypothermia in mice. It is well known that nutrient limitation in microbes promotes their cytodifferentiation [15]. It can be suggested that effects comparable to nutrient limitation can be caused by limited permeabilization of the cytoplasmic membrane. Otherwise, disturbance of ion fluxes of synaptic cells in the presence of peptaibols could explain the observed induction of hypothermia in mice and neuroleptic effects in rats [16].

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Detection of new amino acid sequences of alamethicins F30 by nonaqueous capillary electrophoresis–mass spectrometry

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Abstract: The microheterogeneous alamethicin F30 (ALM F30) isolated from the fermentation of *Trichoderma viride* strain NRRL 3199 was analyzed by nonaqueous capillary electrophoresis coupled to electrospray ion-trap mass spectrometry (ESI-IT-MS) and electrospray time-of-flight mass spectrometry (ESI-TOF-MS). Tandem ESI-IT-MS was used for elucidation of the amino acid sequence based on the fragmentation pattern of selected parent ions. The MS/MS spectra using the $[M+3H]^{3+}$ or $[M+2H]^{2+}$ ions as precursor ions displayed the respective b- and the y-type fragments resulting from cleavage of the particularly labile Aib–Pro bond. The MS^3 of these fragments generated the b acylium ion series, as well as internal fragment ion series. Eleven amino acid sequences were identified, characterized by the exchange of Ala to Aib in position 6, Gln to Glu in positions 7 or 19 as well as the loss of the *C*-terminal amino alcohol. In addition, two truncated pyroglutamyl peptaibols were found. Overall, seven new sequences are reported compared to earlier LC–MS studies. The composition of the components was confirmed by on-line ESI-TOF-MS detection. Mass accuracy well below 5 ppm was observed. Quantification of the individual components was achieved by a combination of UV and TOF-MS detection. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: nonaqueous capillary electrophoresis; mass spectrometry detection; peptaibiotics; polypeptide antibiotics; peptaibols; alamethicin F30; α-aminoisobutyric acid; pyroglutamic acid

INTRODUCTION

Alamethicins are 20-residue peptaibol peptides isolated from the culture broth of the mold *Trichoderma viride* [1,2] exhibiting interesting physicochemical and biological activities, such as the formation of voltage-dependent ion channels in bilayer lipid membranes, as well as antibiotic activities [3]. The voltage-dependent ion channel formation by alamethicin can be described by the dipole flip-flop gating model of Boheim and Jung [4,5] based on electrical field–induced transbilayer orientational movements of single molecules. The conductance states of the ion conductivity pores vary with the number of parallelly arranged α-helices. Recently, single pore states could be stabilized by *C*-terminal conjugation of alamethicin with fullerene or a membrane-anchoring lipopeptide [6].

Peptaibols are linear peptides composed of 5–20 amino acids [7]. These compounds are exclusively biosynthesized by fungicolous, plant or entomopathogenic fungi, thus assuming potential importance in the parasitic life cycle of the producers [8]. The nonribosomal biosynthesis includes nonproteinogenic amino acids, in particular α-aminoisobutyric acid (Aib). Aib residues are conformationally restricted and favor the formation of 3_{10}- and α-helical structures. Peptaibols are amphiphilic because of the acylated, nonpolar *N*-terminus and the more polar, *C*-terminal amino alcohol. The name 'peptaibol' reflects the characteristics of this class of compounds being peptides containing Aib and a *C*-terminal amino alcohol.

Depending on the fermentation conditions, *T. viride* produces the neutral alamethicins F50 (ALM F50) or the acidic peptaibols alamethicins F30 (ALM F30) [2]. Both are microheterogeneous mixtures of closely related sequential analogs that possess a phenylalaninol (Pheol or Fol) at the *C*-terminus, while the *N*-terminus is acetylated. ALM F50 and ALM F30 differ in the amino acid in position 18, which is the (neutral) glutamine in the case of ALM F50 and the (acidic) glutamate residue in the case of ALM F30 [2,9]. The structure of ALM F30 has been confirmed by total synthesis [10].

Tandem mass spectrometry, especially electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) coupled to quadrupole, ion-trap (IT) or time-of-flight (TOF) mass analyzers have been proven useful for structural studies of peptaibols [8]. The separation of the individual components of ALM F50 and ALM F30 has been achieved by reversed-phase high performance liquid chromotography (HPLC) and the structure of the components was determined

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by HPLC–ESI-MS^n by Brückner and coworkers [2]. The acidic ALM F30 and the neutral ALM F50 were isolated from the culture broth by XAD-2 column chromatography and separated by silica gel chromatography. The composition of the individual peptaibols was subsequently determined by HPLC–ESI-MS^n. According to this study, ALM F30 consists of two major components that differ in the amino acid in position 6 (Ala or Aib) and eight minor components.

Nonaqueous capillary electrophoresis (NACE) using solvents such as methanol, acetonitrile or *N*-methylformamide, instead of water, for the preparation of the background electrolytes is increasingly applied to analytical problems [11]. Important parameters such as efficiency and selectivity can be effectively modified when aqueous buffers are replaced by nonaqueous electrolyte solutions. Organic solvents favor interactions that are weak in aqueous media. Furthermore, the solubility and stability of many analytes and additives are enhanced in nonaqueous solvents. Moreover, nonaqueous solvents may be preferable for electrochemical detection as well as for electrospray ionization mass spectrometry (ESI-MS) detection [12].

Traditionally, peptides as hydrophilic compounds are analyzed by capillary electrophoresis (CE) using aqueous background electrolytes. However, beneficial effects of organic solvents for the separation of hydrophobic peptides in aqueous CE media have been described. For example, an acidic aqueous buffer containing 20% 2-propanol as organic modifier was applied to the analysis of isomeric *N*-palmitoylated bradykinin and *O*-palmitoylated gonadorelin as well as (cysteinyl-4,5)-palmitoylated peptide SP-C14 [13]. Hodges and coworkers described the separation of 18-residue α-helical amphipathic peptide diastereomers by capillary zone electrophoresis with highly concentrated (up to 400 mM) perfluorinated acid ion-pairing reagents in aqueous solution [14]. The authors attributed the successful separation to conformational changes between the peptide diastereomers and differences in hydrophobicity of the nonpolar face of the amphipathic α-helices and their interactions with the hydrophobic anionic ion-pairing reagent. On-line NACE–MS of hydrophobic peptides gramicidin S and bacitracin has been demonstrated [15]. The separations were achieved in an acetonitrile/methanol-based system containing ammonium acetate and formic acid. ESI-MS allowed the determination of three minor components in the case of gramicidin S and one minor component in the case of bacitracin. The current status of CE–MS for the analysis of proteins and peptides including NACE–MS has been recently summarized [16].

The separation of peptaibols by CE has not been described previously. However, because of their lipophilic nature, NACE seems to be a suitable electromigration technique for this class of compounds. Thus, the present study was conducted in order to evaluate the potential of NACE coupled to MS for the analysis of peptaibols including the determination of their amino acid sequence. The natural ALM F30 was selected as model peptaibols because these compounds contain a glutamic acid residue in position 18 that can be deprotonated for electrophoretic analysis.

MATERIALS AND METHODS

Chemicals

Methanol, acetonitrile, 2-propanol, dichloromethane (all HPLC grade quality), ammonium acetate and silica gel 60 (mesh size <0.063 mm) were purchased from VWR International (Darmstadt, Germany). Ammonium formate was obtained from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate and ammonium formate were dried overnight in a desiccator over silica before use. ALM F30 was isolated from fermentations of *T. viride* strain NRRL 3199 as described previously [2].

The peptides Pyr-Aib-Val-Aib-Gly-Leuol and Glu-Aib-Val-Aib-Gly-Leuol were synthesized by solid-phase synthesis using the Fmoc strategy for the assembly of the peptide on the solid support [17,18]. The Fmoc-protected terminating amino alcohol was directly anchored onto the 2-chlorotrityl chloride resin. Activation of the sterically hindered Aib was achieved using tetramethylfluoroformamidinium hexafluorophosphate (TFFH) as coupling reagent, as described by Carpino *et al.* [18]. The peptides were purified by preparative HPLC, and their identity was confirmed by MALDI-MS.

Capillary electrophoresis

CE with UV detection was performed on a Beckman P/ACE 5510 instrument (Beckman Coulter, Krefeld, Germany) equipped with a diode-array detector at 25 °C using 50-μm i.d. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an effective length of 50 cm and a total length of 57 cm. UV detection was carried out at 215 nm at the cathodic end of the capillary. Sample solutions were introduced at the anodic end by hydrodynamic injections at a pressure of 0.5 psi for 3 s.

CE–MS experiments were performed using a Hewlett Packard ^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany). Separations were performed at 25 °C in 50-μm i.d. fused-silica capillaries with a length of 57 cm by application of a separation voltage of 30 kV (sprayer grounded). A pressure of 50 mbar for 4 s was used for sample injection.

New capillaries were rinsed for 30 min with 0.1 M sodium hydroxide, 5 min with water and 10 min with methanol followed by the separation medium for 10 min. Between analyses, the capillary was flushed with the running buffer for 2 min. When not in use, it was washed with the respective solvent and then stored under dry conditions.

Electrospray Ionization Mass Spectrometry

On-line coupling of the CE instrument to the mass spectrometer detector was achieved with an Agilent coaxial sheath-liquid sprayer interface (Agilent Technologies, Palo Alto, CA, USA). The sheath liquid, 2-propanol : water (1 : 1, v/v) containing 1% formic acid, was supplied at a flow rate of 4 μl/min by a

syringe pump (Cole-Palmer, Vernon Hill, IL, USA). Nebulizer gas pressure was set to 2–3 psi. All ESI-MS experiments were carried out in positive ionization mode at 4500 V.

ESI-IT-MS measurements were performed using an ion-trap mass spectrometer Esquire HCT™ (Bruker Daltonik, Bremen, Germany). Mass spectra were acquired from m/z 200 to 1500 in the scanning mode and automatic switching between MS and MS^n. Ions were scanned at a speed of 8300 m/z per s in the MS mode in order to achieve sufficient resolution for charge attribution of triply charged peptides. The enhanced auto-MS^n settings were optimized to get as many MS^n spectra over a selected time period as possible. This was achieved by scanning at 26 000 m/z per s and active exclusion after two spectra per mass in a given time window of 0.5 min. MS^2 and MS^3 spectra were acquired selecting one (MS^2) or two (MS^3) most abundant precursors or by adding preferred masses in the case of follow-up experiments.

ESI-TOF-MS measurements were performed on an orthogonal TOF mass spectrometer micrOTOF™ (Bruker Daltonik, Bremen, Germany). The mass spectrometer operated in an m/z range 200–1500.

Data processing was performed by DataAnalysis™ software (Version 3.0; Bruker Daltonik). The peptide MS fragments are labeled according to standard rules [19,20].

RESULTS AND DISCUSSION

NACE Separation of ALM F30

NACE separation of the microheterogeneous ALM F30 was evaluated in methanol, acetonitrile and mixtures of these solvents using ammonium acetate– and ammonium formate–based electrolytes. The solvents are widely used in NACE because of their appropriate dielectric constant–to-viscosity ratio [11]. While electrolyte solutions in acetonitrile and acetonitrile–methanol mixtures did not afford satisfactory separations because of the low mobility of the analytes in these solvents, good separation selectivity was obtained using methanol-based electrolytes. 12.5 mM ammonium formate in methanol yielded six separated peaks using UV detection (data not shown).

Subsequent NACE–ESI-MS was performed using this background electrolyte and a sheath liquid consisting of 1% formic acid in a 1 : 1 mixture of 1-propanol and water, which provided stable spray and protonation conditions for the peptaibols. Figure 1 shows the base peak electropherogram of

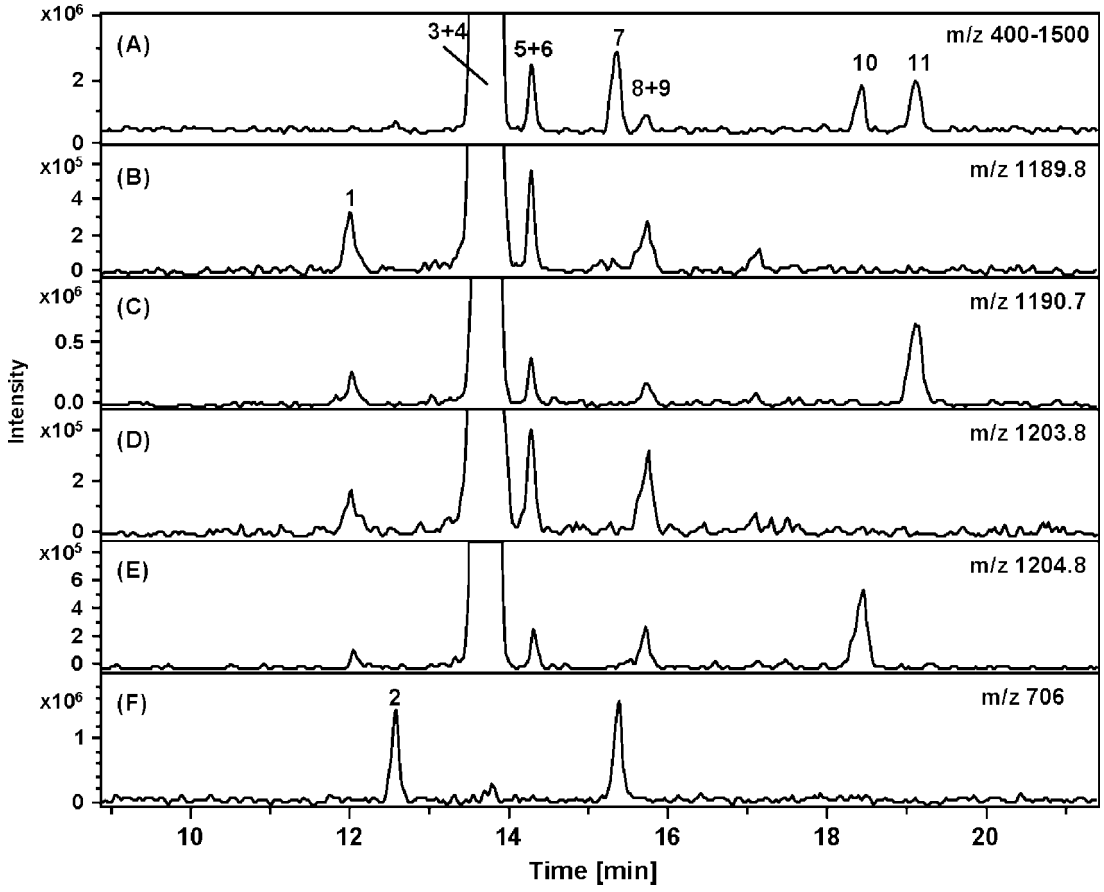

Figure 1 Extracted ion electropherograms of NACE with full-scan ESI-IT-MS detection. (A) Base peak electropherogram. (B–E) Mass traces of the different b_{13} fragments (see Table 2). (F) $[M + 2H]^{2+}$ pseudomolecular ion of the Pyr-containing peptides. Experimental conditions: capillary dimensions, 57 cm × 50 μm i.d.; running electrolyte, 12.5 mM ammonium formate in methanol; separation voltage, 30 kV (18 μA); for ESI-MS conditions see Experimental Section. Sample concentration: 500 μg/ml in methanol. Peak identity: numbers refer to peptide sequences shown in Table 1.

ALM F30 (Figure 1A) as well as electropherograms of selected mass traces of the characteristic b_{13} fragment ions obtained with full-scan ESI-IT-MS (Figure 1B–E). The identification of the individual components and the determination of their amino acid sequences are discussed below. The extracted ion electropherogram (EIE) of the b_{13} fragments at *m/z* 1189.9 (Figure 1B) and 1203.8 (Figure 1D) as well as at *m/z* 1190.7 (Figure 1C) and 1204.8 (Figure 1E) with mass differences of 14 Da correspond to the exchange of Ala by Aib in the peptaibols. Most of the respective compounds comigrated, as the mass difference of 14 Da is apparently not large enough to be translated into electrophoretic separations of otherwise identical peptaibols with molecular masses of about 1900–1950 Da. The EIE of *m/z* 706 (Figure 1F) corresponds to the doubly charged pseudomolecular $[M + 2H]^{2+}$ ions of truncated pyroglutamyl (Pyr) peptaibols.

Amino Acid Sequence Determination

Table 1 summarizes the amino acid sequences of ALM F30 as determined by NACE–ESI-MS analysis. A different numbering as compared to the nomenclature applied in Ref. 2 of the ALM F30 peptides is used for reasons of simplicity. The mass fragments and molecular ions of all components of ALM F30 determined in this study are compiled in Table 2. The majority of the diagnostic ions was identified via NACE–ESI-IT-MS^n in the positive ion mode from specific precursor ions such as the triply and doubly charged pseudomolecular $[M + 3H]^{3+}$ and $[M + 2H]^{2+}$ ions for MS^2 spectra and from the b_{13} and y_7 fragments, respectively, in the case of the MS^3 spectra. Figures 2 and 3 show the ESI-IT-MS^3 spectra of [Aib^6] ALM F30 (**3**) and [desAA(1–6),Pyr^7] ALM F30 (**7**), respectively, as examples.

The MS spectra show the doubly and triply charged pseudomolecular ions, $[M + 2H]^{2+}$ or $[M + 3H]^{3+}$, as well as the corresponding ammonium adducts in some cases. Moreover, b_{13}- and y_7-fragments are generated, resulting from fragmentation of the particularly labile Aib–Pro bond. The tertiary amide bond undergoes a preferential cleavage, leading to an *N*-terminal acylium ion (b-type fragment) and a diprotonated *C*-terminal ion (y-type fragment) [21]. These characteristic fragments were also obtained in the ESI-IT-MS full-scan mode (without MS^n) illustrating the facile cleavage of the Aib–Pro bond. Other peptaibols such as harzianins [21], stilboflavins [22], trichotoxins [23] and trichofumins [24] exhibit similar fragmentation patterns in ESI-MS. The MS^2 spectra using the $[M + 3H]^{3+}$ or $[M + 2H]^{2+}$ ions as precursor ions displayed the respective b- and the y-type fragments resulting from cleavage of the Aib–Pro bond. The selection of the appropriate precursor ions allowed the identification and amino acid sequence determination also in case of comigrating substances.

In the case of the 20- and 19-residue peptaibols, the triply charged pseudomolecular ions $[M + 3H]^{3+}$ were selected as the precursor ions for the MS^2 analysis

Table 1 Sequences and relative quantities (%) of the ALM F30 peptides characterized by NACE–ESI-MS in the microheterogeneous mixture. Exchanged amino acid positions are highlighted in bold letters. Abbreviations of the amino acids are according to the one-letter code, Ac – acetyl, U – Aib, Pyr – pyroglutamic acid. The denotation of the ALM F30 peptides identified by HPLC–MS [2] is listed in the third column

		Nomenclature in Ref. 2	%	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	ALM F50	F50/5	—[a]	Ac	U	P	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	U	**Q**	Q	Fol
2	[desAA(1–6),Pyr^7] ALM F50	—	0.4							**Pyr**	U	V	U	G	L	U	P	V	U	U	**Q**	Q	Fol
3	[Aib^6] ALM F30	F30/7	38.9	Ac	U	P	U	A	U	U	Q	U	V	U	G	L	U	P	V	U	U	E	Q	Fol
4	ALM F30	F30/3	51.0	Ac	U	P	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	U	E	Q	Fol
5	[Glu^{19}] ALM F30	—	1.4	Ac	U	P	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	U	E	**E**	Fol
6	[Aib^6,Glu^{19}] ALM F30	—	1.2	Ac	U	P	U	A	U	**U**	Q	U	V	U	G	L	U	P	V	U	U	E	**E**	Fol
7	[desAA(1–6),Pyr^7] ALM F30	—	2.9							**Pyr**	U	V	U	G	L	U	P	V	U	U	E	Q	Fol
8	[desPheol] ALM F30	—	0.6	Ac	U	P	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	U	E	Q	
9	[Aib^6,desPheol] ALM F30	—	0.7	Ac	U	P	U	A	U	**U**	Q	U	V	U	G	L	U	P	V	U	U	E	Q	
10	[Aib^6,Glu^7] ALM F30	—	1.3	Ac	U	P	U	A	U	**U**	**E**	U	V	U	G	L	U	P	V	U	U	E	Q	Fol
11	[Glu^7] ALM F30	F30/6	1.7	Ac	U	P	U	A	U	A	**E**	U	V	U	G	L	U	P	V	U	U	E	Q	Fol

[a] Not quantified, because the uncharged ALM F50 comigrates with the EOF.

Table 2 Fragment ions, pseudomolecular ions and adducts of pseudomolecular ions (m/z ratio) of ALM F30 determined by NACE–ESI-MS. The numbering of the components corresponds to Table 1

Diagnostic ion	m/z							Diagnostic ion	m/z		Diagnostic ion	m/z	
	1	**3**	**4**	**5**	**6**	**10**	**11**		**2**	**7**		**8**	**9**
b_2	225.1	225.1	225.1	225.1	225.1	225.1	225.1	b_2	n.d.	197.1	b_2	225.1	225.1
b_3	310.2	310.2	310.2	310.2	310.2	310.2	310.2	b_3	296.2	296.2	b_3	310.2	310.2
b_4	381.2	381.2	381.2	381.2	381.2	381.2	381.2	b_4	381.2	381.2	b_4	381.2	381.2
b_5	466.3	466.3	466.3	466.3	466.3	466.3	466.3	b_5	438.2	438.2	b_5	466.3	466.3
b_6	537.3	551.3	537.3	537.3	551.3	551.3	537.3	b_6	551.3	551.3	b_6	537.3	551.3
b_7	665.4	679.4	665.4	665.4	679.4	680.4	666.3	b_7	636.4	636.4	b_7	665.4	679.4
b_8	750.4	764.4	750.4	750.4	764.4	765.4	751.4	—	—	—	b_8	750.4	764.4
b_9	849.5	863.5	849.5	n.d.	n.d.	864.5	850.5	—	—	—	b_9	849.5	863.5
b_{10}	934.5	948.5	934.5	934.5	948.5	949.5	935.5	—	—	—	b_{10}	934.5	948.5
b_{13}	1189.7	1203.7	1189.7	1189.7	1203.7	1204.7	1190.7	—	—	—	b_{13}	1189.7	1203.7
$[b_{13} + 2H]^{2+}$	595.8	602.9	595.8	595.8	602.9	603.4	596.3	—	—	—	$[b_{13} + 2H]^{2+}$	595.8	602.9
y_7	774.4	775.4	775.4	776.4	776.4	775.4	775.4	y_7	774.4	775.4	y_6	642.3	642.3
$[y_7 - H_2O]$	756.4	757.4	757.4	758.4	758.4	757.4	757.4	$[y_7 - H_2O]$	756.4	757.4	$[y_6 - H_2O]$	624.3	624.3
y_7b_{19}	623.3	624.3	624.3	625.3	625.3	624.3	624.3	y_7b_{13}	623.3	624.3	—	—	—
y_7b_{18}	495.3	496.3	496.3	496.3	496.3	496.3	496.3	y_7b_{12}	495.3	496.3	y_6b_{18}	496.3	496.3
y_7b_{17}	367.2	367.2	367.2	367.2	367.2	367.2	367.2	y_7b_{11}	367.2	367.2	y_6b_{17}	367.2	367.2
y_7b_{16}	282.2	282.1	282.2	282.2	282.2	282.2	282.2	y_7b_{10}	282.2	282.2	y_6b_{16}	282.2	282.2
$b_{13}y_{10}$	256.2	256.2	n.d.	256.2	256.2	256.2	256.2	b_7y_{10}	256.2	256.2	$b_{13}y_9$	256.2	256.2
$b_{13}y_{11}$	341.2	341.2	341.2	341.2	341.2	341.2	341.2	$\underline{b}_7y_{11}$	341.2	341.2	$b_{13}y_{10}$	341.2	341.2
$b_{13}y_{12}$	440.3	440.3	440.3	440.3	440.3	440.3	440.3	b_7y_{12}	440.3	440.3	$b_{13}y_{11}$	440.3	440.3
$b_{13}y_{13}$	525.3	525.3	525.3	525.3	525.3	525.3	525.3	b_7y_{13}	525.3	525.3	$b_{13}y_{12}$	525.3	525.3
$b_{13}y_{14}$	653.4	653.4	653.4	653.4	653.4	654.4	654.4	—	—	—	$b_{13}y_{13}$	653.4	653.4
$b_{13}y_{15}$	724.4	738.4	724.4	724.4	738.4	739.4	725.4	—	—	—	$b_{13}y_{14}$	724.4	738.4
$b_{13}y_{16}$	809.5	823.5	809.5	809.5	823.5	824.5	810.5	—	—	—	$b_{13}y_{15}$	809.5	823.5
$b_{13}y_{17}$	880.5	894.5	880.5	880.5	894.5	895.5	881.5	—	—	—	$b_{13}y_{16}$	880.5	894.5
$[M + 2H]^{2+}$	982.1	989.6	982.6	983.1	990.1	990.0	983.1	$[M + 2H]^{2+}$	705.4	705.9	$[M + 2H]^{2+}$	916.0	923.0
$[M + NH_4 + H]^{2+}$	990.6	998.1	991.1	991.6	998.6	998.5	991.6	$[M + NH_4 + H]^{2+}$	713.9	714.4	$[M + NH_4 + H]^{2+}$	924.5	931.5
$[M + 3H]^{3+}$	655.1	660.1	655.4	655.7	660.4	660.4	655.7	$[M + 3H]^{3+}$	n.d.	n.d.	$[M + 3H]^{3+}$	611.0	615.7
$[M + NH_4 + 2H]^{3+}$	660.8	665.8	661.1	661.4	666.1	666.1	661.4	$[M + NH_4 + 2H]^{3+}$	n.d.	n.d.	$[M + NH_4 + 2H]^{3+}$	616.7	621.4

n.d. – not determined.

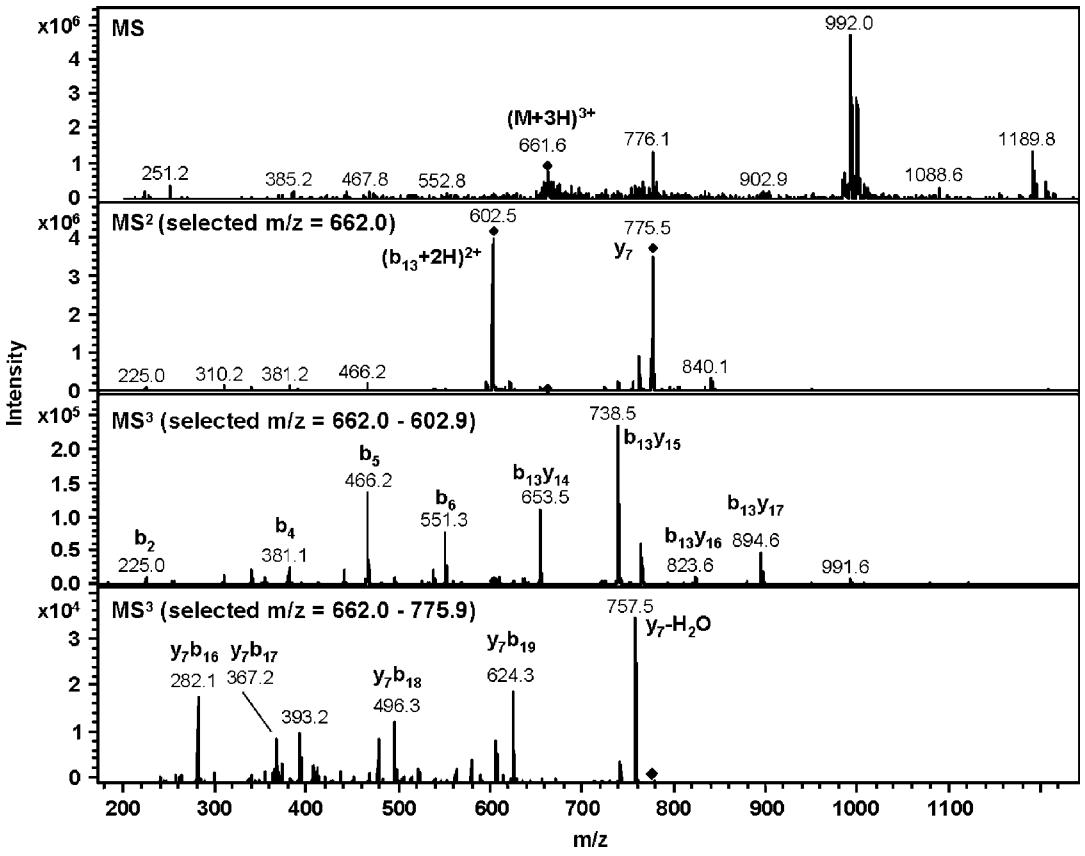

Figure 2 ESI-IT-MS^n ($n = 1–3$) of [Aib^6] ALM F30 (**3**).

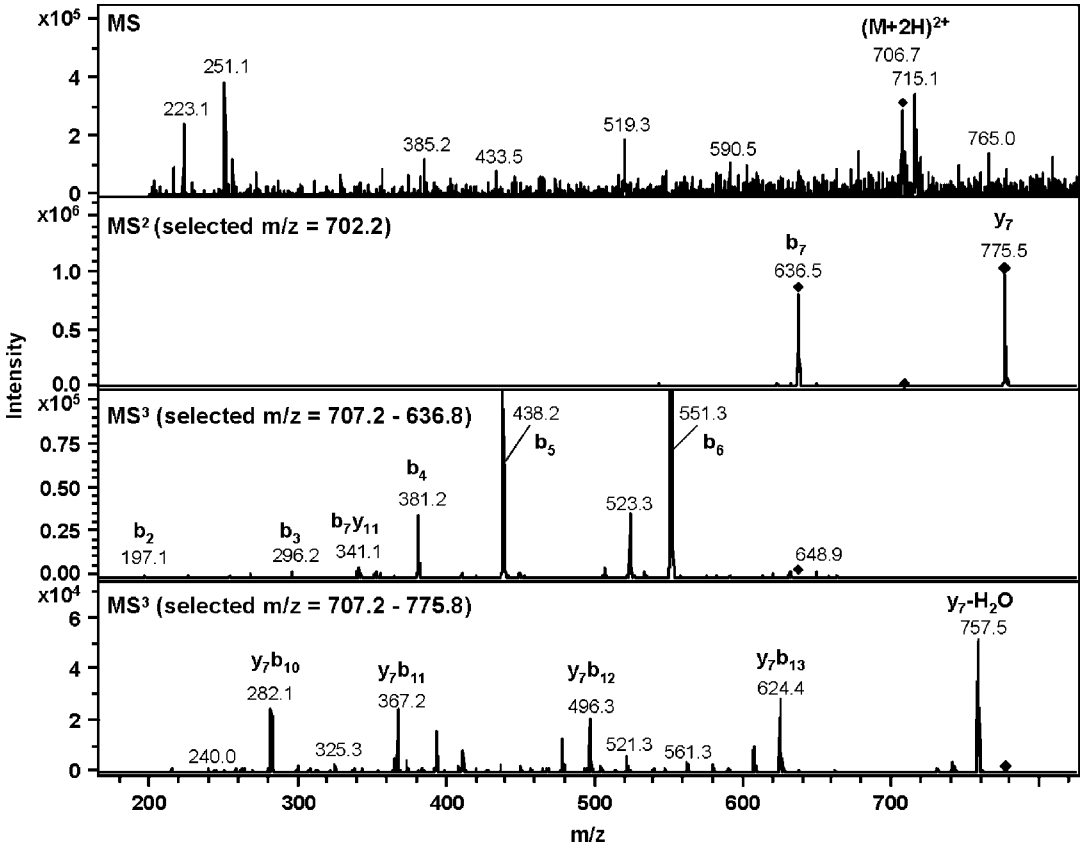

Figure 3 ESI-IT-MS^n ($n = 1–3$) of [desAA(1–6),Pyr^7] ALM F30 (**7**).

(Figure 2). MS^2 generated the b_{13} and the y_7 fragment or, in case of the desPheol ALM F30 peptides, the y_6 fragment (data not shown). The b_{13} fragment is detected as a doubly charged ion. The MS^3 of the diprotonated b_{13} fragment ($[b_{13} + 2H]^{2+}$) generated the $b_2 - b_{10}$ acylium ion series, as well as the monoprotonated internal fragments $b_{13}y_{10} - b_{13}y_{17}$ (Table 2). The *C*-terminal sequences of the peptides were determined by MS^3 of the y_7 fragment and, in the case of the desPheol ALM F30 peptides, of the y_6 fragment. The resulting internal ion series ($y_7b_{19} - y_7b_{16}$) are formed by the cleavage of *C*-terminal residues while the charge remains at the *N*-terminus. Loss of water from the y_7 fragment leads to a $y_7 - H_2O$ fragment ion in the MS^3 spectra. The *C*-terminal position of Pheol was concluded from the mass difference of 150 + 1 Da from diprotonated y_7 and monoprotonated y_7b_{19} fragments. A fragmentation scheme is presented in (Figure 4) showing the series of diagnostic ions and internal fragments used for sequence determination of [Aib^6] ALM F30 (**3**).

The signals of the b_2 ions were recorded at *m/z* 225 corresponding to the fragment Ac-Aib-Pro. The internal fragments $b_{13}y_{10}$ (*m/z* 256) and y_7b_{16} (*m/z* 282) correspond to the Gly-Leu-Aib and the Pro-Val-Aib tripeptides, respectively. The sequences of these three fragments were derived from earlier HPLC–MS investigations of ALM F30 [2,9] assuming sequence analogy.

The sequences of the two pyroglutamyl peptaibols (14-residue peptides) [desAA(1–6),Pyr^7] ALM F50 (**2**) and [desAA(1–6),Pyr^7] ALM F30 (**7**) were concluded from MS^3 analysis of the b_7 and y_7 fragments generated by the cleavage of the Aib-Pro bond of the pseudomolecular ion $[M + 2H]^{2+}$ (Figure 3 for [desAA(1–6),Pyr^7] ALM F30). The internal fragments of b_7 are the $b_2 - b_7$ acylium ion series and the monoprotonated fragments $b_7y_{10} - b_7y_{13}$ analogous to the b_{13} fragment of the 20-residue ALM F30 peptaibols. The y_7 fragment formed the $y_7b_{10} - y_7b_{13}$ internal fragment series. In contrast to the 20-residue ALM F30 peptides, the sequence of the b_7y_{10} fragment at *m/z* 256 corresponding to the Gly-Leu-Aib tripeptide could be confirmed by the $b_5 - b_7$ fragment ions.

Pandey *et al.* reported the formation of pyroglutamyl peptide fragments in an electron-impact mass spectrometry study of ALM F30 [9]. However, the formation of pyroglutamyl peptides during the ESI process has not been described. In order to evaluate the fragmentation pattern of Glu *versus* Pyr peptides, two model peptides containing reduced *C*-termini with the amino acid sequences Glu-Aib-Val-Aib-Gly-Leuol (Glu-hexapeptide) and Pyr-Aib-Val-Aib-Gly-Leuol (Pyr-hexapeptide) were synthesized and subjected to NACE–ESI-MS. The ESI-IT-MS^3 spectra of the two model hexapeptides displayed the respective b-type and the y-type ion series. The $[M + H]^+$ ions were selected as the precursor ions, and the fragment ions are listed in Table 3. In the spectrum of the Glu-hexapeptide, the b_2, b_3 and b_4 fragments were detected and also additional signals corresponding to the loss of water from the $b_2 - b_4$ fragments. These fragments have identical *m/z* values as the $b_2 - b_4$ fragments of the Pyr-hexapeptide so that formation of Pyr peptides in the ESI source can be assumed. However, the Glu-hexapeptide can be clearly distinguished by the presence of the $b_2 - b_5$ fragments 18 mass units higher than those of the Pyr-hexapeptide. In addition, both peptides have different electrophoretic mobilities. Thus, it can be concluded that the truncated Pyr peptaibol components in ALM F30 are in fact present in the investigated sample and not an artefact generated in the ESI ion source because the compounds did not display the respective 18-mass-units-higher fragments in the spectra. Interestingly, [desAA(1–6),Pyr^7] ALM F50 (**2**) displayed a low anodic mobility despite the fact that this compound should be neutral as it does not contain a charged amino acid. Apparently, under the present NACE conditions, a partial negative charge is induced. Moreover, the anodic mobility of [desAA(1–6),Pyr^7] ALM F50 (**2**)

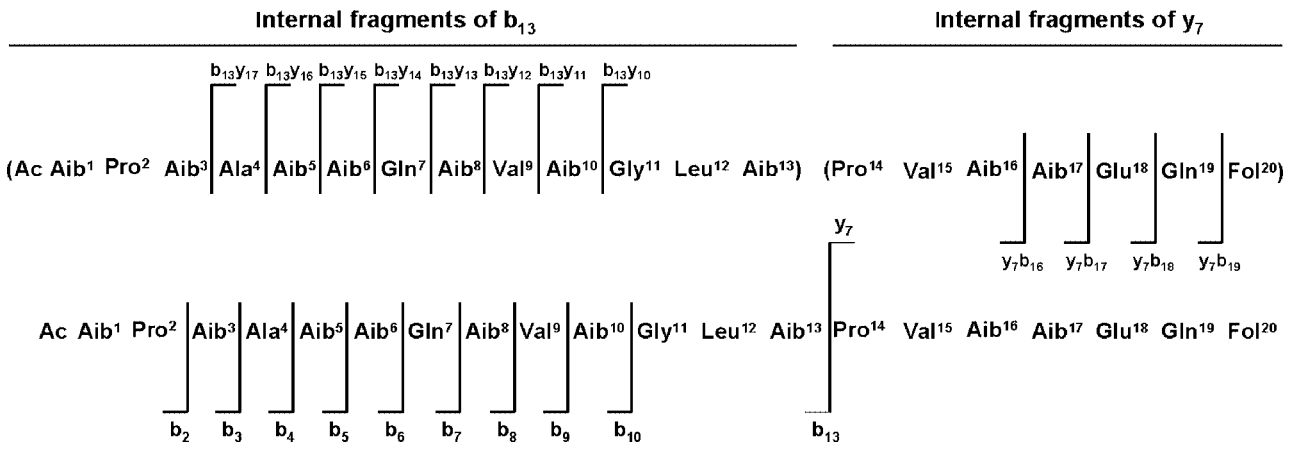

Figure 4 Fragmentation scheme of [Aib^6] ALM F30 (**3**). Fragments were generated by ESI-IT-MS^n ($n = 1–3$).

was confirmed by the anodic mobility of the Pyr model hexapeptide.

The amino acid composition of the ALM F30 components obtained by NACE–ESI-IT-MS^n was confirmed by NACE–ESI-TOF-MS analysis. Doubly or triply charged molecular ions were the most abundant ions in the spectra. Fragmentation was not observed (data not shown). The high resolution of the TOF mass analyzer allowed the analysis of the isotope pattern as shown in Figure 5 for the $[M + 3H]^{3+}$ ion of [desPheol] ALM F30 (**8**) and the $[M + 2H]^{2+}$ ion of [desAA(1–6),Pyr^7] ALM F30 (**7**) including a comparison of the experimental spectra with the simulated isotope pattern obtained by the Bruker DataAnalysis™ software. In both examples, the measured isotope pattern was consistent with the calculated isotope pattern, thus confirming the elemental composition, i.e. the amino acid composition of the identified components. This approach was applied to the confirmation of the amino acid composition of all compounds. Table 4 summarizes the experimentally determined monoisotopic m/z values using ALM F30 as mass calibrant compared to the respective calculated values. With the exception of the neutral ALM F50, mass accuracy was clearly below 5 ppm for the doubly charged ions and below 2 ppm for the triply charged ions. The relative inaccuracy found for ALM F50 may be due to the fact that this compound migrates essentially with the electroosmotic flow (EOF).

Table 3 Fragment ions, pseudomolecular ions and adducts of pseudomolecular ions (m/z ratio) of the model hexapeptides Glu-Aib-Val-Aib-Gly-Leuol (Glu-hexapeptide) and Pyr-Aib-Val-Aib-Gly-Leuol (Pyr-hexapeptide)

Ion	m/z	
	Glu-hexapeptide	Pyr-hexapeptide
b_2	215.1	197.1
b_3	314.2	296.2
b_4	399.2	381.2
b_5	456.2	438.2
y_2	157.1	157.1
y_3	242.2	242.2
y_4	341.2	341.2
y_5	426.3	426.3
$[b_2 - H_2O]$	197.1	—
$[b_3 - H_2O]$	296.2	—
$[b_4 - H_2O]$	381.2	—
$[M + H]^+$	573.3	555.3

Except for the neutral components ALM F50 (**1**) and [desAA(1–6),Pyr^7] ALM F50 (**2**), the compounds possess a Glu residue in position 18 and carry a negative charge under the applied NACE conditions. Not considering [desAA(1–6),Pyr^7] ALM F30 (**7**), the Glu^{18} derivatives can be divided into pairs characterized by the exchange of Ala by Aib in position 6. This exchange is characteristic for many peptaibol peptides [7]. Compared to the pair of the major components ALM F30 (**4**) and [Aib^6] ALM F30 (**3**), the other pairs are characterized by an additional carboxy group resulting from the exchange of Gln in position 19 to Glu, i.e. [Glu^{19}] ALM F30 (**5**) and [Aib^6,Glu^{19}] ALM F30 (**6**), the exchange of Gln to Glu in position 7, i.e. [Glu^7] ALM F30 (**11**) and [Aib^6,Glu^7] ALM F30 (**10**), or loss of the *C*-terminal phenylalaninol, i.e. [desPheol] ALM F30 (**8**) and [Aib^6,desPheol] ALM F30 (**9**). Except for [Glu^7] ALM F30 (**11**) and [Aib^6,Glu^7] ALM F30 (**10**), which are well separated, the peptaibols of the respective pairs comigrate as the mass difference of

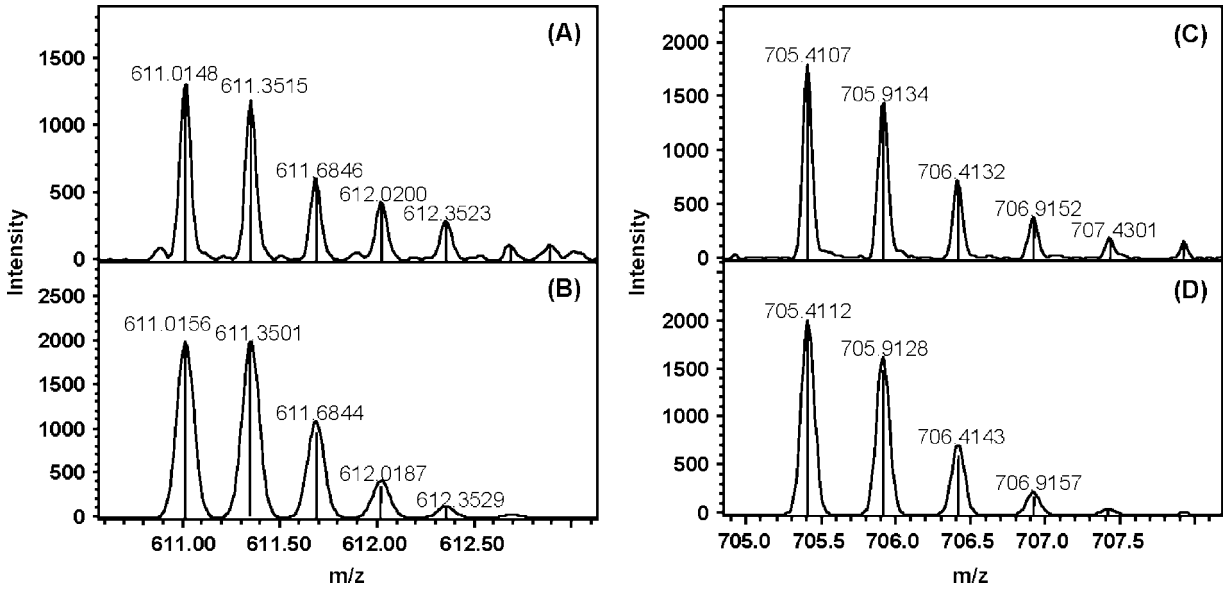

Figure 5 Measured (A, C) and calculated (B, D) isotope pattern of [desPheol] ALM F30 (A, B) and [desAA(1–6),Pyr^7] ALM F30 (C, D).

Table 4 Calculated and measured m/z ratios of the pseudomolecular ions observed in ESI-TOF-MS

		Molecular formula	Monoisotopic molecular mass (Da)	m/z monoisotopic mass					
				$[M+2H]^{2+}$		Δm (ppm)	$[M+3H]^{3+}$		Δm (ppm)
				Measured	Calculated		Measured	Calculated	
1	ALM F50	$C_{92}H_{151}N_{23}O_{24}$	1962.09	982.0784	982.0724	+6.1	655.0483	655.0507	−3.7
2	[des(1–6),Pyr^7] ALM F50	$C_{67}H_{108}N_{16}O_{17}$	1408.77	705.4107	705.4112	−0.7	—	—	—
3	[Aib^6] ALM F30	$C_{93}H_{152}N_{22}O_{25}$	1977.08	989.5723	989.5722	+0.1	660.0507	660.0506	+0.2
4	ALM F30	$C_{92}H_{150}N_{22}O_{25}$	1963.07	Calibrant	982.5644	—	Calibrant	655.3787	—
5	[Glu^{19}] ALM F30	$C_{92}H_{149}N_{21}O_{26}$	1964.05	983.0562	983.0564	−0.2	655.7075	655.7067	+1.2
6	[Aib^6,Glu^{19}] ALM F30	$C_{93}H_{151}N_{21}O_{26}$	1978.06	990.0609	990.0642	−3.3	660.3798	660.3786	+1.8
7	[des(1–6),Pyr^7] ALM F30	$C_{67}H_{107}N_{15}O_{18}$	1409.75	705.9031	705.9032	−0.1	—	—	—
8	[desPheol] ALM F30	$C_{83}H_{139}N_{21}O_{25}$	1830.00	916.0177	916.0198	−2.3	611.0148	611.0156	−1.3
9	[Aib^6,desPheol] ALM F30	$C_{84}H_{141}N_{21}O_{25}$	1844.01	923.0305	923.0276	+3.1	615.6873	615.6875	−0.3
10	[Aib^6,Glu^7] ALM F30	$C_{93}H_{151}N_{21}O_{26}$	1978.06	990.0634	990.0642	−0.8	660.3797	660.3786	+1.7
11	[Glu^7] ALM F30	$C_{92}H_{149}N_{21}O_{26}$	1964.05	983.0557	983.0564	−0.7	655.7066	655.7067	−0.2

14 Da caused by the exchange of Ala *versus* Aib is not sufficient to be translated into CE separations of peptaibols with masses of approximately 1950 Da under the experimental conditions applied.

Peptaibiotics terminating in a free amino acid or amide instead of a *C*-terminal 2-amino alcohol residue have been described previously. They comprise XR 586 (Gly at the *C*-terminus) [25]; trichobrachins TB I A, B, C, and D (Gln at the *C*-terminus) as well as trichobrachins II A, C, and D (Val at the *C*-terminus) [26]; lipohexin (β-Ala at the *C*-terminus) [27,28], pseudokonin KL III (Pro-NH_2 at the *C*-terminus) [29] and cephaibols P and Q (Ser at the *C*-terminus) [30].

N-terminal pyroglutamic acid (Pyr) containing peptaibols have not yet been reported. Whether [desAA(1–6),Pyr^7] ALM F50 (**2**) and [desAA(1–6),Pyr^7] ALM F30 (**7**) are naturally occurring compounds or artifacts caused by the workup or enzymatic degradation of peptaibols in the fermentation process remains to be answered. In order to estimate the possibility of the formation during workup, ALM F30 was heated for 41 h at 100 °C in the crystalline state as well as in dichloromethane/methanol (1 : 1, v/v) in the presence of an equal amount of silica gel G 60 for 4, 8 and 24 h at 70 °C in a closed vial simulating the chromatographic workup [2] of the sample. The samples were analyzed by LC–MS and CE. Neither treatment increased the amount of the truncated pyroglutamyl peptides compared to untreated samples. Thus, formation of these peptides during workup appears to be unlikely. In addition, when specifically searching for the mass trace of the [desAA(1–6),Pyr^7] ALM F30 (**7**) in LC–MS, the compound could also be detected in samples of ALM F30 described in Ref. 2.

To the best of our knowledge, this is the first report on the occurrence of Pyr as a constituent of fungal peptides in nature. Literature search did not reveal any previous publications regarding the isolation of peptide-bound Pyr from a fungal source. Pyr is rather widespread as an *N*-terminal constituent of peptides obtained from bacteria, plants, vertebrates and invertebrates. For example, the enzymatic production of Pyr by thermophilic lactic acid bacteria in Italian cheeses has been described [31,32]. Pyr has been reported as a constituent of antifungal peptides from the bark of *Eucommia ulmoides* [33], as a constituent of an adipokinetic hormone from the corpora cardiaca of the butterfly *Vanessa cardui* (Lepidoptera, Nymphalidae) [34], in gomesin, a defensive peptide found in the hemocytes of the tarantula spider *Acanthoscurria gomesiana* (Theraphosidae) [35], from the abdominal ganglia of the snail *Aplysia californica* [36], as well as from the hemolymph of the shrimp *Penaeus vannamei* (Decapoda) [37]. The *N*-terminus of neurotensins from the European green frog *Rana ridibunda* also contains a Pyr [38] as does the *N*-terminus of bradykinin potentiating peptides (BPPs) from the crude venom of the viper *Bothrops jararaca* [39]. Biosynthetically, Pyr peptides originate from glutaminyl peptides by the action of pyroglutamyl cyclases. Currently, more than 100 pyroglutamyl cyclase-type genes can be found in genomic databases such as BLAST (Basic Local Alignment Tool, National Center for Biotechnology Information), including fungal sources. Thus, the possibility of the formation of the truncated peptaibols before extraction can also not be generally ruled out at present. Future studies have to be performed in order to unequivocally prove the origin of the truncated Pyr peptaibols by *T. viride*.

Further confirmation of the identity of [desAA(1–6),Pyr^7] ALM F30 (**7**) and [desPheol] ALM F30 (**8**) may be derived from the so-called sigma™ value that represents a calculated parameter based on the true isotopic pattern (TIP™; Bruker Daltonik). It considers

the mass and the relative intensities of all isotopes. For both substances, the calculated elemental composition based on the isotopic pattern of the $[M + 2H]^{2+}$ ion belongs to the top 10% of possible hits.

The microheterogeneous ALM F30 isolated from a fermentation broth has previously been analyzed by HPLC–MS and 10 components have been identified [2]. Except for one compound (peptaibol F30/6 in Ref. 2 corresponding to [Glu^7] ALM F30 (**11**) in this study) all components possess only one chargeable function with Glu in position 19. Further amino acid exchanges reported in [2] include Val to Aib or Leu in position 9, Leu to Val in position 12 and Aib to Val in position 17 besides the exchange of Ala with Aib in position 6, which was also found for the peptaibols described in the present study. Seven of these peptaibols were not identified by NACE–ESI-IT-MS. However, careful analysis of the ESI-TOF-MS runs revealed that the peptaibols with the sequences AcUPUAUAQU**U**UGLUPVUUEQFol (F30/1 in Ref. 2) and AcUPUAUAQUVUG**V**UPVUUEQFol (F30/2 in Ref. 2) apparently comigrate with the major components ALM F30 (**4**) and [Aib^6] ALM F30 (**3**) in NACE. Other peptaibols identified in Ref. 2 have identical masses as the major components or masses differing by only 14 Da. As described above, a mass difference of 14 Da between peptides of approximately 1950 Da may not always be sufficient to be translated into a separation by CE. Thus, it appears very likely that most of the minor peptaibol components described in Ref. 2 comigrate with the major components of ALM F30 in NACE. Owing to the large excess of the major component, it was not possible to properly select the appropriate precursor ions for amino acid sequence elucidation of comigrating minor components by NACE–ESI-MS^n.

In contrast, the majority of minor peptaibol components identified by NACE–MS possesses a second carboxy function due to Gln/Glu exchange or loss of the *C*-terminal Pheol. Except for [Glu^7] ALM F30 (component F30/6 in Ref. 2), these compounds were not detected in the earlier HPLC–MS study. Apparently, ALM F30 peptaibols possessing only one carboxy function are separated more efficiently by HPLC, while components with two carboxy functions can be analyzed better by NACE. This illustrates the complementarity of both techniques due to their different separation principles.

Quantification of ALM F30 Components

The relative amounts of the respective peptaibol peptides in a heterogeneous mixture is required in order to judge the relevance of structural variations of individual peptides, in particular, when bioactivities are discussed. In the present NACE assay, the composition cannot be directly obtained from the electropherograms because of the comigration of compounds. Thus, the amount of the identified ALM F30 components in the microheterogeneous mixture was estimated from the NACE-UV traces at 215 nm in combination with the NACE–ESI-TOF-MS results. The relative amount of comigrating or incompletely separated peptides was calculated on the basis of the corrected peak area (peak area divided by the migration time) obtained from the EIE of characteristic isotopic signals of the pseudomolecular $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions, while the absolute amount of comigrating peptides was calculated from the corrected peak area obtained from the NACE-UV trace. Only peptide fragments with similar charges and masses were chosen for the relative

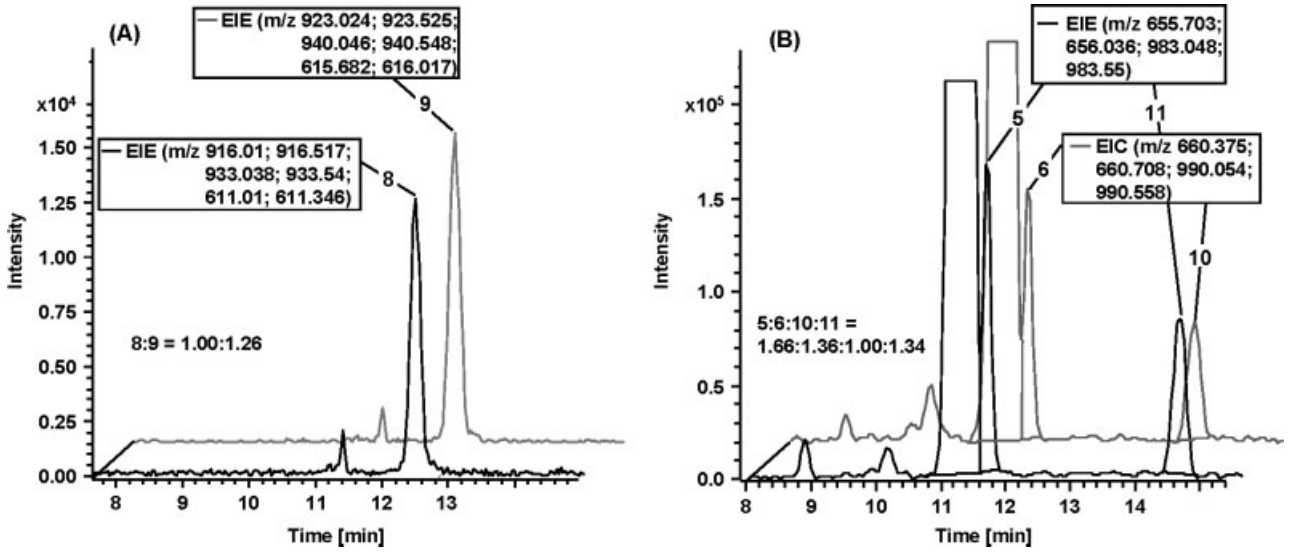

Figure 6 Quantification of (A) [desPheol] ALM F30 (**8**) and [Aib^6,des Pheol], ALM F30 (**9**) and (B) [Glu^{19}] ALM F30 (**5**), [Aib^6,Glu^{19}] ALM F30 (**6**), [Glu^7] ALM F30 (**11**) and [Aib^6,Glu^7] ALM F30 (**10**) by NACE–ESI-TOF-MS. The mass traces shown result from the addition of the unambiguously identified isotope signals of the respective analytes.

quantification, so that comparable ionization yields using ESI can be assumed for the respective analytes. Careful selection of the correct m/z values is important because signal overlapping by ammonium adducts of comigrating peptides occurs. Figure 6A shows the EIE of [desPheol] ALM F30 (**8**) and [Aib^6,desPheol] ALM F30 (**9**). The integration of the peaks resulted in a quantitative relationship of 1.00 : 1.26. The quantification of the other comigrating substances [Glu^{19}] ALM F30 (**5**) and [Aib^6,Glu^{19}] ALM F30 (**6**) as well as [Glu^7] ALM F30 (**11**) and [Aib^6,Glu^7] ALM F30 (**10**) based on the EIE is shown in Figure 6B. Note that the positionally isomeric peptides **5** and **11** form identical pseudomolecular ions as do the positionally isomeric peptides **6** and **10**. The resulting quantitative relationship for these four peptides is 1.66 : 1.36 : 1.00 : 1.34. The relative amount of the pyroglutamyl peptides and of the pair ALM F30 (**4**)/[Aib^6] ALM F30 (**3**) was calculated in a similar manner.

The results of the quantitative analysis are listed in Table 1. In accordance with the results obtained by Brückner and coworkers for ALM F30 using HPLC–MS [2], the main components ALM F30 (**4**) and [Aib^6] ALM F30 (**3**) comprise about 90% of the ALM F30 peptides. The unusual compound [desAA(1–6),Pyr^7] ALM F30 (**7**) was present at a level of about 3% while all other minor components were below 2%. The neutral ALM F50 is transported by the EOF. The quantification of analytes comigrating with the EOF is difficult because of signal quenching by impurities transported by the EOF. Therefore, ALM F50 was not quantified.

CONCLUSIONS

The components of ALM F30 isolated from fermentations of *T. viride* were analyzed by NACE–ESI-IT-MS and NACE–ESI-TOF-MS. A total of 11 compounds were identified. These are characterized by the well-known Ala/Aib exchange in position 6 as well as additional Gln/Glu exchanges in positions 7 or 19, as well as the loss of the *C*-terminal Pheol residue. Additionally, two truncated pyroglutamyl derivatives were detected which have not been described for peptaibols from fungal sources before.

Compared to an earlier study on ALM F30 by HPLC–MS [2], the present results are in agreement with regard to the structure and content of the major components ALM F30 and [Aib^6] ALM F30. However, the discrepancy concerning the minor components is evident. Most of the minor components described in the HPLC study were not detected by NACE–ESI-IT-MS. This may be explained by the fact that the mass differences of $\pm$14 Da between compounds in many cases are not sufficient to translate into a separation in CE. On the other hand, most of the compounds found by NACE–MS were not identified by HPLC–MS. These components are characterized by an additional carboxy group caused by the exchange to Gln *versus* Glu or loss of the *C*-terminal amino alcohol. Apparently, the additional charge makes such compounds more suitable for CE analysis while they were 'missed' by HPLC. This demonstrates that HPLC and CE are complementary techniques due to the different separation mechanisms. For complete characterization of complex peptide mixtures, both techniques should be applied.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

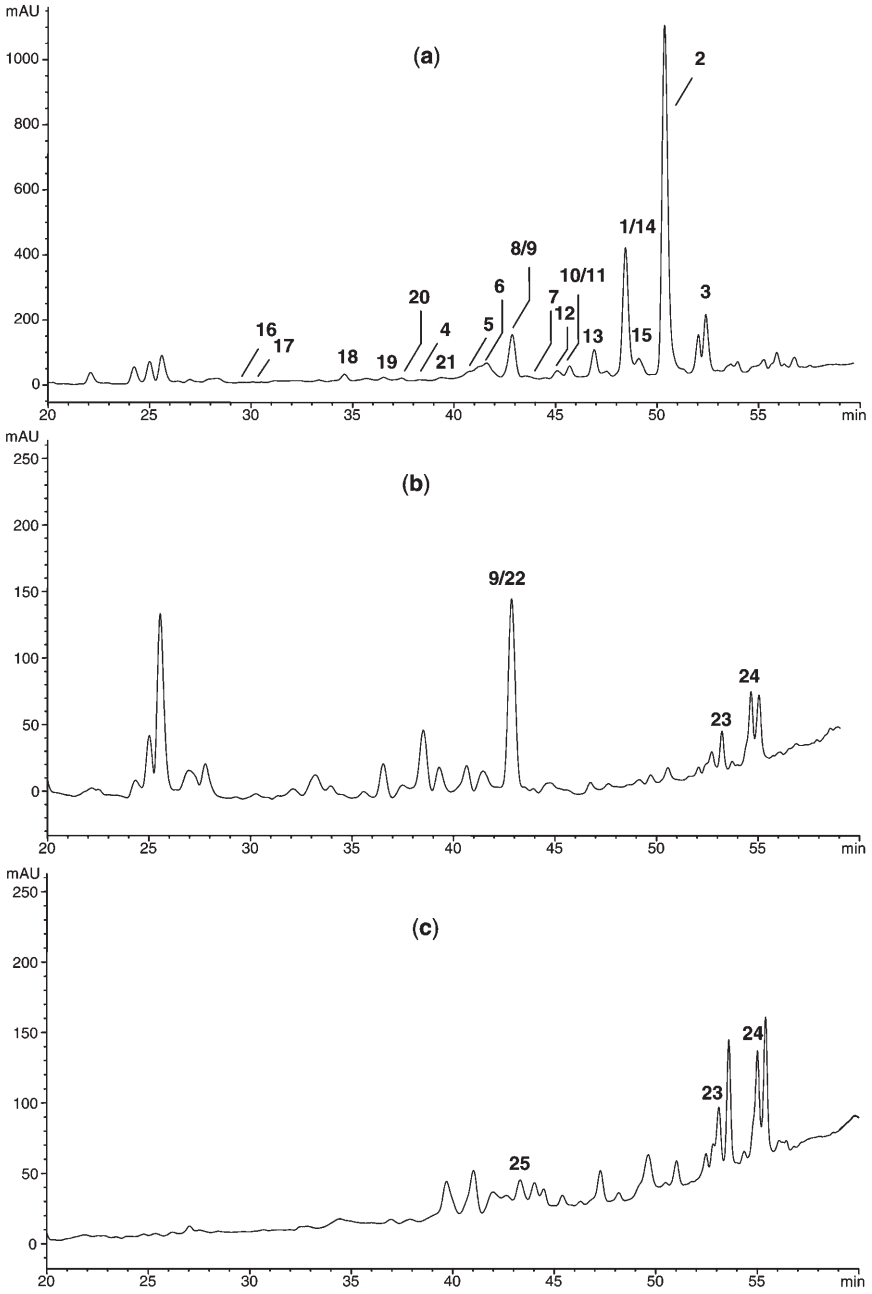

Figure. *HPLC Elution Profiles.* Peptaibiotic-containing fraction of (**a**) *Trichoderma* cf. *strigosum* BBA 69577, (**b**) *Trichoderma strigosum* CBS 348.93, (**c**) *Trichoderma erinaceus* DAOM 230019, (**d**) *Trichoderma* cf. *pubescens* BBA 66989, (**f**) *Trichoderma stromaticum* BBA 70638,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

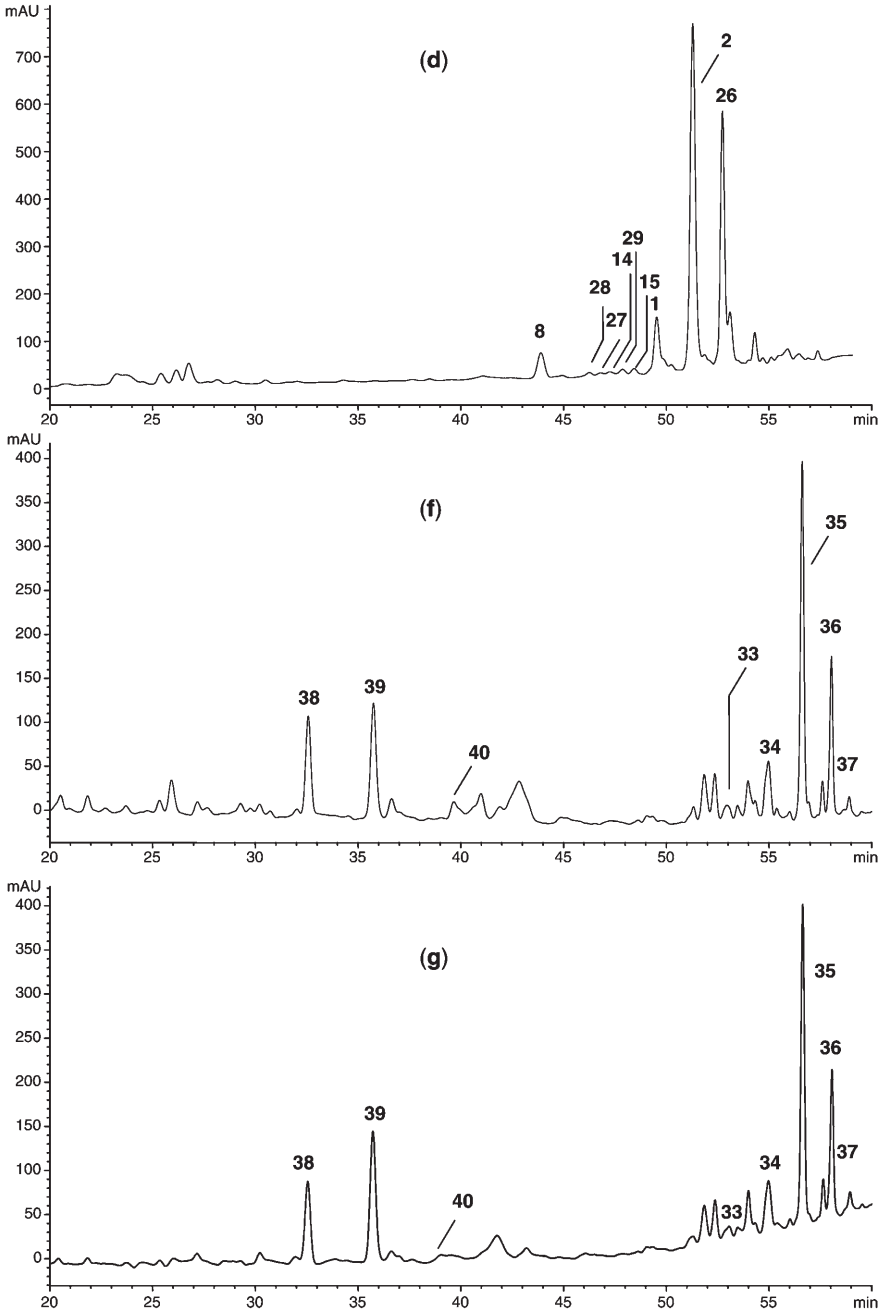

Figure (cont.)

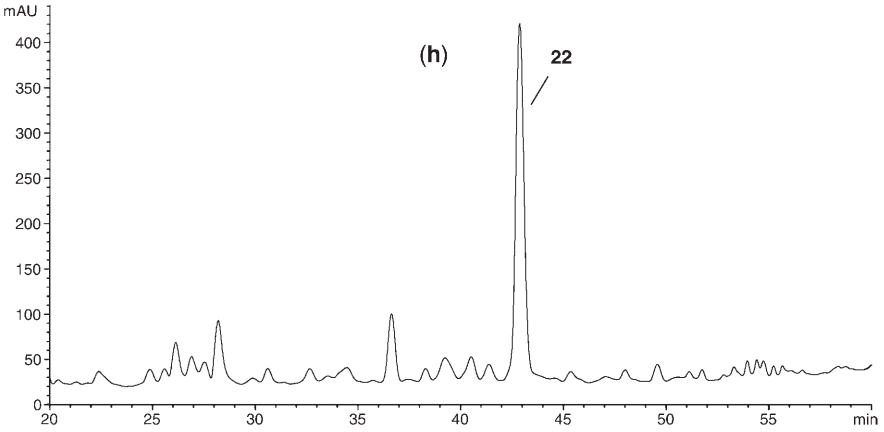

Figure (cont.)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Experimental Part

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

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

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

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

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

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

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

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Trichoderma brevicompactum Complex: Rich Source of Novel and Recurrent Plant-Protective Polypeptide Antibiotics (Peptaibiotics)

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Three strains of *Trichoderma brevicompactum* and another four that are closely related to that species (*Trichoderma* cf. *brevicompactum*) were analyzed for the formation of polypeptide antibiotics (peptaibiotics) by LC/ESI-MS^n. These isolates were selected because of an antagonistic potential against Eutypa dieback and Esca disease of grapevine and have not yet been investigated for the production of peptide antibiotics. Fully grown cultures on potato dextrose agar were extracted with CH_2Cl_2/MeOH, and this extract was subjected to SPE using C_{18} cartridges. The methanolic eluates were analyzed by LC/ESI-MS^n. All strains were found to produce membrane-active alamethicins F30. In addition to that, novel peptaibiotics were detected, namely, 14 12-residue trichocryptins B, 12 11-residue trichocryptins A, 19 11-residue trichobrevins A and B, 6 10-residue trichoferins, and 17 8-residue trichocompactins. These compounds may partially be responsible for the plant-protective action of the producers. Chemotaxonomic considerations also indicated the necessity to introduce another new species that is closely related to *T. brevicompactum*.

KEYWORDS: Peptaibiotic; peptaibol; alamethicin; α-aminoisobutyric acid; electrospray ionization mass spectrometry; peptide sequencing; *Trichoderma*; biocontrol

INTRODUCTION

More than 400 strains of 30 *Trichoderma* species were investigated in the course of a project aimed at preventive plant protection and biocontrol of two fungal diseases in organic viticulture: Eutypa dieback and Esca. These are latent trunk diseases that cause severe economic losses in organic grapevine production (*1*, *2*). The in vitro bioactivity of the *Trichoderma* strains against the causal agents of Eutypa dieback, *Eutypa lata*, and Esca disease, *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*, was evaluated in plate assays using crude extracts. The most active isolates were identified either as *Trichoderma brevicompactum* (*sensu stricto*, *ss*), or as *Trichoderma* cf. *brevicompactum* (*Trichoderma brevicompactum*, *sensu lato*, *sl*). Compared to the bioactivity of isolates representing well-known biocontrol *Trichoderma* species (e.g., *T. atroviride*, *T. harzianum*, *T. koningii*, and *T. viride*), crude extracts of strains belonging to the *T. brevicompactum* complex have been found to inhibit the growth of the above pathogens in vitro far more effectively (*3*). Detailed information about habitat and geographic origin of the seven isolates investigated in this study is given in **Table 1**.

T. brevicompactum, an anamorphic species with a pachybasium-like morphology, has been described from soil and tree bark in North, Central, and South America and southern Asia (*4*). The species was originally proposed to be phylogenetically closely related to *Hypocrea lutea* (*4*), but also discussed to be close to *Trichoderma minutisporum*/*Hypocrea minutispora*. As the alignment of translation−elongation factor (TEF) sequences turned out to be rather difficult, additional sequencing of the second largest RNA polymerase subunit (RPB2) has been performed. These experiments clearly indicated that the fungus known as *T. brevicompactum* comprises two phylogenetically different species that form a new lineage within the genus *Trichoderma*. These findings are further supported by the results of our work as outlined under Discussion.

Species of *Trichoderma* (teleomorphs in *Hypocrea*; *5*) are commercially used as bioprotective agents against many fungal diseases. Most commercial preparations are formulated on the basis of conidia, but application of biomass or chlamydospores

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Table 1. *Trichoderma* Strains Included in This Study

strain[a]	strains investigated[b]	habitat	geographic origin	yield[c] (mg)
1	CBS 109720 (*ex*-type)	soil in sunflower field	Geneva, NY	9.0
2	IBT 40840 (= CBS 119570)	soil	Iran	21.8
3	IBT 40839 (= CBS 119569)	soil	Qazvin, Iran	43.2
4	CBS 112445	soil	Costa Rica	4.0
5	IBT 40863 (= CBS 119577)	soil	Shar-e Kord, Chahar Mahall va Bakhtiari, Iran	14.5
6	ATCC 90237 (= CBS 119576)	micaceous clay from stream bed	Windhoek, Namibia	2.5
7	NRRL 3199[d]	unknown	unknown	17.4

[a] 1-4, *T. brevicompactum*; 5-7, *T.* cf. *brevicompactum.* [b] Abbreviations: ATCC, American Type Culture Collection, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DSM, Deutsche Stammsammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IBT, BioCentrum, DTU, Kgs. Lyngby, Denmark; NRRL, ARS Culture Collection, Northern Regional Research Laboratory, National Center for Agricultural Utilization Research, Peoria, IL. [c] Dry weight of the methanolic extracts obtained after cleanup over Sep-Pak C_{18} cartridges (see Experimental Procedures). [d] Alamethicin patent strain of Upjohn Co., Kalamazoo, MI.

has also been described. Choice of propagule for the preparation depends on both production system and intended application (*6*). The antifungal action of *Trichoderma* is complex. It depends on the species/strain studied and may involve competition for nutrients, plant root colonization, biofertilization, stimulation of plant resistance and defense mechanisms, rhizosphere modification, and different types of mycoparasitism. The latter may involve morphological changes such as coiling of parasite hyphae around the host and the formation of specialized appressorium-like structures (*7*−*11*).

The species of *Trichoderma* are known as saprotrophs, rare plant pathogens (*10*), or polyphagous mycoparasites, which are common in soil ecosystems. During the recent years, fungicolous fungi have attracted particular interest because of their bioactivity against economically important fungal diseases of crop plants, which cannot effectively be controlled by methods of classical plant protection (*12*).

Secondary metabolites of *Trichoderma* have extensively been reviewed (*13, 14*). Synergistic interactions between extracelluar metabolites such as wall-degrading chitinases, glucanases, and proteases, on the one hand, and antibiotics, on the other, have clearly been demonstrated in the past. The parallel formation of hydrolytic enzymes together with a group of membrane-active polypeptide antibiotics, named "peptaibiotics", and their synergistic action play an important role in mycoparasitism between *T. harzianum* and its fungal hosts such as *Botrytis cinerea* (*15, 16*). The term "peptaibiotic" was introduced by Brückner et al. (*17*) and reconsidered by Degenkolb et al. (*18*). It describes linear peptide antibiotics that (i) range from 500 to 2200 Da in molecular mass; (ii) show a high content of α-aminoisobutyric acid; (iii) are characterized by the presence of other nonproteinogenic amino acids and/or lipoamino acids; and (iv) possess an acylated N terminus, whereas the C terminus may consist of a free or methoxy-substituted 2-amino alcohol, amine, amide, free amino acid, diketopiperazine, or sugar alcohol. "Peptaibols" are regarded as a subgroup of the peptaibiotics, the N terminus of which is acetylated, whereas the C terminus is reduced to a 2-amino alcohol.

Recently, the term "peptaibiomics" was proposed by Krause et al. (*19*), describing—in analogy to proteomics—the approach to the analysis of the entirety of peptaibiotics, the so-called "peptaibiome", produced by a certain strain under defined conditions.

Peptaibiotics show interesting physicochemical and biological activities depending on particular structural properties, such as formation of pores in bilayer lipid membranes as well as antibacterial, antifungal, occasionally antiviral, insecticidal, and antiparasitic activities. Inhibition of mitochondrial ATPase, uncoupling of oxidative phosphorylation, immunosuppression, inhibition of platelet aggregation, and induction of fungal morphogenesis and neuroleptic effects have been reported (summarized in refs *18* and *19*).

Detailed information on structures of peptaibiotics and their classification into subfamilies (*20*) can be obtained from public Internet resources such as the "Peptaibol Database" (*21*). More than 250 peptaibiotics produced by members of the genus *Trichoderma* are described in the literature. Recently, a review comprising structures and properties of 186 different peptaibiotics from *Trichoderma* has been published (*14*).

Screening and sequencing of peptaibiotics with a molecular mass up to 2000 Da can be accomplished by advanced methods of tandem mass spectrometry, especially electrospray ionization (ESI-MS) techniques (for a review see ref *18*) and completed by GC/EI-MS and HPLC approaches (*22*).

As species identification of *Trichoderma* strains was demonstrated to be possible by image analysis of HPLC chromatograms (*23*), on-line coupling of HPLC and ESI-ion-trap-MS^n should therefore combine the advantages of both analytical techniques, thus providing a more reliable structural identification of compounds produced by a certain strain.

To date, 88 *Trichoderma* species have been characterized by sequencing of ribosomal DNA, and these data suggest that many species recognized on the basis of morphology have probably been misidentified in the past (*5, 24*). Recently, the species *Trichoderma viride* was subdivided into two species—*T. viride* and *T. asperellum*—on the basis of ribosomal DNA. Antibiotic production was exclusively restricted to *T. asperellum*, whereas *T. viride* (*ss*) produced no antibiotics (*25, 26*).

Therefore, it may be hypothesized that the production of peptaibiotics under standardized conditions might be used as a chemotaxonomic marker in support of morphological, molecular, and other (bio)chemical data for the differentiation between species of the genus *Trichoderma*.

None of the strains used in this study has been screened for peptaibol production, although six of them were previously shown to produce trichothecene-type mycotoxins, such as harzianum A and/or trichodermin (*27*). Recently, harzianum A was also detected in cultures of NRRL 3199, which is *T.* cf. *brevicompactum*.

The present study was aimed at (i) screening of selected plant-protective strains for the production of peptaibols and peptaibol-like antibiotics (peptaibiotics), (ii) sequencing of new and recurrent peptides found, and (*iii*) testing the above hypothesis concerning a possible use of the pattern of peptaibiotics for chemotaxonomy.

EXPERIMENTAL PROCEDURES

Chemicals. Acetonitrile (MeCN; Chromasolve for HPLC, far UV, 99.9%) and dichloromethane (ACS reagent, 99.6%) were obtained from Sigma-Aldrich (Steinheim, Germany); methanol (MeOH; 99.8%, gradi-

Table 2. Structural Variations of Peptaibiotics from the *T. brevicompactum* Complex[a]

	residue																			
peptaibiotic[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ALM F30	Ac	Aib	Pro	Aib	Ala	Aib	Ala	*Gln* *Glu*	Aib	*Aib* *Vxx* *Lxx*	Aib	Gly	*Vxx* *Lxx*	Aib	Pro	Vxx	Aib	Aib	Glu	Gln	Pheol
TCP	Ac	Aib	Gly	Ala	Lxx	*Aib* *Vxx*	*Gly* *Ala* *Ser*	*Vxx* *Lxx*	Vxx											
TBV	Ac	Aib	*Ala* *Ser*	*Aib* *Vxx*	*Aib* *Vxx* *Lxx*	Aib	Pro	Lxx	Lxx	Aib	Pro	*Alaol* *Aibol* *Vxxol* *Lxxol*								
TCT-A	Ac	*Vxx* *Lxx*	Aib	Pro	*Vxx* *Lxx*	Aib	Pro	*Aib* *Lxx*	*Aib* *Vxx* *Lxx*	Aib	Pro	Lxxol								
TCT-B	Ac	*Vxx* *Lxx*	Aib	Pro	Vxx	*Vxx* *Lxx*	Aib	Pro	*Aib* *Lxx*	*Aib* *Vxx* *Lxx*	Aib	Pro	Lxxol							
TF	MDA	Pro	*AHMOD* *desmethyl-AHMOD*	Ala	Aib	*Aib* *Vxx*	*Aib* *Vxx* *Lxx*	*Gly* *Ala* *Aib*	Aib	Aib	*AAE* *AMAE*									

[a] Exchangeable positions in a general sequence are italicized. A list of sequences of peptaibiotics detected in the individual strains is presented in the captions to **Figures 1** and **2**. [b] ALM F30, alamethicin F30; TCP, trichocompactin; TBV, trichobrevin; TCT-A, trichocryptin A; TCT-B trichocryptin B; TF, trichoferin.

ent grade, for HPLC) and trifluoroacetic acid (TFA, 98.0%) were purchased from Fluka (Steinheim, Germany). Toluene (SupraSolv, 99%, for gas chromatography) was bought from VWR International (Darmstadt, Germany). Anhydrous KH_2PO_4 and $Na_2HPO_4 \cdot H_2O$ were from Fluka, and methyl orange (helianthin) was from Riedel-de Haën (Seelze, Germany). Bidistilled water was freshly prepared from demineralized tap water prior to analysis using a quartz distil (Heraeus, Kleinostheim, Germany).

Cultivation of Strains. Cultures were grown at room temperature (23−26 °C) under ambient daylight on Difco potato dextrose agar (PDA, lot 4300389) obtained from Becton Dickinson (BD, Heidelberg, Germany). The medium was prepared according to the directions of the manufacturer and autoclaved at 121 °C for 15 min without pH adjustment. A final pH of 5.6 ± 0.2 was measured after sterilization.

Subcultures were inoculated from PDA slants used for preservation of strains, and a loop of conidia was streaked on 9.5 cm diameter plastic Petri dishes containing 20 mL of PDA. Subcultures were grown for 4 days and used for inoculation of the main culture.

Extraction of Peptaibiotics. After 6 days of cultivation, fungal cultures were extracted with a mixture of CH_2Cl_2/MeOH, 1:1. To prevent any possible contamination of the extracts by plasticizers, a ring of aluminum with a small spout on its upper margin was punched into the agar before application of the solvent mixture. A 5 mL aliquot of the solvent was applied onto the surface of each plate culture and spread with a Drigalski spatula, and the extract was poured off through the spout. This procedure was repeated twice; the combined extracts from each agar plate were transferred into Pyrex tubes and centrifuged at 2400*g* for 30 min. The supernatant was filtered and evaporated to dryness in vacuo.

Cleanup was performed using Sep-Pak C_{18} cartridges (Waters Corp., Milford, MA) as previously described (*19*). Briefly, each cartridge (dimensions: 1.5 cm × 1 cm i.d.) was conditioned by successive addition of MeOH, H_2O, and H_2O/MeOH, 2:1 (10 mL each). The sample was redissolved in H_2O/MeOH, 2:1, and centrifuged at 2400*g* for 30 min, and the supernatant was filtered; the filtrate was applied to the conditioned cartridge. The cartridge was rinsed with H_2O and H_2O/MeOH, 2:1 (10 mL each). Finally, peptaibiotics were eluted with 10 mL of MeOH. The eluate was evaporated to dryness in vacuo. The dry weight of the residue (see **Table 1**) was determined using an analytical balance. A 10 μL aliquot of a 1% methanolic solution that had been freshly prepared from the dried residue of the methanolic eluate prior to analysis was used for HPLC or ion-trap ESI-LC-MS measurements, respectively.

HPLC and Ion-Trap-ESI-LC-MS Measurements. For HPLC, a HP 1100 series instrument was used. ESI mass spectra were recorded on an LCQ instrument (Thermo Finnigan MAT, San Jose, CA). The gradient used for HPLC and ion-trap-ESI-LC-MS measurements was described previously (*19*); further details concerning the analytical equipment were given in an earlier paper (*28*). A CID energy of 45 or 65 eV was applied to generate sequence-specific *b*- and *y*-type fragments from putative $[M + H]^+$, $[M + Na]^+$, or sequence-specific fragment ions, respectively. The collision energy for MS/MS and MS^n measurements was set between 25 and 65 eV, typically at 45 eV.

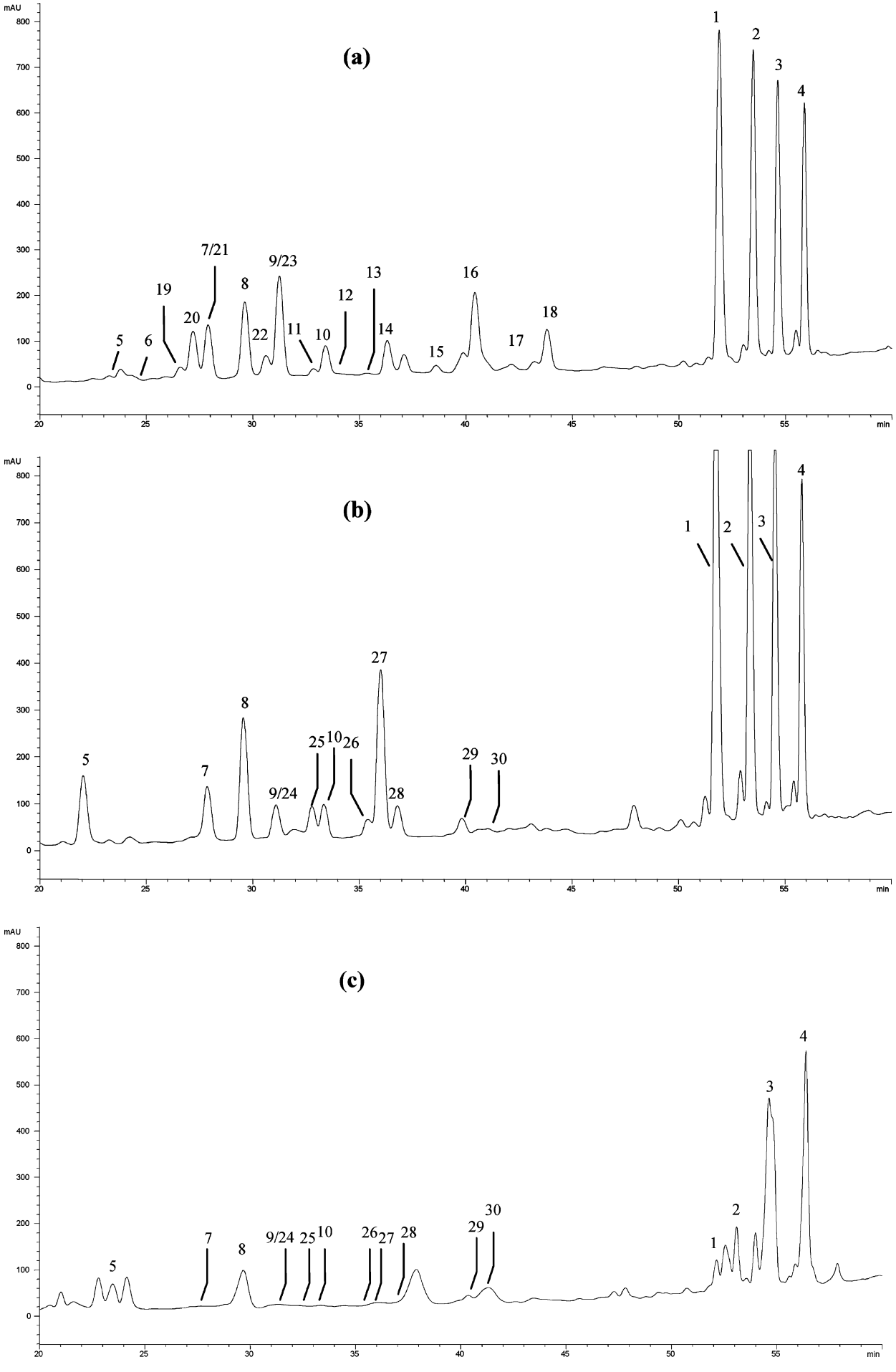
Fragment ion series were assigned in accordance with the Roepstorff/Fohlman−Biemann nomenclature used previously. In cases when the isomeric amino acids Leu/Ile or Val/Iva (Iva, isovaline) could not be distinguished, the abbreviations Lxx and Vxx were used instead (*29*, *30*).

RESULTS

Possible structural variations of all peptaibiotics investigated in this study are summarized in **Table 2**. HPLC elution profiles (detection wavelength $\lambda = 205$ nm) of the peptaibiotic-containing fraction from all strains are shown in **Figures 1** and **2**. In the following section, the HPLC/ESI-MS^n-based sequencing and structural characterization of peptaibiotics produced by *T. brevicompactum* and *T.* cf. *brevicompactum* are described.

***T. brevicompactum* CBS 109720.** The HPLC elution profile of this strain (**Figure 1a**) is dominated by four major peaks. Furthermore, MS/MS, MS^n, and CID-MS investigations and comparison of these results with the recent literature (*28*) and data obtained from experiments with authentic material from *T. viride* NRRL 3199 confirmed the structures of these compounds as the acidic alamethicins (ALM): **1**, F30/3; **2**, F30/5; **3**, F30/7; and **4**, F30/9. The strains of the *T. brevicompactum* group were not screened for the presence of neutral alamethicins F50 (ALM F50) in the course of this study. Analysis of that subgroup would have required the same conditions as described above but without TFA in the eluents. Voltage-dependent pore formation and antimicrobial activity of alamethicins have been reviewed (*31*). Alamethicins are, so far, only known from *T. viride* NRRL 3199 (*28*), which now can be classified as *T.* cf. *brevicompactum* (*3*).

A second group of six novel eight-residue peptaibiotics from ***Tricho**derma brevi**compact**um* was detected. We name these compounds **trichocompactins** (TCP) **5**, Ia; **6**, Ib; **7**, IIa; **8**, IIb;


(a)
mAU
1
2
3
4
7/21
9/23
8
19
20
13
16
12
18
5
6
22
11
10
14
15
17
min
(b)
mAU
1
2
3
4
27
8
5
7
25
10
26
29
30
9/24
28
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29
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min

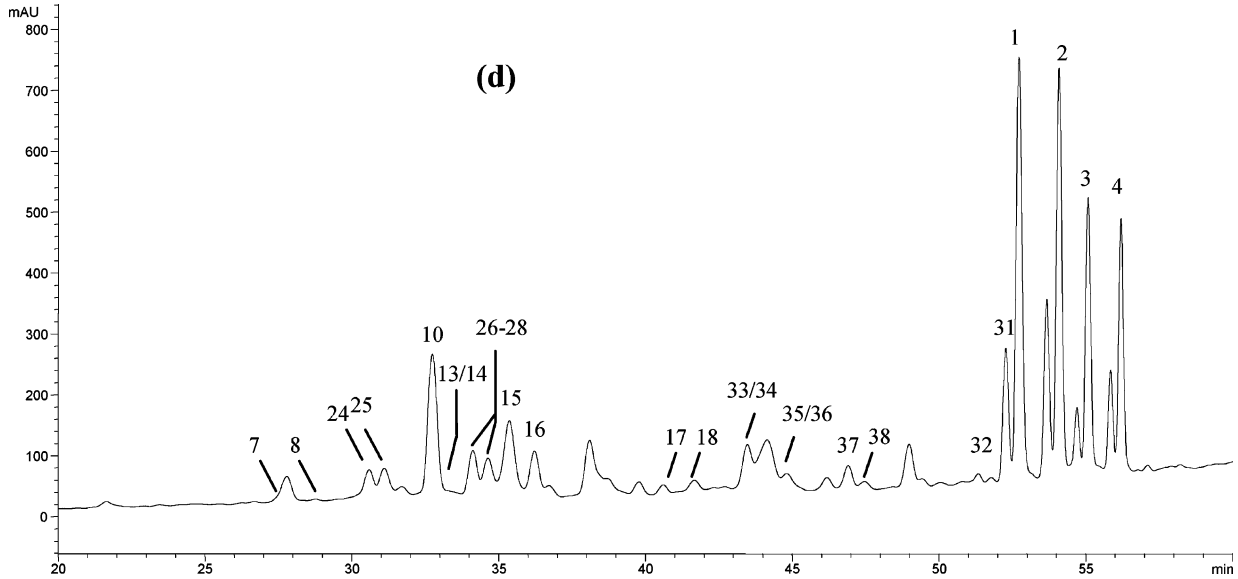

Figure 1. HPLC elution profiles of the peptaibiotic-containing fraction of *T. brevicompactum* strains (**a**) CBS 109720, (**b**) IBT 40839, (**c**) IBT 40840, and (**d**) CBS 112445. Annotations refer to consecutive numbering of peptides used in the text. Numbers separated by a slash refer to coeluting peptides. (**a**) Alamethicins (**1**–**4**), trichocompactins (**5**–**10**), trichocryptins B (**11**–**18**), trichocryptins A (**19**–**23**); (**b**) alamethicins (**1**–**4**), trichocompactins (**5**–**10**), trichocryptins A (**24**–**30**); (**c**) alamethicins (**1**–**4**), trichocompactins (**5**–**10**), trichocryptins A (**24**–**30**); (**d**) alamethicins (**1**–**4**, **31**, and **32**), trichocompactins (**5**–**10**), trichocryptins A (**23**–**28**), trichocryptins B (**13**–**18** and **33**–**38**).

9, IIIa; and **10**, IIIb. Their fragmentation patterns and sequences are listed in **Tables 3** and **4**.

The N-terminal sequence Ac-Aib-Gly-Ala-Leu-Aib was previously described for the trichovirins—peptaibols with a C-terminal Gln-Leuol motif from *T. viride* NRRL 5243 (*30*). That strain is currently deposited as *T. harzianum* sl. Furthermore, valine as a C-terminal residue is known from the trichobrachins TB IIa A and B, only, which have been isolated from *T. longibrachiatum* CBS 936.69 (*32*), a strain now reclassified as *T. paceramosum*/*T. ghanense*.

A third group of homologous peptaibols exhibited *m*/*z* 1210, 1224, 1238, and 1252, which were accompanied by *m*/*z* 1226, 1240, 1254, and 1268, respectively. It was demonstrated by CID-MS^n that the former series of ions represents the predominant $[M+ Na]^+$, whereas the latter corresponds to the $[M + K]^+$ adduct, which is present in smaller amounts. Because the $[M + H]^+$ ions of any of these compounds were never observed, the intensive sodiated adducts had to be selected as precursors for sequencing. Compounds **11**−**18** are novel 12-residue peptaibols from ***Tricho****derma*, which we name **trichocryptins B** (TCT-B) I, II, III, and IV—owing to the **crypti**c behavior of their $[M + H]^+$ ions.

Fragmentation of $[M + Na]^+$, as illustrated in **Table 5**, exclusively generated a sodiated *y*-type series of daughter ions (y_2−y_8), which was dominated by the corresponding series of sodiated *x*-type ions, thus leading to complete suppression of N-terminal fragments. Loss of water from the $[M + Na]^+$ ions indicated the presence of a C-terminal amino alcohol. The first sequence-specific pair of fragment ions is y_2/x_2. The diagnostic difference of either *m*/*z* 201 or 215 supports the presence of a C-terminal Pro-Vxxol or Pro-Lxxol, which is followed by an Aib residue. The extremely labile tertiary Aib−Pro bond is preferably cleaved (*33*), thus explaining the absence of y_1/x_1 fragments. Cleavage of the Aib−Pro bond between positions 6 and 7 is the reason for the generation of an additional intensive sodiated *y*-type fragment comprising amino acids 7−12 (cf. **Tables 2** and **6**). Further sequence information was obtained from CID-MS experiments: Application of a CID energy of 45 and 65 eV generated the diagnostic fragments b_2−b_6 and their corresponding *y*-type ions. The structure of these *b*- and *y*-type ions was confirmed by CID-MS^n experiments. However, attempts to detect the b_1 fragment by CID-MS^n of the ions b_6 and b_5 were unsuccessful. Moreover, the intensity of b_2−b_4 was insufficient to perform further CID-MS^n investigations. Despite this, literature data revealed a single sequence, corresponding only to the pair of b_2/b_3 ions *m*/*z* 241/338 present in compounds **12** and **14**−**18**: the N-terminal fragment Ac-Leu-Aib-Pro has previously been described for the cervinins I and II—12-residue peptaibol antibiotics from *Mycogone cervina* A09-02, parasitizing *Helvella* (*Paxina*) *acetabulum* (*34*). Assuming structural homology, the b_2/b_3 ion pair *m*/*z* 227/324 could represent Ac-Val-Aib-Pro as an N-terminal sequence of compounds **11** and **13**. The sequence Ac-Val-Aib is known from the protonophoric bergofungin A from *Emericellopsis donezkii* HKI 0059 (*35*) as well as from the antiprotozoic/antihelminthic antiamoebins XIII and XIV from *Stilbella fimetaria* (syn. *Stilbella erythrocephala*) ATCC 28144 (*22*). The corresponding isoforms Ac-Ile-Pro and Ac-Iva-Pro have not been described as N termini of peptaibiotics, yet.

The partial sequence Pro-Aib-Leu-Aib-Pro-Leuol is known as the C terminus of harzianins HC I, HC VI, HC XI, and HC XIV from *T. harzianum* M-903614 and M-903603 (*33*), whereas the other C-terminal sequences listed in **Table 6** represent new structural variations.

The strain produces a fourth group of homologous peptaibols displaying *m*/*z* 1125 (compounds **19**−**21**) and 1139 (compounds **22**/**23**, all $[M + Na]^+$). Basically, the mass spectrometric fragmentation of these substances follows the same general scheme described above for compounds **11**−**18**. The CID-MS experiments generated a series of the diagnostic fragments b_2−b_5. Briefly, the—presumably invariable—Vxx residue at position 4 of the peptide chain is lost, thus leading to the appearance of novel 11-residue peptaibols, which we name **trichocryptins A** (TCT-A) I and II. Fragmentation patterns and sequences of these compounds are listed in **Tables 5** and **6**, respectively. Additional homologues and positional isomers of compounds **11**−**23** are

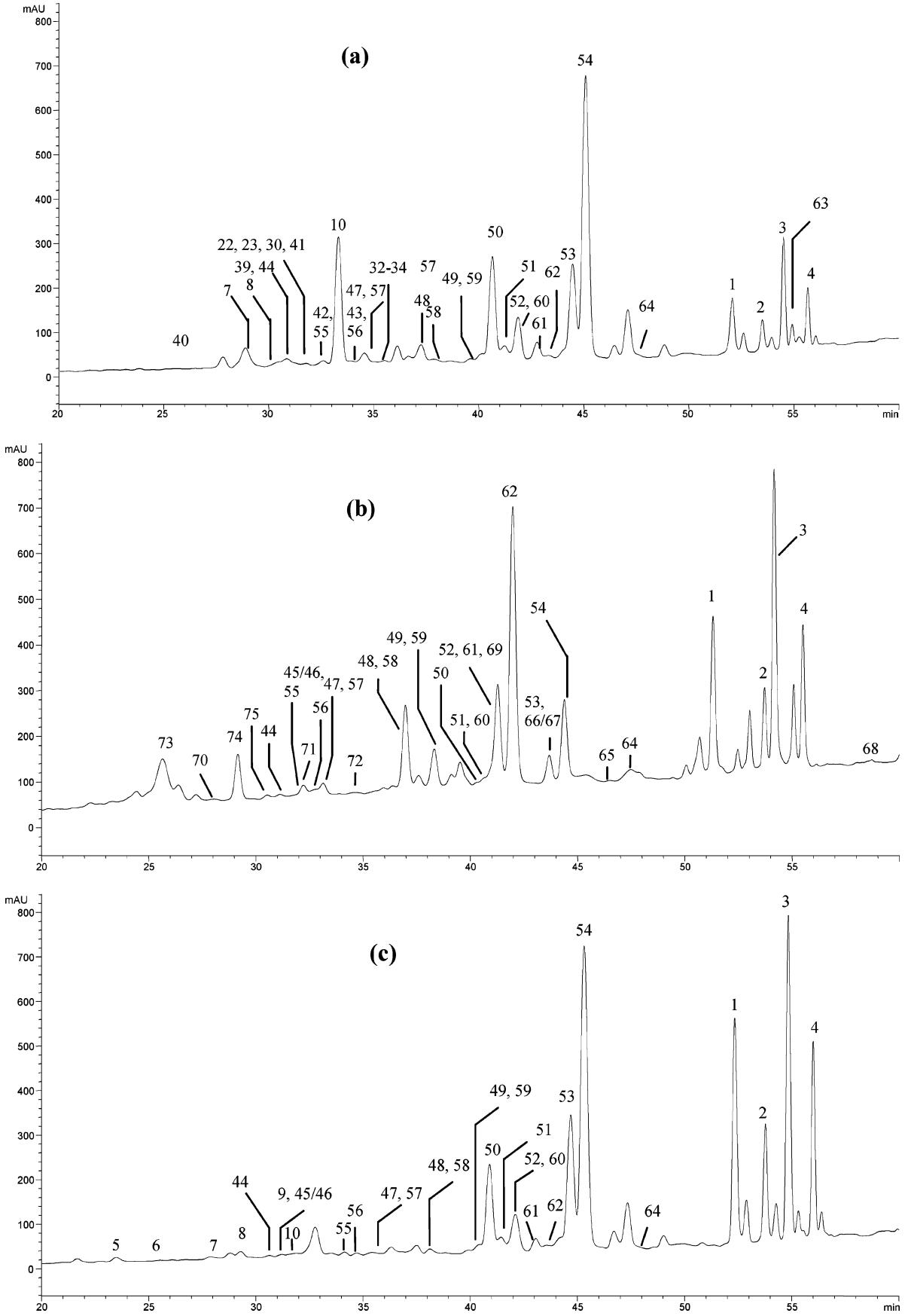

Figure 2. HPLC elution profiles of the peptaibiotic-containing fraction of *T.* cf. *brevicompactum* strains (**a**) ATCC 90237, (**b**) NRRL 3199, and (**c**) IBT 40863. Annotations refer to consecutive numbering of peptides used in the text. Numbers separated by a slash refer to coeluting peptides. (**a**) Alamethicins (**1**–**4** and **63**), trichocompactins (**5**–**8**, **10**, and **39**–**43**), trichocryptins B (**15**–**18** and **33**–**36**), trichobrevins A and B (**44**–**62**), trichoferin A (**64**); (**b**) alamethicins (**1**–**4**), trichocompactins (**70**–**75**), trichobrevins (**44**–**62**); (**c**) alamethicins (**1**-**4**), trichocompactins (**5**–**10**), trichocryptins B (**13**–**18**), trichcryptins A (**22**–**28**), trichobrevins A and B (**44**–**62**), trichoferin A (**64**).

Table 3. Diagnostic Fragment Ions (*m*/*z*) of Trichocompactins[a] Produced by Members of the *T. brevicompactum* Complex

	5	**6**	**7**	**8**	**9**	**10**	**40**	**39**	**41**	**42**	**43**
ion	Ia	Ib	IIa	IIb	IIIa	IIIb	IV	Va	Vb	VIa	VIb
$[M + H]^+$	726	726	740	740	754	754	756	770	770	784	784
$[M - H_2O]^+$	708	708	722	722	nd	736	738	752	752	766	766
$[M + Na]^+$	748	748	762	762	776	776	778	792	792	806	806
b_1	nd[b]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
b_2	184	nd	184	184	184	nd	184	184	184	nd	184
b_3	255	269	255	255	255	nd	255	255	255	nd	255
b_4	368	354	368	368	368	368	368	368	368	381	368
b_5	453	439	453	453	467	467	453	453	453	467	467
$b_6 - H_2O$	nd	nd	nd	nd	nd	nd	522	522	522	nd	nd
b_6	510	496	510	510	524	524	540	540	540	nd	554
$b_7 - H_2O$	nd	nd	nd	nd	nd	nd	621	635	635	649	649
b_7	609	609	623	623	637	637	639	653	653	667	667
a_4	340	nd	340	nd	340	340	340	340	340	nd	nd
$y_3 - H_2O$	nd	nd	nd	nd	nd	nd	nd	300	nd	nd	nd
y_3	nd	nd	287	nd	nd	287	nd	318	nd	nd	nd
$y_4 - H_2O$	nd	nd	nd	nd	nd	nd	nd	nd	385	nd	nd
y_4	nd	nd	372	nd	nd	nd	nd	nd	nd	nd	nd
y_5	nd	nd	nd	485	nd	nd	nd	516	nd	nd	nd

	70	**71**	**72**	**73**	**74**	**75**
ion	VII	VIIIa	VIIIb	IX	Xa	Xb
$[M + H]^+$	740	754	754	756	770	770
$[M - H_2O]^+$	722	736	736	nd	752	752
$[M + Na]^+$	762	776	776	778	792	792
b_1	nd	nd	nd	nd	nd	nd
b_2	184	184	184	184	184	184
b_3	255	255	255	255	255	255
b_4	368	368	368	368	368	368
$b_5 - H_2O$	nd	nd	nd	nd	nd	nd
b_5	453	453	453	453	453	453
$b_6 - H_2O$	nd	nd	nd	522	522	522
b_6	524	524	524	nd	540	540
$b_7 - H_2O$	nd	nd	nd	621	635	635
b_7	623	637	637	nd	653	653
a_4	340	nd	nd	340	340	340
$y_3 - H_2O$	nd	nd	nd	nd	nd	300
y_3	nd	302	nd	nd	nd	nd
y_5	485	nd	nd	nd	516	nd
y_6	nd	nd	nd	nd	nd	601

[a] Arabic numbers in bold refer to the consecutive numbering of peptides used throughout the text and in **Figures 1** and **2**. Roman numerals followed by lower case Arabic letters in **Tables 3**–**8** refer to the abbreviations used for the individual trichocompactins, trichocryptins, and trichobrevins. Capital letters in **Tables 9** and **10** refer to the abbreviations used for the individual trichoferins. Capital letters for characterization of trichoferins are introduced for conformity reasons in the nomenclature of lipoaminopeptides. [b] Not detected.

expected from the corresponding TIC traces. However, their structures could not be determined due to their low abundance in the mixture.

***T. brevicompactum* IBT 40839.** Analysis of the four major peaks displayed in the HPLC elution profile (**Figure 1b**) and comparison of these data with those obtained for strain CBS 109720 confirmed the presence of compounds **1**−**5** and **7**−**10**.

In contrast to what was found in strain CBS 109720, strain IBT 40839 did not produce any of the compounds **11**−**23**, but a mixture of peptaibols with molecular masses *m*/*z* 1153 (**24**/**25**), 1167 (**26**−**28**), and 1181 (**29**/**30**, all $[M + Na]^+$), representing higher homologues of trichocryptins A I and A II. Fragmentation and sequences of these trichocryptins A III, IV, and V are listed in **Tables 5** and **6**. The C-terminal motif of trichocryptins A IV b, IV c, V a, and V b, that is, Pro-Leu-Leu-Aib-Pro-Leuol, has previously been described for harzianin HK VI from *T. pseudokoningii* MVHC 662 (*36*) and the hypomurocins A I, A II, A IV, and A V—hemolytic peptaibols

Table 4. Sequences of Trichocompactins I–X Produced by Members of the *T. brevicompactum* Complex[a]

			residue								
			1	2	3	4	5	6	7	8	$[M + H]^+$
5	Ia	Ac	Aib	Gly	Ala	Lxx	Aib	Gly	Vxx	Vxx	726
6	Ib				[269]	Aib	Aib	Gly	Lxx	Vxx	726
7	IIa	Ac	Aib	Gly	Ala	Lxx	Aib	Gly	Lxx	Vxx	740
8	IIb	Ac	Aib	Gly	Ala	Lxx	Aib	Gly	Lxx	Vxx	740
9	IIIa	Ac	Aib	Gly	Ala	Lxx	Vxx	Gly	Lxx	Vxx	754
10	IIIb	Ac	Aib	Gly	Ala	Lxx	Vxx	Gly	Lxx	Vxx	754
40	IV	Ac	Aib	Gly	Ala	Lxx	Aib	Ser	Vxx	Vxx	756
39	Va	Ac	Aib	Gly	Ala	Lxx	Aib	Ser	Lxx	Vxx	770
41	Vb	Ac	Aib	Gly	Ala	Lxx	Aib	Ser	Lxx	Vxx	770
42	VIa	Ac	Aib	Gly	Ala	Lxx	Aib	Ser	Lxx	Vxx	784
43	VIb	Ac	Aib	Gly	Ala	Lxx	Vxx	Ser	Lxx	Vxx	784
70	VII	Ac	Aib	Gly	Ala	Lxx	Aib	Ala	Vxx	Vxx	740
71	VIIIa	Ac	Aib	Gly	Ala	Lxx	Aib	Ala	Lxx	Vxx	754
72	VIIIb	Ac	Aib	Gly	Ala	Lxx	Aib	Ala	Lxx	Vxx	754
73	IX	Ac	Aib	Gly	Ala	Lxx	Aib	Ser	Lxx	Vxx	756
74	Xa	Ac	Aib	Gly	Ala	Lxx	Aib	Ser	Lxx	Vxx	770
75	Xb	Ac	Aib	Gly	Ala	Lxx	Aib	Ser	Lxx	Vxx	770

[a] Bold numbers in the first column refer to consecutive numbering of peptides used throughout the text. Abbreviations of compound names used in the second column refer to the individual compounds introduced in the text.

from strain IFO 31288 (*37*). That strain was originally described as *Hypocrea muroiana*, but recently demonstrated to be *Trichoderma atroviride*/*Hypocrea atroviridis* by internal transcript spacer (ITS) and elongation factor (EF) sequencing.

***T. brevicompactum* IBT 40840.** Analysis of the four major peaks displayed in the HPLC elution profile (**Figure 1c**) and comparison of these data with those obtained for strain CBS 109720 confirmed the presence of compounds **1**−**9**. The strain also produces peptaibols with molecular masses *m*/*z* 1153, 1167, and 1181 (all $[M + Na]^+$), having the same retention time(s) and thus supposed to be identical or positionally isomeric with compounds **24**−**30** described for strain IBT 40839.

***T.* cf. *brevicompactum* CBS 112445.** Analysis of the four major peaks displayed in the HPLC elution profile (**Figure 1d**) and comparison of these data with those obtained for strain CBS 109720 confirmed the presence of compounds **1**−**4**. Additional ALMs are present, the sequence of which could only partially be assigned by MS/MS, which is due to their low abundance in the mixture. For example, fragmentation of *m*/*z* 1950 $[M + H]^+$ at $t_R = 50.4$ and comparison with literature data reported for ALMs F30 (*28*) indicated that alamethicin F30/2 (compound **31**) could be present as a minor compound. Another novel minor compound, **32**, was detected during fragmentation of *m*/*z* 1964 ($[M + H]^+$) at $t_R = 50.9$. Again, assuming structure homology with literature data deduced for the ALM F30 peptides (*28*), including invariability of amino acid residues 1 and 2, the following possible sequences are proposed for this new ALM F30/11 as the intensity of the fragment ions obtained during MS^3 was insufficient to perform further MS^n experiments. According to structure homologies with compounds **1**−**4**, the variable positions 3, 5, and 8 in compound **32** consist of either Ala or Aib, respectively, whereas positions 9−20 are invariable. Theoretically, three positional isomers are possible. Compounds **5**−**10** are also present—the latter displaying a particularly intense peak in that part of the HPLC elution profile. Sodiated molecular ions *m*/*z* 1139, 1153, and 1167 were detected, which may represent compounds **22**−**28** or homologues thereof.

A fourth group of peptaibols with molecular masses *m*/*z* 1224, 1238, and 1252 may consist of homologues and positional isomers of compounds **13**−**18**. In contrast to what has been

Table 5. Diagnostic Fragment Ions (*m*/*z*) of Trichocryptins A and B Produced by Members of the *T. brevicompactum* Complex[a]

ion	**11** B-Ia	**12** B-Ib	**13** B-IIa	**14** B-IIb	**15** B-IIIa	**16** B-IIIb	**17** B-IVa	**18** B-IVb	**33** B-Va	**34** B-Vb	**35** B-Vc	**36** B-Vd	**37** B-VIa	**38** B-VIb
$[M + Na]^+$	1210	1210	1224	1224	1238	1238	1252	1252	1266	1266	1266	1266	1280	1280
$[M + K]^+$	1226	1226	1240	1240	1254	1254	1268	1268	1282	1282	1282	1282	1296	1296
$[M + Na - H_2O]^+$	1192	1192	nd	nd	nd	1220	1234	1234	nd	nd	nd	nd	1262	1262
b_1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
b_2	227	nd	227	241	nd	241	nd	241	241	241	241	241	241	241
b_3	324	nd	324	338	338	338	nd	338	338	338	338	338	338	338
b_4	423	nd	423	437	437	437	437	437	437	437	437	437	437	437
b_5	522	536	522	536	536	536	550	550	550	550	536	550	550	550
b_6	607	621	607	621	621	621	635	635	635	635	621	635	635	635
$[Pro\text{-}Vxx + H]^+$	197	nd	197	197	197	197	197	197	197	197	197	197	197	197
$[Pro\text{-}Vxx\text{-}Vxx + H]^+$	296	nd	296	296	296	296	nd	nd	nd	nd	296	296	296	296
$[Pro\text{-}Vxx\text{-}Lxx + H]^+$	nd	nd	nd	nd	nd	nd	310	310	310	310	nd	nd	310	310
$[Pro\text{-}Vxx\text{-}Vxx\text{-}Aib + H]^+$	381	nd	381	381	381	381	nd	nd	nd	nd	381	nd	nd	nd
$[Pro\text{-}Vxx\text{-}Lxx\text{-}Aib + H]^+$	nd	nd	nd	nd	nd	nd	395	395	395	395	nd	nd	395	nd
$[Pro\text{-}Vxx\text{-}CO + H]^+$	169	nd	169	169	169	169	169	169	169	169	169	169	nd	nd
$[Pro\text{-}Vxx\text{-}Vxx\text{-}CO + H]^+$	268	nd	268	268	268	268	nd	nd	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Vxx\text{-}Lxx\text{-}CO + H]^+$	nd	nd	nd	nd	nd	nd	282	282	282	282	nd	nd	282	nd
$[Pro\text{-}Vxx\text{-}Vxx\text{-}Aib\text{-}CO + H]^+$	nd	nd	353	nd	nd	353	nd	nd	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Vxx\text{-}Lxx\text{-}Aib\text{-}CO + H]^+$	nd	nd	nd	nd	nd	nd	nd	367	nd	nd	nd	nd	367	nd
$[Pro\text{-}Aib\text{-}Aib\text{-}Aib\text{-}Pro\text{-}Lxxol + Na]^+$	nd	589	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Aib\text{-}Vxx\text{-}Aib\text{-}Pro\text{-}Lxxol + Na]^+$	603	nd	nd	603	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Aib\text{-}Lxx\text{-}Aib\text{-}Pro\text{-}Lxxol + Na]^+$	nd	nd	617	nd	617	617	617	617	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Lxx\text{-}Lxx\text{-}Aib\text{-}Pro\text{-}Vxxol + Na]^+$ or $[Pro\text{-}Vxx\text{-}Lxx\text{-}Aib\text{-}Pro\text{-}Lxxol + Na]^+$	nd	nd	nd	nd	nd	nd	nd	nd	631	631	nd	631	nd	nd
$[Pro\text{-}Lxx\text{-}Lxx\text{-}Aib\text{-}Pro\text{-}Lxxol + Na]^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	645	nd	645	645
$[Pro\text{-}Aib + H]^+$	nd	nd	183	183	nd	183	183	183	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Aib\text{-}Lxx + H]^+$	nd	nd	296	nd	296	296	296	296	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Aib\text{-}Lxx\text{-}Aib + H]^+$	nd	nd	381	nd	381	381	381	381	nd	nd	nd	nd	nd	nd
$(y_8 + Na)^+$	nd	nd	nd	nd	nd	445	459	459	459	459	nd	nd	459	459
$(y_7 + Na)^+$	nd	nd	544	558	558	558	572	572	572	572	558	nd	572	nd
$(y_6 + Na)^+$	629	nd	629	643	643	643	657	657	657	657	643	nd	657	nd
$(y_5 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	754	nd
$(y_4 + Na)^+$	811	825	811	825	825	825	839	839	853	867	853	867	867	867
$(y_3 + Na)^+$	910	910	924	924	938	938	952	952	966	980	966	980	980	980
$(y_2 + Na)^+$	995	995	1009	1009	1023	1023	1037	1037	1051	1065	1051	1065	1065	1065
$(y_1 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$(x_8 + Na)^+$	417	nd	nd	nd	nd	417	431	431	nd	nd	431	431	431	nd
$(x_7 + Na)^+$	516	530	516	530	530	530	544	544	544	544	530	544	544	544
$(x_6 + Na)^+$	601	615	601	615	615	615	629	629	629	629	615	629	629	629
$(x_5 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$(x_4 + Na)^+$	783	797	783	797	797	797	811	811	825	839	825	839	839	839
$(x_3 + Na)^+$	882	882	896	896	910	910	924	924	938	952	938	952	952	952
$(x_2 + Na)^+$	967	967	981	981	995	995	1009	1009	1023	1037	1023	1037	1037	1037
$(x_1 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

ion	**19** A-Ia	**20** A-Ib	**21** A-Ic	**22** A-IIa	**23** A-IIb	**24** A-IIIa	**25** A-IIIb	**26** A-IVa	**27** A-IVb	**28** A-IVc	**29** A-Va	**30** A-Vb
$[M + Na]^+$	1125	1125	1125	1139	1139	1153	1153	1167	1167	1167	1181	1181
$[M + K]^+$	1141	1141	1141	1155	1155	1169	1169	1185	1185	1185	1197	1197
$[M + Na - H_2O]^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
b_1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
b_2	227	227	241	241	241	241	241	241	241	241	241	241
b_3	324	324	338	338	338	338	338	338	338	338	338	338
b_4	423	423	437	437	437	437	437	451	437	437	451	451
b_5	508	508	522	522	522	522	522	536	522	522	536	536
$[Pro\text{-}Vxx + H]^+$	197	197	197	197	197	197	197	197	197	197	nd	nd
$[Pro\text{-}Vxx\text{-}Aib + H]^+$	282	282	282	282	282	282	282	nd	282	282	nd	nd
$[Pro\text{-}Vxx\text{-}Vxx + H]^+$	nd	nd	nd	nd	nd	nd	nd	296	nd	nd	nd	nd
$[Pro\text{-}Vxx\text{-}Lxx + H]^+$ or $[Pro\text{-}Lxx\text{-}Vxx + H]^+$	nd	nd	nd	nd	nd	nd	310	nd	nd	nd	nd	nd
$[Pro\text{-}Lxx\text{-}Lxx + H]^+$	nd	nd	nd	nd	nd	324	nd	nd	324	324	nd	nd
$[Pro\text{-}Lxx\text{-}Lxx\text{-}Aib + H]^+$	nd	nd	nd	nd	nd	nd	nd	nd	409	409	409	nd
$[Pro\text{-}Vxx\text{-}Vxx\text{-}Aib + H]^+$	nd	nd	nd	nd	nd	nd	nd	381	nd	nd	nd	nd
$[Pro\text{-}Vxx\text{-}Lxx\text{-}Aib + H]^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Vxx\text{-}CO + H]^+$	169	169	169	169	169	169	169	169	169	169	nd	nd
$[Pro\text{-}Vxx\text{-}Aib\text{-}CO + H]^+$	254	nd	nd	nd	254	254	254	nd	nd	nd	nd	nd
$[Pro\text{-}Vxx\text{-}Vxx\text{-}CO + H]^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Vxx\text{-}Lxx\text{-}CO + H]^+$	nd	nd	nd	nd	nd	nd	282	nd	282	282	nd	nd
$[Pro\text{-}Lxx + H]^+$	nd	nd	nd	nd	nd	211	211	nd	211	211	211	211
$[Pro\text{-}Lxx\text{-}Aib + H]^+$	nd	nd	nd	nd	nd	nd	296	nd	nd	nd	296	296
$[Pro\text{-}Lxx\text{-}CO + H]^+$	nd	nd	nd	nd	nd	183	183	183	183	183	183	nd
$[Pro\text{-}Lxx\text{-}Aib\text{-}CO + H]^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$[Pro\text{-}Vxx\text{-}Vxx\text{-}Aib\text{-}CO + H]^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 5 (Continued)

ion	**19** A-Ia	**20** A-Ib	**21** A-Ic	**22** A-IIa	**23** A-IIb	**24** A-IIIa	**25** A-IIIb	**26** A-IVa	**27** A-IVb	**28** A-IVc	**29** A-Va	**30** A-Vb
[Pro-Vxx-Lxx-Aib-CO + H]$^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
[Pro-Aib-Aib-Aib-Pro-Lxxol + Na]$^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
[Pro-Aib-Vxx-Aib-Pro-Lxxol + Na]$^+$	nd	nd	631	nd	nd	nd	nd	nd	nd	nd	nd	nd
[Pro-Aib-Lxx-Aib-Pro-Lxxol + Na]$^+$	617	617	nd	617	617	nd	nd	nd	nd	nd	nd	nd
[Pro-Lxx-Vxx-Aib-Pro-Vxxol + Na]$^+$	nd	nd	nd	nd	nd	nd	nd	617	nd	nd	nd	nd
[Pro-Lxx-Lxx-Aib-Pro-Vxxol + Na]$^+$ or [Pro-Vxx-Lxx-Aib-Pro-Lxxol + Na]$^+$	nd	nd	nd	nd	nd	631	631	nd	nd	nd	nd	nd
[Pro-Lxx-Lxx-Aib-Pro-Lxxol + Na]$^+$	nd	nd	nd	nd	nd	nd	nd	nd	645	645	645	645
[Pro-Aib + H]$^+$	nd	nd	183	183	nd	183	183	183	nd	nd	nd	nd
[Pro-Aib-Lxx + H]$^+$	nd	nd	296	nd	296	296	296	296	nd	nd	296	296
[Pro-Aib-Lxx-Aib + H]$^+$	nd	nd	381	nd	381	381	381	381	nd	nd	nd	nd
(y_8 + Na)$^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
(y_7 + Na)$^+$	nd	nd	nd	nd	431	431	431	nd	nd	nd	nd	nd
(y_6 + Na)$^+$	530	530	516	530	516	544	544	nd	nd	nd	nd	nd
(y_5 + Na)$^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
(y_4 + Na)$^+$	712	712	726	726	726	754	754	754	nd	754	768	nd
(y_3 + Na)$^+$	825	825	825	839	839	867	853	867	881	867	881	881
(y_2 + Na)$^+$	910	910	910	924	924	952	938	952	966	952	966	966
(y_1 + Na)$^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
(x_8 + Na)$^+$	nd	nd	nd	nd	nd	403	nd	nd	nd	431	445	nd
(x_7 + Na)$^+$	502	502	nd	502	nd	516	516	516	nd	516	530	530
(x_6 + Na)$^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
(x_5 + Na)$^+$	684	684	698	698	698	726	726	726	740	726	740	740
(x_4 + Na)$^+$	797	797	797	811	811	839	825	839	853	839	853	853
(x_3 + Na)$^+$	882	882	882	896	896	924	910	924	938	924	938	938
(x_2 + Na)$^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
(x_1 + Na)$^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a] See **Table 3** footnote.

Table 6. Sequences of Trichocryptins A and B Produced by Members of the *T. brevicompactum* Complex[a]

		residue												
	A		1	2	3	4	5	6	7	8	9	10	11	[M + Na]$^+$
19	Ia	Ac	Vxx	Aib	Pro	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1125
20	Ib	Ac	Vxx	Aib	Pro	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1125
21	Ic	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Aib	Vxx	Aib	Pro	Lxxol	1125
22	IIa	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1139
23	IIb	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1139
24	IIIa	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1153
25	IIIb	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Lxxol	1153
26	IVa	Ac	Lxx	Aib	Pro	Vxx	Vxx	Pro	Lxx	Vxx	Aib	Pro	Vxxol	1167
27	IVb	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1167
28	IVc	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1167
29	Va	Ac	Lxx	Aib	Pro	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1181
30	Vb	Ac	Lxx	Aib	Pro	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1181

		residue													
	B		1	2	3	4	5	6	7	8	9	10	11	12	[M + Na]$^+$
11	Ia	Ac	Vxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Vxx	Aib	Pro	Lxxol	1210
12	Ib	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Aib	Aib	Pro	Lxxol	1210
13	IIa	Ac	Vxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1224
14	IIb	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Vxx	Aib	Pro	Lxxol	1224
15	IIIa	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1238
16	IIIb	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1238
17	IVa	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1252
18	IVb	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol	1252
33	Va	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1266
34	Vb	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxxol	1266
35	Vc	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1266
36	Vd	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1266
37	VIa	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1280
38	VIb	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1280

[a] See **Table 4** footnote.

described for strain CBS 109720, strain CBS 112445 did not produce peptaibols with molecular masses *m*/*z* 1210. However, additional higher homologues displaying *m*/*z* 1266 and 1280 (all [M + Na]$^+$) are present. Fragmentation patterns and sequences of these trichocryptins (**33**, B-Va; **34**, B-Vb; **35**, B-Vc; and **36**, B-Vd; as well as **37**, B-VIa, and **38**, B-VIb) are listed in **Tables 5** and **6**. The C-terminal motif, Pro-Iva-Leu-Aib-Pro-Leuol, is known from harzianin HB I (*38*) and from

some of the previously described harzianins HC from *T. harzianum* M-903603 and M-903614 (*33*). The C-terminal motif, Pro-Ile-Leu-Aib-Pro-Valol, was reported for the trichorozins I and II isolated from a strain of *T. harzianum* (*39*).

***T.* cf. *brevicompactum* ATCC 90237.** This strain shows a quite different and much more diverse pattern (**Figure 2a**) compared to the other isolates investigated in this study: The CID-MS and MS/MS of *m/z* 756, 770, and 784 (all $M + H^+$) revealed the presence of homologous trichocompactins, which have been sequenced as follows below. The peak of compound **39**, named TCT Va, is comparatively intense in the total ion current (TIC), although its UV absorbance is rather low. Compared to compounds **5**−**10**, these pseudomolecular ions display a mass difference of +30 Da. This fact leads to the hypothesis that a Gly residue in the molecule might be substituted by a Ser residue.

Serine-containing peptaibiotics often tend to display additional b'_n fragments resulting from the loss of water from the corresponding b_n fragments—a feature that is important for the detection of that particular amino acid in CID-MS, MS/MS, and MS^n experiments. The proposed fragmentation pattern has, in fact, been observed, leading to the assignment of the structures indicated in **Tables 3** and **4**. Assuming structural homology of *m/z* 184, the N-terminal fragment of these compounds could consist of Ac-Aib-Gly. Thus, compound **40**, named TCT IV, is a homologue of compound **5**. Compounds **39** and **41** (TCT Vb) might be interpreted as homologues of compounds **7** and **8**, whereas compounds **42** and **43** (TCT VIa and VIb) may represent homologues of compounds **9** and **10** with Gly in position 6 substituted by Ser. However, compounds **5**−**8** and **10** are also present, with **10** displaying the most abundant peak of this part of the HPLC elution profile. Fragmentation patterns and sequences of all trichocompactins are described in **Tables 3** and **4**.

Small amounts of substances with molecular masses of *m/z* 1238, 1252, and 1266 have also been detected, which may represent compounds **15**−**18** and **33**−**36**, whereas *m/z* 1210, 1224, and 1280 (all $[M + Na]^+$) have not been observed. The pattern of peptaibiotics with molecular masses between 1100 and 1200 Da is completely different from those of *T. brevicompactum* CBS 109720, IBT 40840, IBT 40839, and CBS 112445: *T.* cf. *brevicompactum* ATCC 90237 produced two additional series of homologous peptaibols—the former comprising *m/z* 1099, 1113, 1127, and 1141 and the latter, *m/z* 1129, 1143, and 1157. The fragmentation behavior of these ions was very similar to that observed for trichocryptins A and B. The molecular ions mentioned above again represented $[M + Na]^+$ adducts. They generated a series of sodiated *y*-type ions dominated by the corresponding sodiated *x*-type fragments, as proven by MS^n investigations. As previously observed for trichocryptins A and B, any diagnostic N-terminal fragments were completely suppressed in the collision chamber. However, CID-MS revealed the ions b_2-b_5, but the b_1 fragment could not be detected. Despite this, a difference of *m/z* 199 most probably corresponds to the N-terminal sequence Ac-Aib-Ala, which is very common among peptaibols produced by *Trichoderma* spp. (*20*). For instance, an alanyl residue in position 2 has previously been described for the trichocellins from *T. viride* ATCC 20672 (*40*). Compounds from ***Tricho*derma** cf. ***brevicompactum*** displaying *m/z* 1099 (**44**−**47**), 1113 (**48**/**49**), 1127 (**50**−**52**), and 1141 (**53**/**54**, all $[M + Na]^+$) contain Ala in position 2 and were named **trichobrevins A**. The latter two compounds, **53**, trichobrevins A-IVa, and **54**, A-IVb, as well as compound **50**, trichobrevin A-IIIa, are the most abundant peaks in the HPLC elution profile.

In the case of *m/z* 1129, 1143, and 1157, the CID fragments b_2-b_4 are accompanied by the corresponding $b_n - H_2O$ ions. This diagnostic phenomenon has previously been observed for the Ser-containing trichocompactins described above. Thus, the Ala residue in position 2 is exchanged by a seryl residue. These compounds exhibiting $[M + Na]^+$ ions *m/z* 1129 (**55**−**57**), 1143 (**58**/**59**), and 1157 (**60**−**62**) were named **trichobrevins B**. The fragmentation scheme and sequences of trichobrevins A and B are shown in **Tables 7** and **8**.

The C termini of trichobrevin compounds **45**, A-Ib, and **47**, A-Id, consist of a Vxxol residue. Interestingly, MS/MS data indicate that compound **46**, trichobrevin A-Ic, carries a C-terminal Aibol residue, whereas compound **44**, trichobrevin A-Ia, terminates in Alaol. However, the occurrence of Aibol and Alaol remains tentative. Detailed investigations, preferably on the isolated compounds, are required to unequivocally prove the presence of these distinctive structural elements as such C termini have not been previously reported in the literature.

Compounds **1**−**4** have also been detected. Partial sequences of a minor compound *m/z* 1992 were determined. Diagnostic fragments observed in the MS/MS and MS^n spectra give reason for the assumption that ALM F30/8 (compound **63**, cf. ref *28*) could be present.

***T.* cf. *brevicompactum* IBT 40863.** *T.* cf. *brevicompactum* IBT 40863 (**Figure 2c**) produces compounds **1**−**4** as main components and a number of minor ALMs, the structures of which have not been investigated in detail. Pseudomolecular ions *m/z* 1139, 1153, and 1167 could represent compounds **22**−**28** or their positional isomers. Homologues displaying *m/z* 1125 and 1181 $[M + Na]^+$ were not detected. Minor amounts of *m/z* 1224, 1238, 1252, 1266, and 1280 (all $[M + Na]^+$) are present, which could represent compounds **13**−**18** and **33**−**38** or positional isomers thereof. The pattern of compounds **44**−**62** is supposed to be identical or very closely related to that of *T.* cf. *brevicompactum* ATCC 90237—as deduced from the elution order of the respective pseudomolecular ions.

***T.* cf. *brevicompactum* NRRL 3199.** As previously mentioned, this strain is known as the "classical" source of alamethicins (*28*), mostly producing compounds **1**−**4** (**Figure 1b**). Further alamethicin-like compounds are present in minor amounts, the sequence of which could not be determined due to their comparatively low abundance in the mixture. To date, several hundred studies dealing with research on this particular peptaibol have been published. Thus, alamethicin is regarded as the most thoroughly investigated peptaibiotic. Compound **64** displays a rather low UV absorption at 205 nm, but a remarkably good ionization in positive ESI-MS. MS/MS studies on the pseudomolecular ion *m/z* 1207 revealed obvious structural homology to helioferins (*41*) and roseoferins (*42*), nine-residue lipoaminopeptides from the fungicolous *Mycogone rosea* strains DSM 8822 and DSM 12973.

An additional diagnostic fragment, *m/z* 266, is found in CID-MS spectra recorded at a CID energy of 45 and 65 eV, respectively, as well as in MS^3 spectra of the MS^2 fragment ion *m/z* 550. Assuming structural homology with helio- and roseoferins, the difference of *m/z* 213 could correspond to an AHMOD residue. At present, this lipoamino acid is known as a unique constituent of most of the leucinostatins, of trichopolyns, helioferins, roseoferins, and acremostatins (reviewed in ref *18*). Thus, the fragment *m/z* 1135 should indicate the loss of the *n*-butyl side chain ($[M + H - 72]^+$) from the AHMOD residue by α-cleavage—a diagnostic feature observed in positive

Table 7. Diagnostic Fragment Ions (*m*/*z*) of Trichobrevins A and B Produced by Members of the *T. brevicompactum* Complex[a]

	44	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**
ion	A-Ia	A-Ib	A-Ic	A-Id	A-IIa	A-IIb	A-IIIa	A-IIIb	A-IIIc	A-IVa	A-IVb
$[M + Na]^+$	1099	1099	1099	1099	1113	1113	1127	1127	1127	1141	1141
$[M + K]^+$	1115	1115	1115	1115	1129	1129	1143	1143	1143	1157	1157
$[M + H]^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$[M + Na - H_2O]^+$	1081	1081	1081	1081	1095	1095	1109	1109	1109	1123	1123
b_1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
b_2	199	nd	nd	nd	199	199	199	199	199	199	199
b_3	298	nd	nd	284	298	284	298	298	298	298	298
b_4	411	nd	nd	383	397	383	411	397	397	411	411
b_5	496	468	nd	468	482	468	496	482	482	496	496
[Pro-Lxx-Lxx-Aib-Pro-Alaol + Na]$^+$	603	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
[Pro-Lxx-Lxx-Aib-Pro-Aibol + Na]$^+$	nd	nd	617	nd	nd	nd	nd	nd	nd	nd	nd
[Pro-Lxx-Lxx-Aib-Pro-Vxxol + Na]$^+$ or [Pro-Vxx-Lxx-Aib-Pro-Lxxol + Na]$^+$	nd	631	nd	631	631	nd	631	nd	nd	nd	nd
[Pro-Lxx-Lxx-Aib-Pro-Lxxol + Na]$^+$	nd	nd	nd	nd	nd	645	nd	645	645	645	645
$(y_7 + Na)^+$	nd	nd	nd	405	419	nd	433	418	418	433	433
$(y_6 + Na)^+$	nd	nd	nd	490	504	nd	518	504	504	518	nd
$(y_5 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$(y_4 + Na)^+$	728	nd	nd	700	714	700	728	714	714	728	728
$(y_3 + Na)^+$	841	nd	827	813	827	813	841	827	827	841	841
$(y_2 + Na)^+$	926	nd	nd	898	912	898	926	912	912	926	926
$(y_1 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$(x_7 + Na)^+$	nd	nd	nd	377	nd	377	405	nd	nd	405	405
$(x_6 + Na)^+$	490	462	476	462	476	462	490	476	476	490	490
$(x_5 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$(x_4 + Na)^+$	700	672	686	672	686	672	700	686	686	700	700
$(x_3 + Na)^+$	813	785	799	785	799	785	813	799	799	813	813
$(x_2 + Na)^+$	898	870	884	870	884	870	898	884	884	898	898
$(x_1 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

	55	**56**	**57**	**58**	**59**	**60**	**61**	**62**
ion	B-Ia	B-Ib	B-Ic	B-IIa	B-IIb	B-IIIa	B-IIIb	B-IIIc
$[M + Na]^+$	1129	1129	1129	1143	1143	1157	1157	1157
$[M + K]^+$	1145	1145	1145	1159	1159	1173	1173	1173
$[M + H]^+$	nd	nd	nd	nd	nd	nd	nd	nd
$[M + Na - H_2O]^+$	1111	1111	1111	1125	1125	1139	1139	1139
b_1	nd	nd	nd	nd	nd	nd	nd	nd
$b_2 - H_2O$	197	nd	197	197	197	197	197	197
b_2	nd	nd	215	215	215	215	215	215
$b_3 - H_2O$	296	nd	296	296	296	296	296	296
b_3	314	nd	314	314	314	314	314	314
$b_4 - H_2O$	409	nd	nd	409	395	409	409	409
b_4	427	399	413	427	413	427	427	427
$b_5 - H_2O$	nd	nd	nd	494	nd	nd	nd	494
b_5	512	484	498	512	498	512	512	512
[Pro-Lxx-Lxx-Aib-Pro-Aibol + Na]$^+$	617	nd	nd	nd	nd	nd	nd	nd
[Pro-Lxx-Lxx-Aib-Pro-Vxxol + Na]$^+$ or [Pro-Vxx-Lxx-Aib-Pro-Lxxol + Na]$^+$	nd	nd	631	631	nd	nd	nd	nd
[Pro-Lxx-Lxx-Aib-Pro-Lxxol + Na]$^+$	nd	645	nd	nd	645	645	645	645
$(y_7 + Na)^+$	nd	nd	nd	nd	nd	nd	449	nd
$(y_6 + Na)^+$	nd	506	520	nd	nd	534	534	nd
$(y_5 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd
$(y_4 + Na)^+$	744	716	730	744	730	744	744	744
$(y_3 + Na)^+$	857	829	843	857	843	857	857	857
$(y_2 + Na)^+$	942	914	928	942	928	942	942	942
$(y_1 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd
$(x_7 + Na)^+$	nd	nd	nd	431	nd	421	421	421
$(x_6 + Na)^+$	506	nd	492	516	492	nd	506	nd
$(x_5 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd
$(x_4 + Na)^+$	716	688	702	716	702	716	716	716
$(x_3 + Na)^+$	829	801	815	829	815	829	829	829
$(x_2 + Na)^+$	914	886	900	914	900	914	914	914
$(x_1 + Na)^+$	nd	nd	nd	nd	nd	nd	nd	nd

[a] See **Table 3** footnote.

ES-MS of the lipoaminopeptide antibiotics mentioned above. The C terminus of helio- and roseoferins consists of either a 2-[(2′-**a**minopropyl)-**m**ethyl**a**mino]-**e**thanol (**AMAE**, *m*/*z* 132) or a 2-(2′-**a**minopropyl)**a**mino-**e**thanol (**AAE**, *m*/*z* 118) residue. Furthermore, the presence of a C-terminal AMAE is indicated by the loss of C_3H_9NO from the pseudomolecular ion. Consequently, $[M + H - 75]^+$ should be formed—a fragment that is present at *m*/*z* 1132. Moreover, *m*/*z* 1189 indicates the loss of water from $[M + H]^+$—a typical feature of C-terminal (amino) alcohols. CID-MS/MS investigations on *m*/*z* 266 revealed its corresponding *a*-type fragment *m*/*z* 238. Further diagnostic fragments were not observed, due to the comparatively low

Table 8. Sequences of Trichobrevins A and B Produced by Members of the *T. brevicompactum* Complex[a]

		residue											
			1	2	3	4	5	6	7	8	9	10	11	$[M + Na]^+$
44	A-Ia	Ac	Aib	Ala	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol	1099
45	A-Ib	Ac	Aib	Ala	Aib	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1099
46	A-Ic	Ac	Aib	Ala	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Aibol	1099
47	A-Id	Ac	Aib	Ala	Aib	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1099
48	A-IIa	Ac	Aib	Ala	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1113
49	A-IIb	Ac	Aib	Ala	Aib	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1113
50	A-IIIa	Ac	Aib	Ala	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1127
51	A-IIIb	Ac	Aib	Ala	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1127
52	A-IIIc	Ac	Aib	Ala	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1127
53	A-IVa	Ac	Aib	Ala	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1141
54	A-IVb	Ac	Aib	Ala	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1141
55	B-Ia	Ac	Aib	Ser	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Aibol	1129
56	B-Ib	Ac	Aib	Ser	Vxx	Aib	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1129
57	B-Ic	Ac	Aib	Ser	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1129
58	B-IIa	Ac	Aib	Ser	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1143
59	B-IIb	Ac	Aib	Ser	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol	1143
60	B-IIIa	Ac	Aib	Ser	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1157
61	B-IIIb	Ac	Aib	Ser	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1157
62	B-IIIc	Ac	Aib	Ser	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	1157

[a] See **Table 4** footnote.

abundance of *m*/*z* 266, which was not sufficient to perform further CID-MS^n experiments. Considering the obvious structural homologies with helio- and roseoferins, the ion *m*/*z* 266 could consist of a Pro linked to an α-methyldecanoic acid residue. Positional isomers or higher homologues of compound **64** have not been detected. However, homologues displaying a mass difference of −14 Da (*m*/*z* 1193, $[M + H]^+$) are present. The C-terminal AMAE residue of compound **64** is replaced by AAE in compound **65**—as previously described as a structural variation of helio- and roseoferins (*40*, *41*). The Lxx residue in position 6 of compound **64** is exchanged by Vxx in compound **66**, which carries a C-terminal AMAE residue. The presence of *m*/*z* 1118 indicates a C-terminal AMAE residue for compound **67**. Interestingly, MS^3 of the MS^2 ions *m*/*z* 536 and 621 proved that *m*/*z* 465 and 266 are formed. Isomerism is therefore located in the lipoamino acid residue, as also observed for compound **68**. Assuming structural homology with the previously described compounds **64**−**66**, sequences containing a novel desmethyl-AHMOD residue (*m*/*z* 199) are proposed for compounds **67** and **68**. The Ala residue in position 7 of compound **64** is exchanged by Gly in compound **69**, which carries AHMOD in position 3 and a C-terminal AMAE residue. Compounds with a strongly basic secondary or tertiary amine such as AAE or AMAE, respectively, which is bound to a lipophilic backbone, give a positive reaction in the two-phase vertical stacking assay (*43*). This diagnostic feature, which has previously been described for helio- and roseoferins (*41*, *42*), was also observed for extracts from *T.* cf. *brevicompactum* NRRL 3199. The positive reaction of helioferin in the two-phase vertical stacking assay is correlated with a strong ionophoric activity of this antibiotic (*44*). Therefore, these seven novel compounds from ***Trichoderma*** cf. *brevicompactum* NRRL 3199, which promote the trans**fer** of water-soluble helianthin to a toluene layer, were named **trichoferins** (TFR) **A**−**F**. Their fragmentation patterns and structures are illustrated in **Tables 9** and **10**. *T.* cf. *brevicompactum* strains ATCC 90237 and IBT 40863 also produced compound **64**, trichoferin A, as proven by MS/MS and CID-MS experiments. Minor amounts of an ion *m*/*z* 1207 that could be identical with compound **64** were also found in *T. brevicompactum* CBS 109720, whereas only trace amounts of *m*/*z* 1207 are present in *T. brevicompactum* CBS 112445, IBT 40840, and IBT 40839. It should be mentioned that compounds **65**−**69** (trichoferins B−F) have been detected only in *T.* cf. *brevicompactum* NRRL 3199.

Table 9. Diagnostic Fragment Ions (*m*/*z*) of Trichoferins Produced by Members of the *T. brevicompactum* Complex[a]

ion	**64** A	**65** B	**66** C	**67** D	**68** E	**69** F
$[M + H]^+$	1207	1193	1193	1193	1193	1193
$[M + Na]^+$	1229	1215	1215	1215	1215	1215
$[M - H_2O]^+$	1189	1175	1175	1175	nd	nd
$[M - C_4H_7O]^+$	1135	1120	1120	1120	1120	1120
$[M - C_3H_8NO]^+$	1132	nd	1060	1060	1118	nd
$[M - C_2H_6NO]^+$	nd	1132	nd	nd	nd	nd
b_1	nd	nd	nd	nd	nd	nd
b_2	266	266	266	266	266	266
$b_3 - CO$	nd	nd	nd	nd	419	nd
$b_3 - H_2O$	nd	nd	nd	nd	447	nd
b_3	479	479	479	465	465	nd
$b_4 - H_2O$	nd	nd	nd	nd	518	nd
b_4	550	550	550	536	536	550
b_5 - CO	nd	nd	nd	nd	593	nd
$b_5 - H_2O$	nd	nd	nd	nd	603	nd
b_5	635	635	635	621	621	635
b_6	720	720	720	706	706	720
b_7	833	833	819	805	819	833
b_8	904	904	890	890	890	890
b_9	989	989	975	975	975	975
b_{10}	1074	1074	1060	1060	1060	1060
a_2	238	238	238	238	238	238

[a] See **Table 3** footnote.

Furthermore, the strain produces novel sequences of trichocompactins VII, VIIIa, and VIIIb with Gly in position 6 replaced by Ala (compounds **70**−**72**). In addition to that, three serine-containing trichocompactins IX, Xa, and Xb were detected (compounds **73**−**75**). Interestingly, compounds **5**−**10** could not be found. Fragmentations and sequences of all trichocompactins described in this study are listed in **Tables 2** and **3**. Compounds **44**−**62** were detected again and their structures proven by CID-MS and MS^n experiments. In contrast to what was found for *T.* cf. *brevicompactum* ATCC 90237 and IBT 40863, compounds **61** and **62** were the most prominent ions besides the compounds **1**−**4**.

Table 10. Sequences of Trichoferins Produced by Members of the *T. brevicompactum* Complex[a,b]

		residue										
			1	2	3	4	5	6	7	8	9	10	$[M + H]^+$
64	A	MDA	Pro	AHMOD	Ala	Aib	Aib	Lxx	Ala	Aib	Aib	AMAE	1207
65	B	MDA	Pro	AHMOD	Ala	Aib	Aib	Lxx	Ala	Aib	Aib	AAE	1193
66	C	MDA	Pro	AHMOD	Ala	Aib	Aib	Vxx	Ala	Aib	Aib	AMAE	1193
67	D	MDA	Pro	desmethyl-AHMOD	Ala	Aib	Vxx	Aib	Aib	Aib	Aib	AMAE	1193
68	E	MDA	Pro	desmethyl-AHMOD	Ala	Aib	Aib	Lxx	Ala	Aib	Aib	AMAE	1193
69	F	MDA	Pro	AHMOD	Ala	Aib	Aib	Lxx	Gly	Aib	Aib	AMAE	1193

[a] See **Table 4** footnote. [b] Abbreviations: MDA, 2-methyldecanoic acid; AHMOD, 2-amino-4-methyl-6-hydroxy-8-oxodecanoic acid.

DISCUSSION

Screening of recently described species of *Trichoderma* greatly enhances the possibility to find new peptaibiotics. Remarkably, 69 of the 75 peptides (93%) analyzed in this study represent new sequences. The strains produced 14 12-residue trichocryptins B, 12 11-residue trichocryptins A, 19 11-residue trichobrevins A and B, 6 10-residue trichoferins, and 17 8-residue trichocompactins. The number of new compounds described in this study clearly illustrates the impressive potential of a peptaibiomic approach.

Obviously, there are structural homologies of the new 11- and 12-residue compounds with previously reported peptaibiotics, such as harzianins (*33*, *36*, *38*), antiamoebins (*22*), hypomurocins (*37*), and bergofungins (*35*). Thus, comparable biological activities could be expected, although the decrease in chain length may lead to a reduction in efficacy.

As alamethicins are present in every strain investigated, they should considerably contribute to the biological activity against the causal agents of Eutypa dieback and Esca disease of grapevine. The exceptional antimicrobial activity of alamethicins can be explained by the dipole flip-flop gating model of Boheim and Jung (*45*). Alamethicins, as long-chain, 20-residue peptaibols, may form larger and more stable pores than shorter chain peptaibiotics, thus remarkably lowering the minimal inhibitory concentration (MIC) to microorganisms (for a review see ref *31*).

Structural homologies of trichoferins with the protonophoric roseo- and helioferins and the positive reaction of trichoferin-containing extracts in the two-phase vertical stacking assay indicate an ionophoric activity that may amplify the biocontrol potential of the trichoferin-producing strains. However, the importance of trichocompactins for the bioactivity of the producing strains remains doubtful.

Generally, a decrease in chain length is correlated with a loss of bioactivity as exemplified in the case of the 19-residue chrysospermins (*46*) and the 5-residue peptaibolin (*47*) from *Sepedonium chrysospermum* (teleomorph: *Hypomyces chrysospermus*). Chrysospermins may form nongated membrane channels (*48*), thus exhibiting strong antimicrobial activity against Gram-positive bacteria, yeasts, and fungi. They also accelerate cytodifferentiation of the coelomycete *Phoma destructiva* and cause neuroleptic activity in mice (*49*). For peptaibolin, however, no significant bioactivities have been reported.

Notably, the distribution of peptaibiotics among taxonomic groups/species clusters of *Trichoderma* is currently under investigation in order to explain and correlate their antagonistic properties (*50*).

According to our data, the alamethicins are restricted to the *T. brevicompactum* group, being the most abundant peptaibiotic metabolites of *T. brevicompactum* (*ss*).

When grown on PDA at 25 °C, *T. brevicompactum* also biosynthesized diterpene mycotoxins of the trichothecene group: strains CBS 109720, IBT 40839, and IBT 40840 produced trichodermin, whereas harzianum A was detected in strain CBS 112445 as well. However, phylogenetic analyses suggested the classification of all of these strains as *T. brevicompactum* (*ss*). In contrast, *T.* cf. *brevicompactum* ATCC 90237, IBT 40863, and NRRL 3199 mainly biosynthesized harzianum A. Notably, 17 strains belonging to the *T. brevicompactum* complex consistently produced trichothecenes on all media tested. In contrast to that, formation of trichothecenes has not been observed for any other of the more than 250 *Trichoderma* strains screened (*3*, *27*).

This leads to the conclusion that the pattern of characteristic nonpeptidic mycotoxins (trichothecenes) and peptaibiotics (alamethicins and trichocompactins) might be used in addition to morphological and molecular data to separate the *brevicompactum* complex from other taxa of the genus *Trichoderma*. Morphological, molecular, and chemical data of strain NRRL 3199 support its affiliation with *T.* cf. *brevicompactum* rather than *T. viride* (*3*). Taken together, the differential patterns of alamethicin production as well as the production of two different trichothecene-type mycotoxins clearly support DNA sequencing results. Both molecular and chemotaxonomic approaches clearly indicate the existence of two phylogenetic species within what has been called *T. brevicompactum*, so far. Both trichocryptins and trichobrevins are more widespread in the genus *Trichoderma*, illustrating the limitations of chemotaxonomic conclusions focused exclusively on the pattern of peptaibiotics. Nevertheless, the taxonomy of *T. brevicompactum* remains a rather complex topic and is the subject of an ongoing study.

Summarizing the sequences presented in this paper, it can be concluded that peptaibiotics still seem to be of questionable chemotaxonomic importance. Literature data clearly support this opinion, because fungi belonging to divergent taxonomic groups may produce closely related sequences of peptaibiotics.

The biosynthesis of lipoaminopeptides, for example, has been described for strains of the fungicolous species *Paecilomyces lilacinus* and *Paecilomyces marquandii*, but was also observed in cultures of *Trichoderma polysporum* isolated from infested fruit bodies of *Lentinula edodes* and in the mycoparasite *M. rosea* (reviewed in ref *18*).

To continue, 16-residue peptaibols, antiamoebins, were obtained from *Emericellopsis synnematicola*, *Emericellopsis poonensis*, *Verticillium epiphytum* (syn. *Cephalosporium pimprina*), and *Stilbella fimetaria* CBS 548.84 and ATCC 28144 (syn. *Stilbella erythrocephala*), but have also been isolated from *Clonostachys rosea* f. *catenulata* (syn. *Gliocladium catenulatum*) CBS 511.66 (*21*, *22*).

Consequently, the formation of peptaibiotics should rather be defined as an adaptation to highly specialized modes of life of the producers, mostly being facultative or obligate plant

pathogens or fungicolous fungi occupying some particular ecological niches.

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REVIEW

New Sequences, Constituents, and Producers of Peptaibiotics: An Updated Review

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To date, 18 genera of imperfect and ascomycetous fungi have been recognized to produce *ca.* 700 individual sequences of peptaibiotics. These are linear polypeptide antibiotics which *i*) have a molecular weight between 500 and 2,200 Dalton, thus containing 5–21 residues; *ii*) show a high content of α-aminoisobutyric acid; *iii*) are characterized by the presence of other nonproteinogenic amino acids and/or lipoamino acids; *iv*) possess an acylated N-terminus, and *v*) have a C-terminal residue that, in most of them, consists of a free or acetylated amide-bonded 1,2-amino alcohol, but might also be an amine, amide, free amino acid, 2,5-dioxopiperazine, or sugar alcohol. From April 2003 until present, *ca.* 300 new individual sequences of peptaibiotics have been published in the literature, but most of them have not yet been included in databases. To summarize these new sequences and novel constituents, as well as to introduce fungal species hitherto unknown as producers of peptaibiotics, the relevant literature is reviewed. Furthermore, ecophysiological and taxonomic aspects of the producing fungi are discussed.

1. Introduction. – Peptaibiotics constitute a constantly growing family of peptide antibiotics of fungal origin. The term 'peptaibiotics' was introduced in [1] and reconsidered in [2]. Peptaibiotics are defined as linear peptide antibiotics which *i*) have a molecular weight between 500 and 2,200 Dalton, thus containing 5–21 residues; *ii*) show a high content of α-aminoisobutyric acid (Aib); *iii*) are characterized by the presence of other nonproteinogenic amino acids and/or lipoamino acids; *iv*) possess an acylated N-terminus, and *v*) have a C-terminal residue that, in most of them, consists of a free or acetylated amide-bonded 1,2-amino alcohol, but might also be an amine, amide, free amino acid, 2,5-dioxopiperazine, or sugar alcohol. Since the majority of Aib-containing peptides carries a C-terminal residue representing a 1,2-amino alcohol, this subgroup is referred to as **peptaibols**. Very lipophilic peptaibols, the N-terminus of which is acylated by octanoic, decanoic, or *cis*-dec-4-enoic acid, are named **lipopeptaibols** [2][3]. In the third subfamily of **lipoaminopeptides** (also reported as **aminolipopeptides**), the N-terminus is substituted by unbranched, α- or γ-methyl-branched, saturated, or unsaturated C_4–C_{15} fatty acids. An L-proline-, *trans*-4-hydroxy-L-proline, or *cis*-4-methyl-L-proline residue is found in position 1 of the peptide chain, and, in most cases, it is followed by a lipoamino acid residue in position 2. To our

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present knowledge, this compound, 2-amino-6-hydroxy-4-methyl-8-oxo-decanoic acid (AHMOD), has only been recorded from this subfamily. A fourth subfamily comprises all other peptaibiotics that cannot be classified in any of the other three preceding subfamilies. A review introducing nine subfamilies according to structural homologies of peptaibiotics has been published [4].

Usually, peptaibiotics are classified according to their main chain length, as long-chain (17–21 residues), medium-chain (11–16), and short-chain (5–10) sequences. Although not yet reported in literature, the detection of very short chain (<5) peptaibiotics that may represent drastically truncated sequences is basically possible. Notably, sequences comprising more than 21 amino acids have not been reported yet. The number of Aib residues found may range from one as in some of the trichocompactins [5] or two as in the five-residue peptaibolin [6] up to nine in some of the 20-residue stilboflavins [7], or even ten in the 21-residue peptaibiotic SCH 643432 [8]. Trichobrachins TB III A and TB III B are peptides, the N-termini of which have not been assigned yet [9]. Nonribosomal peptide synthetases from *Trichoderma virens* [10] and *Sepedonium ampullosporum* [11] have recently been characterized.

Peptaibiotics show interesting physicochemical and biological activities depending on particular structural properties, such as formation of pores in bilayer lipid membranes as well as antibacterial, antifungal, occasionally antiviral, and antiparasitic activities. Inhibition of mitochondrial ATPase, uncoupling of oxidative phosphorylation, immunosuppression, inhibition of platelet aggregation, induction of fungal morphogenesis, and neuroleptic effects have been reported and reviewed in [2].

From April 2003 until present, *ca.* 300 new individual sequences of peptaibiotics were published in the literature, but most of them have not yet been included in databases such as the '*Peptaibol Database*' [12]. To summarize these new sequences and novel constituents, as well as to introduce fungal species hitherto unknown as producers of peptaibiotics, the relevant literature is reviewed. Furthermore, ecophysiological and taxonomic aspects of the producing fungi are discussed.

2. New Sequences, Constituents, and Producers of Peptaibiotics: General Remarks. – The first three paragraphs, *i.e.*, *2.1–2.3*, of this section comprise these new peptaibiotics that have been isolated from their fungal producers as pure individual substances, or mixtures of homologues and/or positional isomers in preparative amounts. In contrast, those new peptaibiotics that have been exclusively discovered and sequenced by a so-called '*peptaibiomic*' approach [5][13][14] will be introduced separately in *2.4* of this section. Representative sequences of new peptaibiotics are listed in the *Table*, whereas uncommon and new constituents of peptaibiotics are illustrated in the *Figure*.

2.1. *Peptaibols. Alamethicin*. The 20-residue alamethicins (ALM) can be considered as the most thoroughly investigated peptaibol antibiotics. The first report on the isolation and partial structural characterization of a polypeptide antibiotic (*Upjohn Company*, Kalamazoo; U-22,324) from the culture broth of the fungal strain NRRL 3199, having originally been misidentified as *Trichoderma viride*, was published almost 40 years ago [15]. The taxonomy of ALM-producing *Trichoderma* species has recently been revised. All strains investigated were assigned to belong to the so-called *T. brevicompactum* complex [5]. An investigation by TLC on silica *H* (*i.e.*, acidic silica

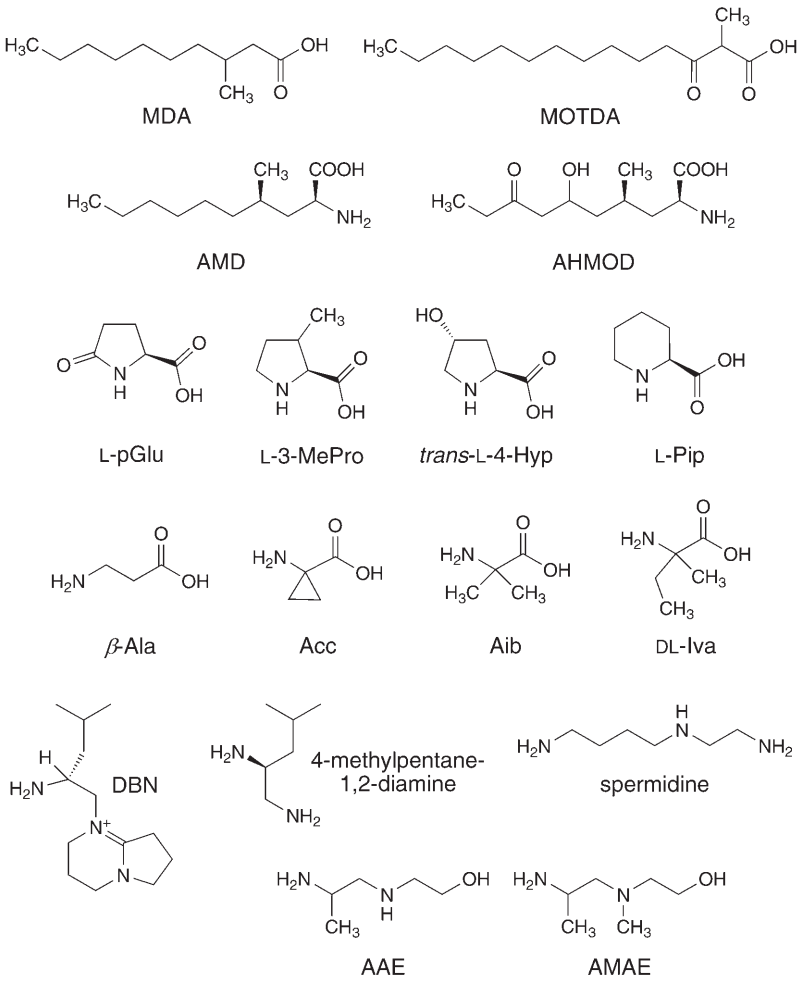

Figure. *Selected uncommon constituents of peptaibiotics*

gel) of the *Upjohn* ALM revealed that this material was composed of two major groups named, according to R_f 100 values: ALM F30 (*ca.* 85%), ALM F50 (*ca.* 12%), and a minor component ALM F20 (2%). However, minor or trace components of ALMs, numbered accordingly F40, F60, and F70, were also detected by TLC [16]. In 1984, a sequence of the acidic ALM F30 and a sequence of the neutral ALM F50 were published [17]. The former was recognized to carry a Glu-Gln residue in positions 18 and 19, which is exchanged by Gln-Gln in the latter. In 2003, the sequences of ALM F30 and ALM F50 were thoroughly reconsidered and reconciled [18].

HPLC Separation of ALM F30 using an acidic gradient revealed ten individual sequences of ALM F30/1–10. Basically, the same gradient was used to separate ALM F50 but omitting the addition of TFA (CF_3COOH) to the eluents. As a result, 13

Table. *Representative Sequences of New Peptaibiotics*[a])

Peptaibiotic		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
ALM F30/3	Ac	Aib	Pro	Aib	Ala	Aib	*Ala*	*Gln*	Aib	*Aib*	Aib	Gly	*Val*	Aib	Pro	Val	Aib	Aib	*Glu*	Gln	Pheol	
ALM F50/5	Ac	Aib	Pro	*Aib*	Ala	Aib	*Ala*	Gln	Aib	*Aib*	Aib	Gly	*Val*	Aib	Pro	*Val*	Aib	Aib	*Gln*	Gln	Pheol	
[desAA(1–6),Pyr] ALM F30								**Glp**	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	*Glu*	Gln	Pheol	
[desAA(1–6),Pyr] ALM F50								**Glp**	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	*Gln*	Gln	Pheol	
SZ-A4	Ac	*Aib*	Ala	Aib	Ala	Aib	*Ala*	Gln	Aib	*Lxx*	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	*Vxx*	Gln	Gln	Pheol	
Cervinin I	**Ac**	**Leu**	Aib	Aib	Leu	Aib	Pro	Ala	Aib	Val	Leu	*Leuol*										
Cervinin II	**Ac**	**Leu**	Aib	Aib	Leu	Aib	Pro	Ala	Aib	Val	Leu	*Leuol-OAc*										
Culicinin D	**BTA**	Pro	**AHMOD**	*Aib*	*Aib*	**AMD**	Leu	Aib	Leu	*β*Ala	**AAE**											
SCH 643432	**MOTDA**	Pro	Aib	Aib	Aib	*Aib*	Ala	Ala	Aib	Leu	Ala	Aib	Ala	Ala	Aib	**Arg**	Ala	Aib	Gly	Aib	Aib	Ala
EFR-G	Ac	**Pip**	Aib	**Pip**	**L-*Iva***	*Aib*	Leu	*β*Ala	Gly	*Aib*	Aib	***Pip***	Aib	Ala	Leu	**L-*Iva***	**PIHPPE**					
ACR-2	Ac	**Pip**	Aib	**Pip**	**D-*Iva***	**D-*Iva***	Leu	*β*Ala	Gly	***Acc***	Aib	***Pip***	Aib	Gly	Leu	*Aib*	**PIHPPE**					
Neoefrapeptin E	Ac	**Pip**	Aib	**Pip**	**D-*Iva***	**D-*Iva***	Leu	*β*Ala	Gly	***Acc***	**L-*Iva***	***Pip***	Aib	Gly	Leu	**L-*Iva***	**PIHPPE**					
Neoefrapeptin F	Ac	**Pip**	Aib	**Pip**	*Iva*	*Aib*	Leu	*β*Ala	Gly	***Acc***	Aib	***3MePro***	Aib	Gly	Leu	**L-*Iva***	**PIHPPE**					
Neoefrapeptin H	Ac	**Pip**	Aib	**Pip**	**D-*Iva***	**D-*Iva***	Leu	*β*Ala	Gly	***Acc***	*Aib*	***Pip***	Aib	Gly								
Cicadapeptin1	**Dec**	**Hyp**	**Hyp**	Val	Aib	Gln	Aib	*Leu*	**DAMP**													
Integramin B	**Ac**	**D-Iva**	**Hyp**	Ile	**L-Iva**	Leu	Aib	*Aib*	***Iva***	Hyp	Leu	**D-Iva**	Aib	Hyp	**Iva**	**Iva**	Gly					
TCP-IIa/-IIb	Ac	Aib	Gly	Ala	Lxx	*Aib*	*Gly*	*Lxx*	**Vxx**													
TCT-AIIa/-IIb	**Ac**	***Lxx***	Aib	Pro	*Vxx*	Aib	Pro	*Aib*	*Lxx*	Aib	Pro	Lxxol										
TCT-BIVa/-IVb	**Ac**	***Lxx***	Aib	Pro	Vxx	*Lxx*	Aib	Pro	*Aib*	*Lxx*	Aib	Pro	Lxxol									
TFR-A	**MDA**	Pro	***AHMOD***	Ala	Aib	*Aib*	*Lxx*	*Ala*	Aib	Aib	***AMAE***											
TFR-B	**MDA**	Pro	***AHMOD***	Ala	Aib	*Aib*	*Lxx*	*Ala*	Aib	Aib	***AAE***											
TFR-E	**MDA**	Pro	***Desmethyl-AHMOD***	Ala	Aib	*Aib*	*Lxx*	*Ala*	Aib	Aib	***AMAE***											

[a]) Abbreviations: ALM, alamethicin; SZ, suzukacillin; EFR, efrapeptin; ACR, acretocin; TCP, trichocompactin; TCT, trichocryptin; TFR, trichoferin. Exchangeable positions are italicized, new and uncommon constituents are highlighted in bold. Abbreviations of uncommon constituents are explained in the text and the *Figure*. Vxx, valine/isovaline; Lxx, leucine/isoleucine. Note, that only peptaibiotics displaying new or uncommon constituents are listed.

individual peptides, ALM F 50/2, 3a–3c, 4a, 4b, 5, 6a, 6b, 7, and 8a–8c, could be sequenced.

Recently, the same material used in [18] was further analyzed by nonaqueous capillary electrophoresis/mass spectrometry (NACE/MS): eleven amino acid sequences were identified, characterized by the exchange of Ala to Aib in position 6, Gln to Glu in position 7 or 19. To continue, two novel ALM were detected, which are characterized by the loss of the C-terminal Pheol residue, thus terminating in Gln. Notably, two truncated 14-residue sequences carrying an N-terminal pyroglutamyl group (commonly abbreviated as Glp, pGlu, or Pyr) were found. Overall, seven new minor sequences are reported compared to [18]. To the best of our knowledge, this is the first report on the occurrence of Glp as a constituent of fungal peptides in nature. Literature search did not reveal any previous publication regarding the isolation of peptide-bound Glp from a fungal source. To exclude the artificial formation of the Glp-peptaibols during workup or long-term storage, degradation studies were performed. Although neither treatment increased the amount of the truncated Glp peptides compared to untreated samples, further studies have to be performed in order to unequivocally prove the origin of the truncated Glp peptaibols [19].

Suzukacillin. Suzukacillin (SZ) was isolated from the culture broth of *Trichoderma viride* strain 63C-I and separated by TLC into two fractions, suzukacillins A (R_f 0.18) and B (R_f 0.70) [20][21]. Using the material provided by Dr. *T. Ooka*, the major fraction, SZ-A, was further characterized and a preliminary sequence published [22]. Although it appeared to be uniform in TLC, SZ-A could be further separated by HPLC, yielding 13 individual peaks. Nevertheless, two of them, SZ-A10 and -11, still represented a microheterogeneous mixture. Thus, 15 individual 20-residue peptaibols, SZ-A1–A9, -A10a/b, -A11a/b, and SZ-A12–A13, were shown to be present in total. Despite the microheterogeneity, complete sequences of all SZ-A peptaibols and chirality of the individual amino acids have been determined by HPLC/MS^n and GC/EI-MS approaches [23]. In this context, the previously established presence of D-Iva [24] was confirmed.

Trichobrachin. Trichobrachin (TB) has been isolated from *Trichoderma longibrachiatum* CBS 936.69 – a strain that is now reclassified as *Trichoderma ghanense*. Three major groups designated TB I, TB II, and TB III could be separated and isolated by preparative TLC on silica gel [9]. Recently, the formation of peptaibiotics by *Trichoderma parceramosum* (*ghanense*) CBS 936.69 was thoroughly reinvestigated [25]. The trichobrachin mixture comprises ten 19-residue peptides with free C-terminal Gln residues (TB I peptides), two 18-residue peptides with free C-terminal Gln residues (TB II 1 and TB II 2), seven 20-residue peptides with C-terminal amide-bonded Pheol (TB II 3–10), and thirty-four eleven-residue peptides with either C-terminal Leuol, Ileol, or Valol (TB III 1–34). TLC Analysis of the dynamics of TB formation and degradation unequivocally demonstrated that those two 18-residue TB I and TB II peptides with a free carboxy terminus resulted from enzymatic C-terminal degradation of 20-residue TB II peptides.

Antiamoebin. The 16-residue peptaibols antiamoebins (AAM) I–XVI, known for their antihelminthic and antiprotozoal activities had previously been reported from cultures of *Emericellopsis synnematicola*, *Emericellopsis poonensis*, and *Verticillium epiphytum* (syn. *Cephalosporium pimprina*), but have also been isolated from

Clonostachys rosea f. *catenulata* (syn. *Gliocladium catenulatum*) CBS 511.66, as well as *Stilbella fimetaria* (syn. *Stilbella erythrocephala*) CBS 548.84 and ATCC 28144 [26]. Notably, most of the above producers of AAM are fungicolous or coprophilous fungi. Fruiting bodies of mushrooms and toadstools, but also dung, are generally regarded as highly competitive substrates. In an impressive study [27], it has unequivocally been proven that antiamoebins were responsible for antibiosis in colonized herbivore dung.

Four strains of *Stilbella fimetaria* (syn. *S. erythrocephala*) were isolated from dung of wild rabbits (D 99026, D 01024, and D 03001) or of the tortoise *Testudo hermanni* (D 03012). Furthermore, dung pellets naturally colonized by *S. fimetaria*, were collected in the field, lyophilized, and extracted. The same was carried out with pre-sterilized dung that has been artificially inoculated with the four strains mentioned above. As expected, AAM could be detected in all liquid cultures. To continue, the total AAM concentration – both in wild and artificially inoculated dungs – was 126–624 μg/g fresh weight, with minimum inhibitory concentrations against most other coprophilous fungi being at or below 100 μg/ml. It should be pointed out that this is the first report describing the detection and isolation of peptaibiotics from natural substrates. The diterpene antibiotic myrocin B, not previously described from *S. fimetaria*, was also produced, but only at low, nonfungicidal levels (5.3 μg/g). As no other antifungal substances could be detected, a decisive role of the broad-spectrum antimycotic AAM during colonization of dung was proposed.

Cervinins I and II. The genus *Mycogone* exclusively comprises fungicolous fungi. *M. perniciosa* and *M. rosea* are known as devastating, nonspecific parasites of agarics. These two species have been reported to cause economically relevant losses in commercial mushroom growing [28]. *M. rosea* is known as the producer of nine-residue lipoaminopeptides: strain DSM 8822 was reported as the producer of helioferins A and B [29], whereas strain DSM 12973 produced both helioferins and the closely related roseoferins A–G [30]. Other species of *Mycogone* infect a rather narrow range of host fungi: *M. calospora*, for instance, exclusively parasitizes coral fungi of the genus *Ramaria* (Gomphales, Ramariaceae), whereas *Mycogone cervina* has been reported on the Glazed Cup, *Mycolachnea hemisphaerica* (Pezizales, Pyronemataceae), and on false morels (Pezizales, Helvellaceae) so far [31].

Recently, *M. cervina* strain A09–02 was isolated as a parasite of the Vinegar Cup *Helvella* (*Paxina*) *acetabulum* and shown to produce two twelve-residue peptaibol antibiotics both exhibiting Ac-Leu as a new N-terminal motif [32]. Moreover, Leuol is found as the C-terminal residue of cervinin I. Notably, no attempts were made to determine the configuration of the amino acids. Cervinin II, however, has the same sequence of amino acids, but its C-terminal Leuol residue is acetylated [32]. As no comments were made with respect to the origin of the latter C-terminus it cannot be completely excluded that cervinin I could have been partly acetylated during workup, thus generating cervinin II. Furthermore, the authors stated that peptaibols exclusively contain L-amino acids. The latter assertion is not acceptable because a number of well-known counter-examples concerning the occurrence of D-Iva in peptaibiotics has been published and discussed in [2]. To the best of our knowledge, this is the first report on the isolation and structure elucidation of secondary metabolites from *M. cervina*. Surprisingly, no lipoaminopeptides have yet been recorded for that species.

2.2. *Lipoaminopeptides. Culicinin.* Four ten-residue lipoaminopeptides, culicinins A–D, were recently characterized from an Australian isolate of *Culicinomyces clavisporus* (Hypocreales, Clavicipitaceae). Strain LL-12I252 was isolated from larvae of the biting midge *Forcipomyia marksae* (Diptera, Ceratopogonidae). Culicinins exhibit a number of common structural features: their N-terminal Pro residue is protected by butanoic acid (BTA). In 2-position, 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD) is found. Another lipoamino acid, 2-amino-4-methyl-decanoic acid (AMD), is situated in position 5, while β-alanine (3-aminopropanoic acid: APA) is located in position 9 of the peptide chain. The C-terminus is substituted by 2-(2′-aminopropyl)aminoethanol (AAE), here referred to as APAE [33]. The lipoamino acid AHMOD is present in position 2 of almost all lipoaminopeptides, being the characteristic structural element of this subgroup of the peptaibiotics. C-Terminal AAE has previously been described for trichopolyns, helio- and roseoferins (as reviewed in [2]). Notably, BTA and AMD have been detected in peptaibiotics for the first time, thus representing novel structural elements. Selective inhibitory activity against PTEN-negative MDA468 tumor cells has been observed.

2.3. *Other Peptaibiotics. SCH 466457 and SCH 466456*. Two antifungal peptaibiotics carrying an N-terminal 2-methyl-3-oxotetradecanoyl (MOTDA) residue were isolated from a taxonomically unidentified fungus [34]. The first compound, SCH 466457, represents a 17-residue peptaibiotic which terminates in free Ala. The second compound, SCH 466456, exhibits the same amino acid sequence in positions 1–16, and another Ala residue follows in position 17. Notably, a free Aib residue is found in the C-terminal position 18. To the best of our knowledge, this is the first report in literature describing the occurrence of free Aib residue as the C-terminal constituent of a peptaibiotic. Both compounds were active against *Candida* ssp., *Aspergillus* ssp., and *Trichophyton* dermatophytes.

SCH 643432. Two antifungal 21-residue peptaibiotics, the N-termini of which are also blocked by a MOTDA residue, were isolated from *Paecilomyces variotii* SCF 1559 [8]. They exhibit some structural homology with the 21-residue texenomycins A and B [35], as well as the six-residue lipohexin which represents a deletion sequence of the texenomycins. However, the configuration of the amino acids has not been determined. Both peptaibiotics are produced by *Acremonium lindtneri* (syn. *Moeszia lindtneri*, teleomorph: *Hypomyces chrysostomus*) DSM 11119, *Mariannaea elegans* CBS 120677 (i265), and *M. elegans* CBS 120687 (i910), the latter two strains originally being misidentified as *Paecilomyces* sp. [36]. As in the case of lipohexin, the C-terminus of SCH 643432-(1) is composed of free alanine or β-alanine, respectively. The primary structure of the isobaric compound SCH 643432-(2) has not yet been published. There are some examples of peptaibols, the C-terminus of which are composed of a free amino acid: Gly is known as a constituent XR586 from *Acremonium persicinum* X21488 [37], from both integramides A and B, and from two neoefrapeptins (see below). Ser has been described for cephaibols P and Q from *Acremonium tubakii* DSM 12774 [38], Val is known from the trichobrachins TB IIa A and B [9], and Gln has recently been detected in two minor compounds of alamethicin ([19], see above). Furthermore, the presence of Vxx has recently been reported for trichocompactins I–XII from *Trichoderma brevicompactum* and *T.* cf. *brevicompactum* [4] as well as *T. stromaticum* [14].

Efrapeptins/Elvapeptins. The 16-residue efrapeptins (EFR) [39] have been isolated from *Tolypocladium inflatum* (syn. *T. niveum*, teleomorph: *Cordyceps subsessilis:* [40]). The material referred to as tolypin by *Matha et al.* [41] mainly consists of efrapeptin F. The potent antifungal and insecticidal activity of these peptaibiotics is best explained by inhibition of mitochondrial ATPase [42]. In 1982, a partial sequence of efrapeptin D, the major compound, was published [43]. However, the structure of the C-terminus was not elucidated until 1991, when efrapeptins C, D, E, F, G, and H were shown to be blocked by *N*-peptido-1-isobutyl-2-(2,3,4,6,7,8-hexahydro-1-pyrrolo[1,2-*a*]pyrimidinio)ethylamine [39], subsequently abbreviated as PIHPPE. Furthermore, production of efrapeptins has been demonstrated for *Tolypocladium geodes* [44] and the two entomopathogenic species *T. parasiticum* [45] and *T. cylindrosporum*, as well as for *T. nubicola* and *T. tundrense* [46]. It was suggested to use the pattern of efrapeptins as a chemotaxonomic marker to distinguish between morphologically similar species of *Tolypocladium*. However, efrapeptin production seems to be not only species-, but strain-specific. The remarkable antifungal and insecticidal activity of the efrapeptins may contribute to the ecophysiology of their fungal producers as their *in vivo* formation has also been demonstrated [45]. Further minor compounds designated efrapeptins A, H, and I have been reported, but no attempts were made to elucidate their structures [47].

Recently, two highly methylated linear peptides, as well as efrapeptin G, were isolated from the marine fungus, strain 021172cKZ, associated with the sponge *Teichaxinella* sp. (Halichondria, Axinellidae). The taxonomy of the producer remains, however, confusing: it was identified as *Leucosphaerina indica* (Hypocreales, Sordariomycetes, Ascomycota) *via* molecular methods, but classified as an atypical *Acremonium* sp. through morphology-based examination [48].

To clarify the biosynthetic relationship between efra- and elvapeptins (ELV) that have been obtained as co-metabolites from *Tolypocladium inflatum* IMI 202309 [49], a study was conducted which demonstrated the *in vitro* conversion of elvapeptins to efrapeptins by oxidative cyclization with CuCl/pyridine. From this observation, the authors concluded that elvapeptins may contain a C-terminal spermidine residue that might also be converted to the amine residue of efrapeptins *in vivo* [50].

Acretocin. Another group of peptaibiotics, acretocin (ACR), that is very closely related to the efrapeptins has been detected in *Acremonium croticinigenum* CBS 217.70 – a species that is known as a facultative parasite of a number of polypores (= bracket fungi) and agarics. Unlike efrapeptins which carry L-Iva ((*S*)-Iva), the presence of D-Iva ((*R*)-Iva) has been established for acretocins. Remarkably, acretocins I–VI differ from the efrapeptins C–G by a mass difference of 2 Da, an observation that led to the assumption that an α,β-didehydro amino acid, presumably α,β-didehydro-α-aminobutanoic acid, might be present [51]. Although α,β-didehydro amino acids have not yet been reported as constituents of peptaibiotics, they are frequently found in peptide antibiotics from *Gram*-positive bacteria. The most well-known examples are lantibiotics, such as gallidermin which is produced by the *Gram*-positive bacterium *Staphylococcus gallinarum* (F16/P57) Tü 3928 [52]. Apart from those antibiotics, the presence of α,β-didehydro-α-aminobutanoic acid has recently been established for hassallidin A [53] and B [54] – two glycosylated *cyclo*-lipopeptides from the epiphytic cyanobacterium *Hassallia* sp. (Nostocales, Microchaetaceae). Comparing the sequences of the neoefrapeptins [55], the presence of 1-aminocyclopropane-1-carboxylic acid

(Ac_3c) in acretocins instead of an α,β-didehydro amino acid was established recently by us (to be published).

Neoefrapeptins. Recently, ten 16-residue and two 13-residue neoefrapeptins A–N with insecticidal activity [55] were isolated from *Geotrichum candidum* SID 22780 (Saccharomycetales, Endomycetaceae; teleomorph: *Galactomyces candidus*). Notably, strains of *G. candidum* are of relevance in smear-ripened cheeses. It should be pointed out that this is the first report in the literature regarding the isolation and structural elucidation of a peptaibiotic from a yeast-like fungus. A nonribosomal peptide synthetase (NRPS) has recently been assigned as the key enzyme for production of the cyclohexapeptide siderophore ferrichrome by the fission yeast *Schizosaccharomyces pombe* [56]. Thus, the detection of further peptaibiotics from yeast-like organisms could be postulated.

Neoefrapeptins revealed a number of uncommon structural elements that are newly described for peptaibiotics: Ac_3c (abbreviated as Acc in the *Table* and in [55]) and (2*S*,3*S*)-3-methylproline (*cis*-L-3-MePro) were identified. Configuration analysis of the 16-residue neoefrapeptins revealed L-Iva[15]. Except for the Aib-containing neoefrapeptins D and N, Iva is present in position 4. Some of the neoefrapeptins also contain Iva[5] and Iva[10]. If present in position 4 and/or 5, the Iva residue was assigned the D-configuration, whereas the Iva residues in positions 10 and 15 were shown to possess the L-configuration. The L-configuration was assigned to all pipecolic acid (Pip) residues found in neoefrapeptins.

Cicadapeptins. Some species of *Cordyceps* (Hypocreales, Clavicipitaceae) are widely used in traditional Chinese medicine. Structures of known bioactive secondary metabolites and their pharmacological effects have thoroughly been reviewed [57]. The genus contains mostly entomopathogenic (*C. sinensis*, *C. militaris*, *C. pruinosa*) and fungicolous fungi (*C. ophioglossoides*, a parasite of underground deer truffles, *Elaphomyces* spp.). Recently, two eight-residue peptaibiotics, cicadapeptins I and II, were isolated from *Cordyceps heteropoda* ARSEF#1880, a parasite of the Australian cicada *Cicadetta puer* (Homoptera, Cicadaceae). The N-terminus of these peptaibiotics is blocked by a decanoic acid residue, and the C-terminus is amidated by 1,2-diamino-4-methylpentane (DMAP) [58]. Decanoic acid as N-terminal substituent of peptaibiotics has previously described for the lipopeptaibol LP237/F 7 [59] obtained from the soil-borne *Tolypocladium geodes* LP237, whereas 1,2-diamino-4-methylpentane represents a new structural element of peptaibiotics. Another distinguishing feature is the presence of two consecutive *trans*-L-4-Hyp residues. Notably, such Hyp-Hyp motif has been described for the first time to occur in peptaibiotics.

Integramides. Two novel inhibitors of HIV-1 integrase, the 16-residue integramides A and B, were isolated from extracts of *Dendrodochium* sp. MF 6888 [60]. The taxonomic position of the anamorphic fungal genus is yet unclear but some representatives seem to belong to the family Bionectriaceae of the order Hypocreales. Remarkably, integramides contain an acetylated N-terminal D-Iva and a total of five (A) or six (B) D- or L-Iva residues. Among the natural peptaibiotics, integramides are those containing the largest proportion of Iva described to date. Free Gly is found at the C-terminus of both peptaibiotics.

2.4. *Peptaibiomics – A Novel Methodical Approach in the Search for Peptaibiotics.* Recently, the technical term '*peptaibiomics*' was proposed, describing, in analogy to

proteomics, the approach to analyze the entirety of peptaibiotics, the so-called 'peptaibiome', produced by a certain strain under defined conditions. An advanced, rapid, and selective method was introduced that comprises solid-phase extraction (SPE) of peptaibiotics on C_{18}-cartridges followed by on-line reversed-phase (RP) HPLC coupled to an ion trap electrospray tandem mass spectrometer (ES-MS). The presence of peptaibiotics is indicated by the characteristic mass differences of m/z 85.1 Da, representing Aib residues which can be observed in the *b*-series of acylium fragment ions resulting from ESI-MS. This method was used to analyze the peptaibiome of recently described species of *Hypocrea* and *Trichoderma*. Peptaibiotics produced by the following strains were partially sequenced and compared to published sequences: *Hypocrea muroiana* MUCL 28442, *H. nigricans* MUCL 28439, *H. gelatinosa* CBS 724.87, *H. dichromospora* CBS 337.69, H. *vinosa* CBS 247.63, *H. semiorbis* CBS 244.63, and *H. citrina* (syn. *H. lactea*) CBS 853.70; *Trichoderma asperellum* CBS 433.97, *T. aggressivum* f. *europaeum* CBS 100526, *T. inhamatum* CBS 345.96, and *T. stromaticum* CBS 101875 [13].

Three strains of *Trichoderma brevicompactum* (CBS 109720, CBS 119569, CBS 119570) and another four which are closely related to that species (*Trichoderma* cf. *brevicompactum* CBS 112445, CBS 119576, CBS 119577, and NRRL 3199) were analyzed for the formation of peptaibiotics. These isolates were selected because of an antagonistic potential against Eutypa dieback and Esca, fungal diseases of grapevine (*Vitis vinifera*) trunks, that have not yet been investigated for the production of peptide antibiotics. All strains were found to produce membrane-active alamethicins F 30 (see above). In addition to that, novel peptaibols were detected, namely fourteen twelve-residue trichocryptins B, twelve eleven-residue trichocryptins A, nineteen eleven-residue trichobrevins A and B, six ten-residue lipoaminopeptides – trichoferins, and seventeen eight-residue trichocompactins, the latter terminating in free Vxx [5]. Taken together, the differential patterns of alamethicin production as well as the production of different trichothecene-type mycotoxins clearly support DNA-sequencing results. Both molecular and chemotaxonomic approaches indicate the existence of two species within what has been called *Trichoderma brevicompactum*. The taxonomy of *Trichoderma brevicompactum* is the subject of an ongoing study because the species, as presently circumscribed, is still heterogeneous.

The same approach has been applied to another eight grapevine-protective strains of *Trichoderma* species (*T. strigosum*, *T. erinaceus*, *T. pubescens*, *T. stromaticum*, and *T. spirale* as well as *T.* cf. *strigosum*, *T.* cf. *pubescens*): new seven-, ten-, and eleven-residue lipopeptaibols, with N-terminal alkanoyl, and C-terminal Leuol or Ileol residues were found, and named lipostrigocins and lipopubescins. Furthermore, new 18-residue peptaibols named trichostromaticins, and 19-residue peptaibols, named trichostrigocins, were discovered. One peptaibiotic carrying a free C-terminal Vxx, named trichocompactin XII, was also sequenced. In summary, these compounds may partially be responsible for plant-protective action of the producers [5][14].

3. Discussion. – The number of peptaibiotics published in the literature remarkably increased during the last three years. A first major reason explaining this phenomenon is that the analytical equipment used for screening and structural elucidation of peptide antibiotics is becoming more and more sophisticated [61]. New analytical approaches,

as illustrated by the method of peptaibiomics, target-oriented selection, and optimization of separation techniques, as well as the use of state-of-the-art methods in mass spectrometry, enable the detection of homologues and positional isomers, even if present in trace amounts. In principle, only one fully-grown *Petri* dish or slope is required for a first routine analysis of the peptaibiome of a fungal culture. In this context, it should be clearly pointed out again that Iva is frequently found as D- or L-isomer in peptaibiotics [2]. To continue, both enantiomers of Iva can be present in the same peptaibiotic, as was reported for neoefrapeptins and integramides (see above) as well as for the 15-residue peptaibol clonostachin: the latter contains D-Iva in positions 4 and 13, whereas L-Iva is found in positions 7 and 10 [62]. On total hydrolysis, an apparently racemic mixture (*RS*)-DL-Iva is released. Separation of DL-Iva and DL-Pip, analyzed as *N*-trifluoroacyl amino acid propyl esters on *Chirasil*-L-*Val*™ (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane), is not always satisfactory, as it depends both on the age and quality of the capillary columns provided by various manufacturers. However, Iva enantiomers can be resolved on *Chirasil*-L-*Val*™ after conversion into *N*-acetylisovaline propyl esters [63]. Instead of *Chirasil*-L-*Val*™, a *Lipodex*™ *E* column, representing a functionalized γ-cyclodextrine, can be used providing excellent resolution of Iva and Pro enantiomers [64]. Notably, all eight stereoisomers of 3- and 4-Hyp can be resolved on *Chirasil*-L-*Val*™ [65]. Alternatively, pre-column derivatization with the chiral N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alanine amide (FDAA; *Marfey*'s reagent), and subsequent HPLC or HPLC/ESI-MS analysis of the resulting diastereoisomers is recommended [66]: by this method, DL-Iva and DL-Pip are very well resolved. Last but not least, methanolysis of peptaibiotics, followed by trifluoroacetylation and analysis of the resulting dipeptides by GC/EI-MS, has been introduced as a method to solve the problem to assign the positions of isobaric amino acids Val/Iva and Leu/Ile, respectively [26]. Recent advances in quantitation of DL-amino acids are summarized in [67].

Second, screening of fungi from highly competitive habitats such as fungicolous, coprophilous, and entomopathogenic species remarkably enhances the probability to find new sequences of peptaibiotics. For instance, trichostromaticins A–E [14] may contribute to the potent bioactivity of *Trichoderma stromaticum* [68] (teleomorph: *Hypocrea stromatica* [69]). Hyperparasitic strains of this species that is known as a biologically active cacao endophyte have successfully been introduced in field control of the causal agent of Witches' Broom Disease of cacao (*Theobroma cacao*) in South America. They were shown to effectively suppress basidioma formation of the plant pathogen *Crinipellis perniciosa* (Tricholomataceae, Agaricales) [68][70]. High selection pressure in such environment favors strains capable of producing secondary metabolites with interesting biological activities that may effectively eliminate competitors. Owing to their exceptional membrane-penetrating action as exemplified in [71–74], peptaibiotics may play a decisive role in natural habitat as clearly demonstrated [27]. This observation supports the hypothesis of a parallel formation of hydrolytic enzymes and peptaibiotics, as their synergistic action was attributed an important role in mycoparasitism between *T. harzianum* and its fungal hosts such as *Botrytis cinerea* [75][76]. Depending on the *Trichoderma* strain/species studied, other antibiotic metabolites such as α-pentyl pyrones (6-PAP: 6-pentyl-2*H*-pyran-2-one and related structures [77]), as well as trichothecenes [78], may play an important role.

It can be hypothesized that the number of peptaibiotics described in the near future will increase considerably. For instance, the genus *Trichoderma* (with teleomorphs in *Hypocrea* [79]) is generally regarded as the richest source of peptaibiotic-producing species [80]. At the beginning of 2005, it was shown to comprise 88 species [81]. Two new species, for example, have been reported as endophytes of cocoa [82]. In April 2006, more than 100 *Trichoderma* species could be distinguished by molecular methods [83]. Many more – detected in sapwood of trunks of *Theobroma* spp., *Cola* spp., *Fagus sylvatica* (beech), *Scalesia pedunculata* (Daisy tree, Asteraceae), and in the woody liana *Ancistroderma korupensis* – have recently been described [84][85]. To continue, two new species of *Hypocrea*, *H. voglmayrii* [86] and *H. cristalligena* [87], have also been published.

Modern methods of molecular taxonomy have already led to significant changes in fungal systematics. The contribution of chemotaxonomy to this process is yet unclear but should not be overestimated. Nevertheless, the differential patterns of alamethicin production, as well as the production of different trichothecene-type mycotoxins, clearly supported necessary subdivision that is evident from the DNA-sequencing results in the *Trichoderma brevicompactum* complex. Both molecular and chemotaxonomic approaches indicate the existence of two species within what has been called *Trichoderma brevicompactum*.

With the exception of neoefrapeptins (see above), peptaibiotics have only been reported and sequenced in three families, Hypocreaceae, Clavicipitaceae, and Bionectriaceae of the order Hypocreales. Publications regarding the isolation of peptaibiotics from basidiomycetes should be considered very critically as outlined in [2]. In some cases, an infection of an asco- or basidiomycete's fruiting-body with mycoparasites might not be visible to the naked eye. For that reason, it cannot be excluded that younger stages of infection have accidentally not been recognized macroscopically, thus leading to extraction of infested material. This was first pointed out in [88]. The authors isolated chrysospermins A–D from the new Vietnamese species *Xerocomus langbianensis* (Boletaceae, Boletales) [89]. In contrast, in [35][90][91], the production of the respective peptaibiotics texenomycin, boletusin, and tylopeptin were attributed to the extracted fruiting bodies of the Potato Earthball *Scleroderma texense* (syn. *Scleroderma bovista*, Sclerodermataceae, Boletales), *Boletus* sp., and the Bitter Bolete *Tylopilus neofelleus* (Boletaceae, Boletales). It is likely that an unrecognized infection of *X. langbianensis* with *Sepedonium* sp. was the reason for detection of the four chrysospermins [88]. It should be considered that chrysospermins have originally been described from *S. chrysospermum* [92], a widespread parasite of the order Boletales [31].

To date, 18 genera of imperfect and ascomycetous fungi have been recognized to produce *ca.* 700 sequences of peptaibiotics. Most of the structures reviewed here and in [2] were isolated from the genera *Trichoderma* and its *Hypocrea* teleomorphs, from *Acremonium*, *Tolypocladium*, *Paecilomyces*, *Emericellopsis*, and *Sepedonium*. Less commonly reported producers were found in *Verticimonosporium*, *Stilbella*, *Mycogone*, *Mariannaea*, *Myrothecium*, *Clonostachys*, *Culicinomyces*, *Cordyceps*, *Geotrichum*, and *Dendrodochium*. Regardless of a number of attempts undertaken to validate the previously reported detection of Aib in hydrolyzed extracts of *Penicillium roquefortii* [93] and *P. nalgiovense* [94], both species could not be confirmed as producers of

peptaibiotics. Nevertheless, the abundance of data fully confirm our predictions expressed almost two decades ago [93][94].

By GC-FID and GC/EI-MS approaches, Aib, and pure enantiomers or mixtures of D- and L-Iva were found to occur in HCl hydrolysates of organic extracts of a number of ascomycetes. This comprises the following strains [95]: *Lecythophora mutabilis* CBS 303.62, *Nectriopsis candicans* CBS 627.72 (Bionectriaceae, Hypocreales), *Clonostachys candelabrum* (syn. *Sesquicillium candelabrum*) CBS 205.69 (Bionectriaceae, Hypocreales), *Bionectria pityrodes* CBS 322.78 (syn. *Nectria pityrodes*, Bionectriaceae, Hypocreales), *Niesslia aemula* CBS 556.75, *N. exigua* CBS 152.68, *N. exilis* CBS 560.74, as well as *Niesslia* sp., strains CBS 236.74 and CBS 477.74 (Niessliaceae, Hypocreales), and the related anamorphs *Monocillum mucidum* CBS 306.70 and *M. nordinii* CBS 101.63 (Niessliaceae, Hypocreales), *Wardomyces columbinus* CBS 233.66, *W. humicola* CBS 368.62, and *W. inflatus* CBS 367.62 (Microascaceae, Microascales). From these observations, the formation of peptides containing these nonproteinogenic α-alkyl amino acids can be concluded. Consequently, those strains should be regarded as particularly promising sources for future screening approaches. To continue, free Aib and enantiomeric mixtures of D- and L-Iva have actually been detected to occur in aqueous extracts of cultures of *Nectria cinnabarina* (Nectriaceae, Hypocreales) and a number of *Hypomyces* sp. (personal observation). To understand the importance of the latter finding, additional investigations would be required.

Generally, formation of peptaibiotics should rather be discussed as an adaptation to highly specialized life styles of the producers occasionally being marine symbionts [48][96], but mostly facultative or obligate plant pathogens, fungicolous or entomopathogenic fungi occupying some particular ecological niches.

Overall, fungal biodiversity creates differences in chemical structures that may exhibit new bioactivities, thus leading to the development of new drugs for use in human and veterinary medicine, and in agriculture and forestry.

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Note added in Proof. Four nine-residue peptaibiotics, MS-681a, b, c, and d, which act as inhibitors of myrosin light chain kinase, were isolated from *Myrothecium* sp. KY6568 [97]. Notably, the configuration of the Iva^{8} and the Iva^{2} residue was shown to be 'L', whereas Iva^{5} was assigned the 'D'-configuration. The C-terminus of all four homologues is protected by the same polyamine, (2′*RS*)-N^{1}-(2-amino-3-phenylpropyl)spermidine, being another novel constituent of peptaibiotics. [98]. Two 20-residue peptaibiotics, septocylindrin A and B, have been isolated from the fungus LL-Z1518 [99]. Amino acid residues 1–19 and configurations of the amino acids are identical with those reported for alamethicin F30/3 of alamethicin F50/5, respectively.

Septocylindrins differ from alamethicins only in the structure of the C-terminal alcohol, which is 2-(2-amino-3-phenylpropylamino)ethanol (Phaol). Such C-terminus has only been described for aibellin, a 20-residue peptaibiotic from *Verticimonosporium ellipticum* D1528 before [100]. However, the identification of the producer as *Septocylindrium* sp. is questionable as the authors did not provide taxonomic data to verify its identity [101].

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REVIEW

Recent Advances and Future Prospects in Peptaibiotics, Hydrophobin, and Mycotoxin Research, and Their Importance for Chemotaxonomy of *Trichoderma* and *Hypocrea*

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Fungi of the genus *Trichoderma* with teleomorphs in *Hypocrea* are abundant producers of a group of amphiphilic, non-ribosomal **pept**ide antib**iotics**, which are rich in the non-proteinogenic amino acid **Aib** (α-aminoisobutyric acid). They are referred to as **peptaibiotics**, or **peptaibols**, if a 1,2-amino alcoh**ol** is present at the C-terminus. *Trichoderma*/*Hypocrea*, like other ascomycetous fungi, also produce hydrophobins, a class of small, cysteine-rich proteins. Advanced soft ionization mass spectrometric techniques such as LC-CID-MS, LC-ESI-MS^n, and IC-MALDI-TOF-MS enabled the high-throughput analysis, simultaneous detection and sequence determination of peptaibiotics and hydrophobins from minute quantities of fungal materials. Some *Trichoderma* species have been recognized to produce peptaibiotics as well as simple mycotoxins of the trichothecene group. The combination of sequence data of both groups of peptides with the pattern of low-molecular-weight secondary metabolites, including trichothecene-type mycotoxins, independently confirmed the results of morphological, molecular, and phylogenetic analyses. This approach established a new lineage in *Trichoderma*/*Hypocrea*, the *Brevicompactum* clade, comprising four new and one redescribed species. Notably, commercial preparations of single or mixed cultures of *Trichoderma* species, in particular *T. harzianum*, and *T. koningii*, are registered as biocontrol agents for soil and plant pathogens. In this context, it is emphasized that the four mycotoxin-producing species of the recently established *Brevicompactum* clade (*T. brevicompactum*, *T. arundinaceum*, *T. turrialbense,* and *T. protrudens*) are not closely related to any of the *Trichoderma* species currently used as biocontrol agents. Furthermore, possible health concerns about release of peptaibiotics in the biosphere are discussed with respect to their bioactivities and their use as drugs in human and veterinary medicine. Finally, future prospects regarding novel bioactivities and further research needs, including interdisciplinary taxonomic approaches, are outlined.

Peptaibiotics. – Peptaibiotics/peptaibols constitute a constantly growing family of peptide antibiotics [1–3]. Currently, more than 700 individual sequences of peptai-

biotics, produced by members of 18 fungal genera, are reported in the literature. Of these *ca.* 20 genera, *Trichoderma* and *Hypocrea* are the most abundant sources of peptaibiotics with approximately half of the known peptaibiotics originating from those two genera [4]. *Trichoderma* is the asexual form of, and is phylogenetically indistinguishable from, *Hypocrea*. Thus, in this review we refer to a single genus, *Trichoderma*/*Hypocrea*. Most of the peptaibiotic-producing species formerly included in *Gliocladium* have been reclassified in *Trichoderma* [5]. A continuous problem has been the high rate of reports of biological activities of *Trichoderma*/*Hypocrea* based on misidentified species [6]. However, positive identification can be achieved by DNA sequencing which is currently accepted as the state-of-the-art approach in fungal taxonomy. Complete or partial sequences of peptaibiotics have been reported from *ca.* 25 *Trichoderma*/*Hypocrea* species [7–9], the identity of which has been verified by DNA sequencing. An additional 15 positively identified species are known to produce peptaibiotics [10] by an 'intact cell matrix assisted laser desorption/ionization-time of flight mass spectrometry' (IC-MALDI-TOF-MS) approach [11]. In a pilot study of *ca.* 60 species, we found that *ca.* 75% of these strains produced peptaibiotics on the medium tested. While *ca.* 400 species of *Hypocrea* have been described over the past 175 years, few of them have been seen since they were first described, and even fewer have been linked to *Trichoderma* stages. On the other hand, only few species of *Trichoderma* were described prior to the mid 1990s, when molecular techniques were routinely applied in taxonomy. The consequence is that *Trichoderma* stands as one of the few species-rich genera of microfungi for which all known species have been cultured, and for which sequences of two or more genomes have been deposited in GenBank. The web site of the *International Commission for Taxonomy of Fungi*, *Trichoderma* and *Hypocrea* subcommittee (http://www.isth.info/biodiversity/index.php), currently provides DNA sequences for 104 species of *Trichoderma*/*Hypocrea,* but several new species are in the process of being described. So, while only approximately half of the known *Trichoderma*/*Hypocrea* species have been investigated for the production of peptaibiotics, the full diversity of the genus has only begun to be revealed. The certain discovery and characterization of new *Trichoderma*/*Hypocrea* species in previously unexplored regions, for instance Asia [12], and in ecological niches, such as woody tissues of trees [13–15], will lead certainly to the discovery of new producers, novel constituents, and eventually uncommon building schemes of peptaibiotics.

A multiphasic, interdisciplinary approach to taxonomy has always been most convenient to discern species boundaries and the relationships among species. Taxonomy was initially strictly based on morphology of the sexual or asexual morph, which ever was in the hand of the taxonomist and depended upon the interests of the taxonomist. By the mid 19th century, it was known that ascomycetes such as *Hypocrea* had asexual morphs that were classified in different genera, but rather few taxonomists viewed the organism as a whole. Over the last 30 years of the 20th century, a greater emphasis was placed on amalgamating anamorph and teleomorph taxonomy. This amalgamation not only provided information about the respective morphs, but also, because artificial cultures were involved, taxonomic characters could be drawn from the *Petri* dish. Over time, there have been attempts to use metabolites and physiology in taxonomy of ascomycetes, such as carbon uptake [16], but interspecific variation in

these characters has limited their use in taxonomy. A major difficulty in evaluating the taxonomic utility of metabolic or other physiological characters is the high degree of misidentifications in the literature, as was mentioned above. The mid 1990s brought molecular phylogenetics together with a surge in descriptions or redescriptions of *Trichoderma* species. The DNA sequencing provided a precision in species identification and species boundaries that was lacking in purely morphological taxonomy. Thus, the newly described species were scrutinized through the lens of DNA sequencing, as have been all of the roughly 130 species known today. Prior to 2000, peptaibiotics were attributed to four 'common' species, *T. viride*, *T. koningii*, *T. harzianum*, and *T. longibrachiatum*. However, the identity of those cultures is in doubt, or has already been shown to be incorrect. Certainly, we cannot be criticized, as it has only been in the last few years that sufficient sequences of correctly identified *Trichoderma* species have been generally available through the ISTH web site and GenBank [17]. Nevertheless, statements about *Trichoderma* species must always be accepted with great caution unless the authors of those reports describe how their cultures were identified.

Peptaibiotics such as alamethicins are considered for treatment of plant diseases because of their effect on the walls of pathogens [18][19]. They may also have a role to play in taxonomy and species identification. Peptaibiomics [7–9] and IC-MALDI-TOF-MS [3][10][11] have considerably increased the screening efficiency for peptaibiotics but also contributed to progress in chemotaxonomy of *Trichoderma*/*Hypocrea*. Recently, peptaibiotics have even been isolated from natural habitats, such as dung of rabbits and from the tortoise *Testudo hermanni* [20]; and they were also considered as stable markers for the presence of fungi in the marine environment [21]. Briefly, linear peptaibiotics are polypeptide antibiotics which *i*) have a molecular weight between 500 and 2,200 Da, thus containing 5–21 residues; *ii*) show a high content of α-aminoisobutyric acid (Aib); *iii*) are characterized by the presence of other non-proteinogenic amino acids and/or lipoamino acids; *iv*) possess an acylated N-terminus, and *v*) have a C-terminal residue that, in most of them, consists of a free or acetylated amide-bonded 1,2-amino alcohol, but might also be an amine, amide, free amino acid, 2,5-dioxopiperazine, or sugar alcohol [4]. Many different peptaibiotics may be produced by a single strain of *Trichoderma*/*Hypocrea*. Peptaibiomics is the characterization of the entirety of peptaibiotics produced by a fungal strain under defined culture conditions. It also may include the time-dependent dynamics of biosynthesis and degradation of peptaibiotics [22]. An LC/MS-based peptaibiomics approach is based on the generation of so-called 'in-source fragments' by 'collision-induced decomposition' mass spectrometry (CID-MS) to the skimmer region of an ESI mass spectrometer. Varying voltages are applied to the skimmer, thus providing the possibility of clearly differentiating various types of ions. The CID-MS technique provides a possibility to partially sequence peptaibiotics. The most diagnostic difference to screen for is Δ (m/z) 85, which indicates the presence of the α-aminoisobutyric (Aib) residue being unique to all peptaibiotics. Another diagnostic difference is Δ (m/z) 99, indicative for a Vxx residue (*i.e.*, isomeric valine or isovaline). In this context, it should be pointed out again that non-proteinogenic Iva is frequently found as D- or L-isomer in peptaibiotics, whereas all other chiral constituents known so far have been reported to possess L-configuration. In some cases, both enantiomers of Iva are present in the same

peptaibiotic [4]. Complete sequencing is achieved by a combination of data from CID-MS, MS/MS, QTOF MS, and MS^n (in the case of ion-trap instruments). These data are generally needed to be combined with HPLC elution profiles, the so-called fingerprints of the samples [4][8][9].

Intact cell MALDI-TOF mass spectrometry (ICMS) permits detection of biomolecules in unfractionated biomass down to the attomolar level. Microcolonies of cyanobacteria of 50 to 100 cells have been characterized at the clonal level, differentiating strains by their peptide metabolite inventory [23–25]. We thus could expect to analyze much larger fungal cells down to the single-cell level. Analysis of bacteria at the intact-cell level by ICMS is now well-established, and generally detection of a set of ribosomal proteins as most abundant compounds permits identification at or below the species level at a comparable sensitivity as PCR techniques [26–29], while additional protein markers permit, *e.g.*, the differentiation of drug-resistant strains [30–33]. The standard ICMS sample-preparation process suspending cells in a solvent mixture disintegrates bacterial membranes releasing intracellular proteins. With fungal cells, however, solvent-soluble components of the cell wall as hydrophobins or excreted metabolites are detected [3][10][11][34][35]. Although the metabolite inventory is essential for strain characterization, this information alone is generally insufficient for the identification of microbial species and strains. Peptaibol formation has been comparatively analyzed in 32 strains of 29 species of *Trichoderma*/*Hypocrea* concluding that there is no strict correlation of phylogeny with the types and sequences of the peptaibols produced, but that the production of some groups of peptaibols appears to be found only in some clades or sections of the genus [10]. It is clear from this and another analysis including mycotoxins [36] that relevant phylogenetic information could only be drawn from the respective biosynthetic genes.

Most peptaibols range in size between 1000 and 2000 Da, and are thus easily detected without interference from low-mass metabolite signals or matrix signals. In this region, monoisotopic masses are recorded, and peptide family patterns are visible. Peptaibol production on solid media starts at the onset of sporulation, as has been shown for *H. atroviridis* [35]. In recent studies, more than 40 strains of 32 species of *Trichoderma*/*Hypocrea* have been investigated for the production of 10- to 20-residue peptaibiotics. One study defined 26 groups of characteristic mass peaks, and assigned half of these to known peptaibiotics [10]. This overview identified species with yet undescribed compounds. The purpose of the study has been to search for correlations of peptaibol type production with phylogenetically ascertained species descriptions. Taken together with all available data on peptaibiotics in *Trichoderma*/*Hypocrea*, the complexity of relations between phylogeny and peptaibol synthetases becomes obvious. Certain peptaibols are found throughout all sections and clades, while others are restricted to particular sections. As orthologous peptide synthetase domains and modules are found, and also obvious extensive regional similarities exist, possible relationships would best be analyzed at the peptide synthetase gene level.

ICMS Peptaibol patterns obviously need further analysis of molecular ions to clearly identify the sequences of the respective compounds. With the current MALDI-TOF-MS approach, the presence of these metabolites can be detected extremely rapidly from small amounts of mycelia/spores, thus also facilitating extended expression studies.

HPLC-MS combined with ESI, atmospheric-pressure chemical ionization (APCI), or other atmospheric-ionization techniques as well as UV/VIS diode array detection revolutionized the process of assaying for uncommon mycotoxins. These can be further characterized by high-resolution MS from TOF, and now also with Orbi-Trap instruments, for quickly determining the elementary composition of a given metabolite, which can be identified in databases such as Antibase, comprising 33,557 records in the 2007 version [37]. This is especially true for UV/VIS-active compounds where the UV/VIS data and original strain information can be used for validation [38]. For trace analysis, HPLC-MS/MS on triple quadrupole instruments is state-of-the art, because both the sensitivity (down to low fg levels) and specificity are excellent.

Hydrophobins. – Hydrophobins range in size from *ca.* 75 up to 400 amino acid residues containing eight positionally conserved cysteine residues forming characteristic disulfide bonds, and are divided into two classes with respect to their hydropathy profiles and cystein spacings. Hydrophobins have been considered as suitable phylogenetic markers for several reasons. Fungi contain a set of hydrophobin genes, generally with a developmentally regulated expression reflecting life conditions and interactions. Overproduction of a hydrophobin during endophytic interaction of *T. asperellum* and cucumber roots has recently been demonstrated [39]. The hydrophobins HFB1 and HFB2 have diverse functions in development of *H. jecorina* [40], including surface hydrophobicity, formation of aerial mycelia, and spore properties.

Genome sequencing of *H. jecorina*/*T. reesei* revealed the presence of six hydrophobin genes, and for *H. atroviridis*/*T. atroviride* and *T. virens*, ten hydrophobin genes have been annotated so far, respectively [10][41][42]. These hydrophobic peptides can be selectively dissolved and are thus excellent biomarkers for MALDI-TOF-MS [3][10][11]. Extending genomic information, hydrophobin patterns are diverse due to posttranslational processing, as shown in the pioneering work on *H. jecorina* [43–45]. Processing involves the respective signal sequences for excreted proteins, but also defined N- and C-terminal cleavage sites [10][11]. Due to their fairly small size, sequence information of the hydrophobins can be obtained by mass spectrometry, and the respective genes are accessible by standard PCR methods. It has been noted that the variability of hydrophobin genes is fairly significant and exceeds similarities of other biomarkers proposed, *e.g.*, ubiquitins. In addition, variation of the number of hydrophobin genes provides additional information to housekeeping gene structures. Gain and loss of hydrophobin genes may also play a key role in speciation.

ICMS Patterns of the strain *H. jecorina* (syn. *T. reesei*) QM9414 have been correlated with the respective hydrophobin composition of HFB1 and HFB2 through analysis of knock out mutants [10]. Unexpectedly, two other strains, CPK 618 and CPK 665, showed differing mass peaks, which have been tentatively assigned to the expression of either HFB3 and HFB4 or differing processing of HFB1. Vegetative and sporulating mycelia differed as expected in their hydrophobin patterns. In the same study, 29 species of *Trichoderma*/*Hypocrea* have been compared, and unique patterns have been detected. In case of *H. atroviridis*/*T. atroviride* P1, mass peaks could be correlated with sporulation related hydrophobin SRH1, as also for *T. longibrachiatum* HFB3. As the results indicate proteolytic processing beyond signal peptide cleavage, further work is needed to ascertain cleavage sites and proteases involved.

We successfully linked morphological, molecular taxonomic, and phylogenetic approaches, and IC-MALDI-TOF MS, peptaibiomics, and LC/HR-ESI-MS mycotoxin screening of *Trichoderma*/*Hypocrea* to redescribe a new lineage in *Trichoderma*/*Hypocrea*, the *Brevicompactum* clade. This clade included, in addition to *T. brevicompactum*, four new species in the *T. brevicompactum* complex, and an additional new *Hypocrea* species [36]. This approach clearly supported our previous hypothesis that more than one species was involved when *T. brevicompactum* has originally been described. Combination of classical morphological mycology with DNA sequencing and profiling of mycotoxins and other small metabolites, hydrophobins, and peptaibioitics clearly delimited four new species from within the original morphological construct of *T. brevicompactum* [46]. In summary, the combination of these methods provides information about metabolites that can be used to identify species and strains.

New Bioactivities. – Sophisticated approaches are urgently required to detect novel or previously unrecognized biological activities of peptaibiotics. *Hosotani et al.* [1] reported on the inhibition of amyloid β-peptide formation in primary guinea pig cerebral cortex neuron cell cultures by a 14-residue peptaibol, SPF-5506-A. It is known that increased accumulation of amyloid β-peptides plays a decisive role in the pathogenesis of neurodegenerative dementia such as *Alzheimer*'s disease. Produced by *Trichoderma* sp. SPF-5506, peptaibol SPF-5506-A can be considered as a positional isomer of the 14-residue harzianin HC [47]. Thus, it can be hypothesized that harzianins and structurally related peptaibols might display similar bioactivity.

Biocontrol Agents. – In the past decade, peptaibiotics and the fungi producing them have gained much interest as a possible alternative to xenobiotics such as synthetic pesticides in agriculture and forestry. Recently, it has been demonstrated that five new 18-residue peptaibols, trichostromaticins A–E, produced by *Trichoderma stromaticum* [48] (teleomorph: *Hypocrea stromatica* [49]), strains CBS 101875 and CBS 101730, may contribute to the potent bioactivity of these two strains [9]. This species, which is known only as a mycoparasite of the cacao (*Theobroma cacao*) Witches' Broom disease pathogen *Moniliophthora perniciosa* [48], formerly *Crinipellis perniciosa* [50], has successfully been introduced into field control of the disease in South America. The main effect is the suppression of basidioma formation by the pathogen [51]. These two strains of *T. stromaticum* and another seven strains of *T.* cf. *strigosum*, *T. strigosum*, *T. erinaceus*, *T.* cf. *pubescens*, *T. pubescens*, and *T. spirale* were highly active against the causal agents of *Eutypa* dieback, *Eutypa lata*, and Esca disease, *Phaeomoniella chlamydospora*, and *Phaeoacremonium aleophilum*. These are latent trunk diseases that cause severe economic losses in viticulture [52]. Notably, all of the eight *Trichoderma* strains produced peptaibiotics that may contribute to their potent bioactivity against the above grapevine pathogens [9].

Strains originally identified as *T. harzianum* and *T. koningii* are currently used in commercial biological control agents (BCAs) in Europe and North America. Our own, preliminary studies on five BCAs available on the German market most likely indicate that the bioactivity of these commercial products can clearly be attributed to the presence of peptaibiotics. Taxonomy of the *Trichoderma* strains in these BCAs is currently under revision [53].

Mycotoxin Production. – Concerns have been expressed about potential toxicity of peptaibols/peptaibiotics in biological control, a fear that is reinforced by names such as 'trichotoxin'. Clearly, a biological control agent that enters the food chain should not produce toxins. Species identified as biological control agents, including *T. harzianum* and *T. viride*, are reported to produce trichothecene toxins. However, they have been reidentified as *T. brevicompactum* and *T. arundinaceum*, closely related species, both of which belong to the *T. brevicompactum* complex that was mentioned above. This lineage is distant from any biological control species. Only *T. brevicompactum* and three closely related species within the *T. brevicompactum* complex [8][36] have been proven to produce trichothecene-type mycotoxins (trichodermin, harzianum A), and neither in [54], nor in [9] were trichothecenes reported in any species outside of the *T. brevicompactum* complex.

Note, that the 11- and 14-residue peptaibols harzianins HB [55] and HC [47] must not be confused with the non-peptidic, trichothecene-type mycotoxins harzianum A [54][56][57] and harzianum B [58]. Studies reporting other types of trichothecenes than the one with only a OH group in the 4- and perhaps 15-position are probably wrong, as these other types, *e.g.*, from *Fusarium,* are biosynthesised *via* trichotriol, rather than trichodermol [59], as is probably the case in *Stachybotrys*, *Myrothecium*, *Memnoniella*, and *Podostroma* (= *Hypocrea*) [54][59][60], all of which are members of the Hypocreales along with *Trichoderma*/*Hypocrea*. Gliotoxin is produced by *Trichoderma virens*, which is commonly reported in the literature as *Gliocladium virens* [61].

Toxicity of Peptaibiotics. – Recently, it has been reported [62][63] that the 20-residue peptaibols alamethicin and paracelsin, the 16-residue antiamoebin, and other 11-residue trichobrachins [2][64] were highly toxic in three *in vitro* invertebrate models, *viz*. *Crassostrea gigas*, *Artemia salina*, and *Daphnia magna*. An alternative explanation of the toxicity reported in [62] and [63] might be that the batch of the alamethicin standard used (*Sigma-Aldrich*, product number A-4665) may have been contaminated with the trichothecene mycotoxin harzianum A that is produced by the strain of *Trichoderma* cf. *brevicompactum* used for alamethicin fermentations [8]. In our own work, we have found that this particular *Sigma* alamethicin contains harzianum A; and this group of trichothecene toxins is highly toxic to *Artemia salina* [36].

In contrast, oral administration of antiamoebin [65][66], trichotoxin A [67], aibellin [68][69], ampullosporin [70], and trichofumin [71] to rodents and ruminants revealed a very low toxicity. This was explained by the almost complete resistance of peptaibiotics towards any kind of proteolytic cleavage with the result that their high molecular weight prevented them from passing through the intestinal cell. The microheterogeneous mixtures of antiamoebin or aibellin were administered in huge amounts to cows or goats, respectively. The idea behind these experiments was to inhibit protozoa and other microbial competitors in order to increase cellulose digestibility by rumen bacteria. Antiamoebin was also tested for antiprotozoal and anthelminthic activities in man and against *Trypanosoma evansii* in laboratory animals. While its LD_{50} value was very high: 155–165 mg/kg body weight/animal, only 10 mg/kg killed the pathogen [65], a concentration far below the level that would affect the test animal.

Trichotoxin and alamethicin have been reported to cause haemolysis of erythrocytes [72]. This action, however, is common for many amphiphilic detergent-like molecules, including soaps and saponins, and requires direct contact with cell membranes. Analogously, the reported uncoupling of oxidative phosphorylation in mitochondria caused, for instance, by efrapeptins [73] requires direct interaction under experimental conditions.

From an application point of view, the potential 'toxic' effect of peptaibiotics appears to be more theoretical than real, perhaps no more than that of common amphiphilic detergents, and well below the threshold of human consequence. On the other hand, the reported synergistic effects of peptaibiotics and cellulases by strains of *Trichoderma* and *Hypocrea,* and the antibiotic activities of peptaibiotics which are of interest for plant protection [18][19] enhance their potential for biological control application.

Conclusions. – The introduction of novel methodical approaches during the past two years such as peptaibiomics and IC-MALDI-TOF-MS have remarkably enhanced screening efficiency for peptaibiotics and has extended their application to chemotaxonomic questions. More than half of the currently known sequences of peptaibiotics were determined, between 2002 and 2007, mostly by HPLC-MS approaches. Likewise, HPLC-MS has revolutionized mycotoxin analysis in the past decade, not only lowering detection limits but also with the introduction of high-resolution MS screening, and fast identification/dereplication of mycotoxins without reference standards are now possible. Novel test assays have been used to detect new or previously unrecognized bioactivities of peptaibiotics such as neuroleptic [70], anti-HIV integrase I [74], and anti-*Alzheimer* [1] effects. Combined use of molecular biology, direct-infusion MS, HPLC-MS, and MALDI-TOF-MS has also been proven to be beneficial for characterization of new fungal species [36][38][75].

Certainly, trichothecene-producing strains of *Trichoderma* and *Hypocrea* must not be introduced into biological control of fungal pathogens in the field or greenhouse. Thanks to a growing phylogenetic understanding of *Trichoderma*/*Hypocrea*, we are able to predict biological properties of species, and avoid those that are related to known toxigenic species. Mycotoxin-free *Trichoderma* species such *T. stromaticum* that can be regarded as safe are already in use for crop protection [51].

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Note Added in Proof. Recently, a new subfamily of natural peptaibiotics, named cyclopeptaibiotics, was introduced, currently comprising seven 4-membered and two 7-membered cyclic peptides, which contain at least one Aib or Iva residue [76].

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ORIGINAL ARTICLE

The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptaibiotics, and mycotoxins

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Abstract The Brevicompactum clade is recognized as a separate lineage in *Trichoderma*/*Hypocrea*. This includes *T. brevicompactum* and the new species *T. arundinaceum, T. turrialbense, T. protrudens* and *Hypocrea rodmanii*. The closest relative of the Brevicompactum clade is the Lutea clade. With the exception of *H. rodmanii*, all members of this clade produce the simple trichothecene-type toxins harzianum A or trichodermin. All members of the clade produce peptaibiotics, including alamethicins. Strains previously reported as *T. harzianum* (ATCC 90237), *T. viride* (NRRL 3199) or *Hypocrea* sp. (F000527, CBS 113214) to produce trichothecenes are reidentified as *T. arundinaceum*. The Brevicompactum clade is not closely related to species that have biological application.

Keywords Hypocrea · Hypocreales · Hypocreaceae · Systematics · Hydrophobins · Trichothecene · Harzianum A · Trichodermin · Endophyte · MALDI-TOF · Biocontrol

Taxonomic novelties *Hypocrea rodmanii* Samuels & Chaverri, *Trichoderma arundinaceum* Zafari, Gräfenhan & Samuels, *Trichoderma protrudens* Samuels & Chaverri, *Trichoderma turrialbense* Samuels, Degenkolb, K.F. Nielsen & Gräfenhan.

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Introduction

Visible phenotype, including microscopic and colony characters, has come to occupy a role subsidiary to molecular phylogenetics in *Trichoderma* and its corresponding teleomorph, *Hypocrea*. For example, *T. viride* is the most commonly reported species in the genus, but many, or most, of those reports are likely to have been based on misidentifications because it is now known that *T. viride* has a limited distribution in the northern hemisphere while the morphologically very similar *T. viridescens* is widely distributed at temperate latitudes (Jaklitsch et al. 2006). Similarly, *T. harzianum* also ranks among the most commonly reported species, on the basis of its morphology, but gradually this morphological concept is being refined. Thus, the cause of green mould disease of commercial mushrooms was originally identified as *T. harzianum* but is now known to be *T. aggressivum* (Samuels et al. 2002). While the physical differences between *T. viride* and *T. viridescens* and between *T. harzianum* and *T. aggressivum* are small but consistent, it was only through phylogenetic analysis that the respective pairs were seen to represent distinct species.

Trichoderma brevicompactum could be mistaken for *T. harzianum* on the basis of considerable shared micromorphology, but phylogenetic analysis shows them to be only distantly related (Kraus et al. 2004). Moreover, when it was originally described (Kraus et al. 2004), a combined ITS-*tef1* phylogram indicated two, highly supported, sister groups within *T. brevicompactum*. The two well-supported sister clades at least indicated distinct phylogenetic if not taxonomic species.

In their study of mycotoxin production in *Trichoderma*, Nielsen et al. (2005) found that the ex-type and other cultures of *T. brevicompactum* produced the simple trichothecene-type mycotoxin trichodermin, while isolates in the sister lineage to the ex-type produced harziaum A. Trichodermin is the first trichothecene for which the structure was fully elucidated; it was produced by a culture identified as *T. viride* LEO ND 8 (Godtfredsen and Vangedal 1964, 1965). That culture was reidentified by Nielsen et al. (2005) as *T. brevicompactum*. Harzianum A was isolated from a culture identified as *T. harzianum* (ATCC 90237, Corley et al. 1994) and later reidentified by Nielsen et al. (2005) as *T. brevicompactum*, although in the sister lineage mentioned above. Because the patterns of toxin production by isolates coincided with the phylogenetic lineages observed in the protologue of *T. brevicompactum*, Nielsen et al. (2005) suggested the existence of two phylogenetic species within the morphological species *T. brevicompactum*. However, the trichothecene results were ambiguous in that one of the cultures (CBS 112445) identified by Kraus et al. (2004) as being in the *T. brevicompactum*-type group produced harzianum A, not trichodermin. This culture was separated from the type group in a parsimony tree, but with only modest bootstrap support (69%) (Kraus et al. 2004).

Nielsen et al. (2005) demonstrated the production of alamethicins in extracts of *T. brevicompactum*. Alamethicins are a group of 20-residue polypeptides belonging to the so-called peptaibiotics. Almost 850 individual sequences of linear and 9 of cyclic peptaibiotics are reported to be produced by members of approximately 20 fungal genera. Peptaibiotics are defined as linear or cyclic polypeptide antibiotics which (1) have a molecular weight between 500 and 2,200 Dalton, thus containing 4–21 residues; (2) show a high content of α-aminoisobutyric acid (Aib); (3) are characterized by the presence of other non-proteinogenic amino acids such as isovaline (Iva) and/or lipoamino acids; (4) possess an acylated *N*-terminus, and (5), in the case of the linear compounds, have a *C*-terminal residue that in most of them consists of a free or acetylated amide-bonded 1,2-amino alcohol, but might also be an amine, amide, free amino acid, 2,5-dioxopiperazine, or sugar alcohol. The majority of linear Aib-containing peptides carries a *C*-terminal residue representing a 2-amino alcohol, and this subgroup is therefore referred to as peptaibols. Notably, half of the known peptaibiotics are produced by strains and species of the genus *Hypocrea/Trichoderma* (Degenkolb et al. 2003, 2006a, 2007, 2008a, b; Krause et al. 2006). Peptaibiotics were proposed to contribute to the disruption of the cell walls of fungal pathogens owing to their amphiphilicity and membrane activity (Lorito et al. 1996). Gräfenhan (2006, see also Degenkolb et al. 2006a) reported that cultures of *T. brevicompactum* had unusually high antagonistic activity against the causal agents of two grapevine diseases, Eutypa dieback (*Eutypa lata*) and Esca disease (*Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*) when compared to other *Trichoderma* species so far used in biological control (*T. atroviride*, *T. harzianum*, *T. koningii*, and *T. viride*). In an effort to understand the in vitro antagonistic activity against the grape pathogens and, at the same time, to explore for any link between production of peptaibiotics and phylogeny within what they termed 'the *T. brevicompactum* complex,' Degenkolb et al. (2006a) documented the production of peptaibiotics in *T. brevicompactum*. They also showed that the alamethicin F30 patent strain NRRL 3199, originally identified as *T. viride* (Coats et al. 1974; Brückner and Jung 1980; Kirschbaum et al. 2003; Psurek et al. 2006), belonged to the sister clade of the ex-type strain of *T. brevicompactum*. Degenkolb et al. (2006a) found that in general the patterns of peptaibiotics produced in *Trichoderma*/*Hypocrea* are species-specific. The isolates in the type-group of *T. brevicompactum* did not produce trichobrevins while those belonging to its sister clade, including

NRRL 3199, did. Like other members of this clade, NRRL 3199 produced harzianum A. The peptaibiotics of CBS 112445, which was weakly linked to the type group of *T. brevicompactum* (Kraus et al. 2004), were more comparable to those of the type group than to those of the sister group.

Since the appearance of the publications cited above, we have found additional cultures of the morphological species *T. brevicompactum*. DNA sequence analysis showed that these cultures fall into the respective phylogenetic lineages that were seen in the protologue. In the course of our monographic studies of *Trichoderma* and *Hypocrea* we have found additional cultures of *Trichoderma*/*Hypocrea* that are closely related to *T. brevicompactum* s. lat. but that form independent lineages. In the present work, we re-examine the phylogenetic lineages within the morphological species *T. brevicompactum* and the phylogenetic relationships of *T. brevicompactum* and its closest relatives. We continue an examination of the secondary metabolites produced by members of this clade with descriptions of the peptaibiotics, hydrophobins, and small metabolites like trichothecenes, and the significance of these metabolites to taxonomy.

Materials and methods

Chemicals used

Solvents were HPLC grade and all other chemicals analytical grade unless otherwise stated. Water was 18.2 MΩ cm^{-1} purified.

Morphological analyses

The isolates studied are listed in Table 1. Most were obtained from the indicated culture collections. The strain CBS 121320 was isolated as an endophyte from sapwood of a cacao tree (*Theobroma cacao*) following the protocol described by Evans et al. (2003). Cultures of *Hypocrea rodmanii* were obtained by isolating single ascospores from freshly collected specimens on cornmeal-dextrose agar (CMD; cornmeal agar Difco + 2% dextrose).

Material to be used for microscopic measurements was first immersed in 3% KOH. Herbarium specimens of *Hypocrea* collections were rehydrated briefly in 3% KOH. Sections were made with an IEC-CTF microtome cryostat (International Equipment, Needham Heights, Mass.). Measurements and observations of sectioned perithecia were carried out in lactic acid preparations. Measurements of microscopic characters of the anamorph and teleomorph were made from 3% KOH, which was replaced with distilled water, following the protocol of Samuels et al. (2002). Microscopic characters of conidiophores and conidia were taken from CMD or SNA (synthetic low nutrient agar; Nirenberg 1976) within approximately 7 days, mostly using intermittent light (12 h cool white fluorescent lamps, 12 h darkness). Presence of chlamydospores was determined by examining the underside of colonies after 7–10 days on CMD with the 40× objective. Where possible, 30 units of each character were measured, using Scion Image for Windows™ (www.scioncorp.com). Observations were made with differential interference contrast (DIC), phase contrast (PC), and bright field (BF) microscopy. Helicon Focus™ version 4.21.5 Pro(MP) (Helicon Soft, http://HeliconFocus.com) was used to produce some composite images (e.g. Figs. 7o, 8b, 9i and 12c). Color standards are from Kornerup and Wanscher (1978) and are cited as "K&W."

Growth rates were determined on potato-dextrose agar (PDA, Becton, Dickenson, Sparks, Md.) and SNA following Samuels et al. (2002, 2006).

Statistical analysis

For each strain, descriptive statistics (minimum, mean, median, maximum, standard deviation and 95% confidence intervals of the mean) were computed for the morphological data. These are summarized in Table 8 (see later). Metabolite data were coded using categorical scales. Multidimensional scaling (MDS) was performed using standardized data matrices that included either the morphological data, expressed as means for each strain, or the metabolite data, or both. The characters used in the analysis are shown in Tables 7 and 8 (see later). Computation of descriptive statistics was performed using Systat 11 (Systat Software, San José, Calif.). Only strains for which no data were missing were included in the analysis. The MDS had exploratory character only and was aimed at detecting grouping of strains according to the variables measured. The ALSCAL procedure implemented in SPSS 16 (SPSS, Chicago, Ill.) was used for the MDS analysis.

Phylogenetic analysis

The extraction of genomic DNA was performed as reported previously (Dodd et al. 2002). Sixty-six cultures of diverse *Trichoderma* species were used in the phylogenetic analysis (Table 2).

The gene regions studied were RNA polymerase II subunit (*rpb2*), translation elongation factor 1α (*tef1*) and internal transcribed spacers ITS (ITS1, 5.8S, and ITS2) of the nrDNA gene repeat. The primers for *rbp2* amplification were fRPB2-and fRPB2-7cR (Liu et al. 1999). Primers for *Tef1* amplification were Ef728F (Carbone and Kohn 1999) and Tef1R (Samuels et al. 2002). The primers for ITS amplification were ITS5 and ITS4 (White et al. 1990).

Table 1 Members of the Brevicompactum Clade, their provenances, major peptaibiotics, hydrophobins and trichothecene toxins

Strain	Species	Geographic location	Substratum	Peptaibiotics (LC-ESI-MS)[a]	Peptaibiotics (MALDI-TOF-MS)	Hydrophobin biomarkers[c]	Trichothecene
CBS 113214 = F000527	*T. arundinaceum* (received as *Hypocrea* sp.)	Korea: Daejeon	Soil	n.d.	n.d.	n.d.	Harzianum A
CBS 119573 = IBT 40837	*T. arundinaceum*	Iran: Khormabad, Alshter	Soil	n.d.	n.d.	n.d.	Harzianum A
CBS 119574 = IBT 40836	*T. arundinaceum*	Iran: Khormabad	Soil	n.d.	n.d.	n.d.	Harzianum A
CBS 119575 = IBT 40842 (ex-type)	*T. arundinaceum*	Iran: Hamadan	Soil	ALM F50/F30, TBV, TCP, TFR-A	ALM, TBV, TFR	5605, 7126, 7172, 7225, 8649, 9651	Harzianum A
CBS 119576 = ATCC 90237 = IBT 9471	*T. arundinaceum* (received as *T. harzianum*)	Namibia: Windhoek	Micaceous clay from stream bed	TBV-B, TBV-A, ALM, TCP, TFR, traces of TCT-B	ALM, TFR, TBV	5605, 7197, 7231, 7256, 8644, 9649	Harzianum A
CBS 119577 = IBT 40863	*T. arundinaceum*	Iran: Shar-e Kord, Chahar Mahall va Bakhtiari	Soil	TBV-A, ALM F30, TBV-B, TCP, TFR-A	n.d.	n.d.	Harzianum A
CBS 119578 = IBT 40864	*T. arundinaceum*	Iran: Hamadan	Soil	n.d.	n.d.	n.d.	Harzianum A
CBS 121153 = G.J.S. 90-2	*T. arundinaceum*	USA: Miss.	Soil in soy bean field	ALM F30/F50, TBV-B, TBV-A, TCP, TFR-A	n.d.	n.d.	Harzianum A
NRRL 3199	*T. arundinaceum* (received as *T. viride*)	Not available. Patent strain (Coats et al. 1974)	Not available	ALM F30, TBV-B, TBV-A, TCP, TFR [b]	ALM, TBV	5602, 7143, 7165, 7201, 8648, 9653	Harzianum A
CBS 109720 = DAOM 231232 = IBT 40866 (ex-type)	*T. brevicompactum*	USA: Geneva, N.Y.	Soil under *Helianthus annuus*	ALM F30, TCT-A, TCT-B, TCP	TCT-A, TCT-B, ALM	6658, 6879, 6943, 7118, 7289, 7319, 8755, 9499, 9567	Trichodermin
CBS 112443 = IBT 40867	*T. brevicompactum*	Papua New Guinea: Kuriva Forest	Rhizosphere soil with *Glycosmis sapindoiodes*	n.d.	n.d.	n.d.	Trichodermin
CBS 112444 = IBT 40861	*T. brevicompactum*	St Vincent and the Grenadines: Union Island	Soil, maize field	ALM F30, TCP, TCT-A, TCT-B, TFR-A	TCT-A, TCT-B, ALM	6952, 7112, 7140, 7294, 8761, 9302, 9378, 9576, 10003	Trichodermin
CBS 112446 = IBT 40862	*T. brevicompactum*	India: Trivandrum	Soil in backyard	ALM F30/F50, TCP, TCT-A, TCT-B, TFR	TCT-A, TCT-B, ALM	6945, 6981, 7103, 9367, 9575	Trichodermin
CBS 112447	*T. brevicompactum*	México: Distrito Federal	Soil	ALM F30/F50, TCP, TCT-A, TCT-B, TFR	TCT-A, TCT-B, ALM	5598, 7107, 8813, 9382, 9585	Trichodermin
CBS 112945	*T. brevicompactum*	Not available	Not available	n.d.	n.d.	n.d.	n.d.
CBS 119569 = IBT 40839	*T. brevicompactum*	Iran: Aligoodarz	Soil	ALM F30, TCT-A, TCP	n.d.	n.d.	Trichodermin
CBS 119570 = IBT 40840	*T. brevicompactum*	Iran: Doroud	Soil	ALM F30, TCT-A, TCP	n.d.	n.d.	Trichodermin
CBS 119571 = IBT 40838	*T. brevicompactum*	Iran: Qazvin	Soil	n.d.	n.d.	n.d.	Trichodermin
CBS 119572 = IBT 40865	*T. brevicompactum*	Iran: Khoran Abad	Soil	n.d.	n.d.	n.d.	Trichodermin

CBS 121154 = G.J.S. 05–355	*T. brevicompactum*	Cameroon: Bokito	Soil under *Theobroma cacao*	ALM F30/F50, TCP, TCT-A, TBV-A, TBV-B (the latter two in trace amounts)	TCT-A, ALM	6950, 7115, 7288, 7449, 8750, 8777, 9275, 9300, 9355, 9564	Trichodermin
IBT 40841 = G.J.S. 05–176	*T. brevicompactum*	Iran: Alshter	Soil	n.d.	n.d.	n.d.	Trichodermin
MA 4103 = G.J.S. 04–380	*T. brevicompactum*	USA: N.Y.	Soil with *Cucurbita maxima*	n.d.	n.d.	n.d.	n.d.
CBS 121320 = DIS 119f (ex-type)	*T. protrudens*	India: Kerala	*Theobroma cacao,* trunk endophyte	TBV-B, TBV-A, ALMF30/F50, TCP, TFR	ALM, TBV-A, TBV-B, TFR	5570, 6895, 7191, 7252, 7278, 7665, 8741, 9235, 9619	Harzianum A
CBS 120895 = G.J.S. 91-88 (ex-type)	*Hypocrea rodmanii*	USA: Va.	Branchlets	HCP, HRC, TKO	HRC, TKO	7348, 7375, 7561	None detected
CBS 109719 = G.J.S. 91-91	*Hypocrea rodmanii*	USA: Va.	Decorticated wood	HCP, HRC, TKO	n.d.	n.d.	None detected
CBS 120897 = G.J.S. 91-89	*Hypocrea rodmanii*	USA: Va.	Bark	n.d.	n.d.	n.d.	None detected
CBS 112554 = BBA 72294	*T. turrialbense*	Costa Rica: Turrialba	Soil	ALM F30/F50, TCT-C, TCT-D,TCP, TFR-A, TBV-A, TBV-B (the latter two in small amounts)	TCT-C, TCT-D, ALM	7094, 7115, 7298, 8819, 9310, 9555, 10060	Harzianum A
CBS 112445 = IBT 40868	*T. turrialbense*	Costa Rica: Turrialba	Soil	ALM F30, TCT-A, TCT-B, TCP	TCT-A, TCT-B, ALM	7091, 7112, 7267, 8748, 9142, 9302, 9546	Harzianum A, traces of trichodermin

[a] *ALM F30/F50* Alamethicin F30/F50, *TCP* trichocompactin, *TBV-A* trichobrevin A, *TBV-B* trichobrevin B, *TCT-A* trichocryptin A, *TCT-B* trichocryptin B, *TCT-C* trichocryptin C, *TCT-D* trichocryptin D, *TFR* trichoferin, *HCP* hypocompactin, *HRC* hyporodicin, *TKO* trichokonin, *n.d.* not done, i. e. those strains have not been analyzed for peptaibiotics and hydrophobins

[b] For a complete list of ALMs F30/F50 produced by *Trichoderma arundinaceum* NRRL 3199 see Kirschbaum et al. (2003). Further truncated peptaibiotics from *Trichoderma arundinaceum* NRRL 3199 were listed by Psurek et al. (2006)

[c] MALDI TOF mass data have mass accuracy of 0.1%

Table 2 Isolates and GenBank accession numbers

Isolate	Species	*tef1*	*rpb2*	ITS
CBS 119573	*T. arundinaceum*	EU338280	EU338308	AY154943
NRRL 3199	*T. arundinaceum*	EU338279	EU338307	EU330932
CBS 121153	*T. arundinaceum*	EU338278	EU338306	EU330931
CBS 119577	*T. arundinaceum*	EU338277	EU338305	AY154921
CBS 119578	*T. arundinaceum*	EU338276	EU338304	AY154921
CBS 119575 T	*T. arundinaceum*	EU338275	EU338303	AY154921
CBS 119574	*T. arundinaceum*	EU338274	EU338302	AY154921
ATCC 90237	*T. arundinaceum*	EU338291	EU338326	DQ080074
CBS 1132314	*T. arundinaceum*	EU596602		EU596603
CBS 109720 T	*T. brevicompactum*	EU338299	EU338317	AY324173/AY324183
CBS 112443	*T. brevicompactum*	EU338281	EU338319	AY324174/AY324183
CBS 121154	*T. brevicompactum*	EU338283	EU338310	EU338330
CBS 119570	*T. brevicompactum*	EU338298	EU338316	AY154920
CBS 119569	*T. brevicompactum*	EU338297	EU338315	AY154920
CBS 112444	*T. brevicompactum*	EU338296	EU338314	AY324173/AY324183
CBS 119572	*T. brevicompactum*	EU338295	EU338313	EU330937
G.J.S. 04–380 = MA 4103	*T. brevicompactum*	EU338292	EU338309	EU330935
IBT 40841	*T. brevicompactum*	EU338294	EU338312	AY154920
CBS 119571	*T. brevicompactum*	EU338293	EU338311	AY154920
CBS 112446	*T. brevicompactum*	EU338273	EU338301	AY324173/AY324183
CBS 112447	*T. brevicompactum*	EU338300	EU338318	EU330942
CBS 114249	*H. candida*	AY737742	AY391899	AY737757
CBS 114232	*H. catoptron*	AY737726	AY391900	AY737766
CBS 114245	*H. ceracea*	AY937437	AF545508	EU330953
CBS 114576	*H. ceramica*	AY737738	AF545510	AY737764
G.J.S. 90–97 = IMI 352471	*H. cf. rufa VE*	DQ307530	EU341808	DQ315449
G.J.S. 98-1	*H. chlorospora*	AY737737	AY391906	AY737762
G.J.S. 94-67	*H. chromosperma*	AY737728	AY391912	AY737774
G.J.S. 97-237	*H. cinnamomea*	AY737732	AY391920	AY737759
DAOM 139758	*H. citrinoviride*	EU338334	EU338338	EU330960
P.C. 21	*H. costaricensis*	AY737741	AY391921	AY737754
CBS 111146	*H. cremea*	AY737736	AF545511	AY737760
CBS 111148	*H. cuneispora*	AY737727	AF545512	AY737763
CBS 119053	*H. dingleyae*	AF348117	EU341803	DQ313151
CBS 119089	*H. dorotheae*	DQ307536	EU248602	DQ313144
CBS 111147	*H. estonica*	AY737733	AF545514	AY737767
G.J.S. 02-78	*H. intricata*	EU248630	EU241505	EU264002
G.J.S. 90-22 = IMI 393966	*H. lixii*	AF443933	AY391925	AF443915
CBS 102037	*H. lutea*	AY737731	AY489662	AY737773
CBS 114236	*H. melanomagna*	AY737751	AY391926	AY737770
CBS 114330	*H. nigrovirens*	AY737744	AF545518	AY737777
CBS 115283	*H. pezizoides*	AY937438	EU248608	DQ000632
CBS 114071	*H. phyllostachydis*	AY737745	AF545513	EU330959
CBS 814.68	*H. pilulifera*	AY737747	AF545519	Z48813
CBS 121320 = DIS 119fT	*T. protrudens*	EU338289	EU338322	EU330946
HY 8	*H. psychrophila*	AY737752	AF545520	EU330957
G.J.S. 89–120	*H. rodmanii*	EU338285	EU338323	EU330947
CBS 120895	*H. rodmanii*	EU338286	EU338324	EU330948
CBS 109719	*H. rodmanii*	EU338290	EU338325	EU330949
DAOM 167636	*H. semiorbis*	AY737750	AF545522	AY737758
CBS 112888	*H. stilbohypoxyli*	AY376062	EU341805	AY380915
CBS 114248	*H. straminea*	AY737746	AY391945	AY737765
G.J.S. 85–228	*H. sulawesensis*	AY737730	AY391954	AY737753
CBS 111145	*H. surrotunda*	AY737734	AF545540	AY737769
CBS 114234	*H. thailandica*	AY737748	AY391957	AY737772
CBS 114237	*H. thelephoricola*	AY737735	AY391958	AY737776

Table 2 (continued)

Isolate	Species	*tef1*	*rpb2*	ITS
CBS 112445	*T. turrialbense*	EU338284	EU338321	AY324173/AY324183
BBA 72294	*T. turrialbense*	EU338282	EU338320	EU330944
G.J.S. 99–130	*H. victoriensis*	EU338331	EU338336	EU330952
PC 278	*H. virescentiflava*	AY737749	AY391059	AY737768
DAOM 100525	*T. aggressivum*	AF348095	AF545541	AF057600
G.J.S. 90–7	*T. asperellum*	EU338333	EU338337	EU330956
CBS 142.95	*T. atroviride*	AF456891	EU341801	AF456917
DAOM 167161	*T. fertile*	AY750881	AF545546	DQ083018
DAOM 167652	*T. flavofuscum*	AY750891	AF545547	EU330955
DAOM 167057	*T. hamatum*	AY750893	AF545548	Z48816
CBS 988.97	*T. koningii*	DQ289007	EU248600	DQ323409
DAOM 222105	*T. koningiopsis*	AY376042	EU341810	AY380901
DAOM 166989	*T. longibrachiatum*	EU338335	EU338339	EU330961
DAOM 167069	*T. minutisporum*	AY750883	EU341809	DQ083015
DAOM 167085	*T. oblongisporum*	AY750884	AY545551	DQ083020
DAOM 166162	*T. pubescens*	AY750887	AF545552	DQ083016
G.J.S. 03–74	*T. scalesiae*	DQ841726	EU252007	DQ841742
DAOM 183974	*T. spirale*	AY750890	AF545553	DQ083014
DAOM 166121	*T. erinaceus*	DQ109547	EU248604	DQ109534
DAOM 166121	*T. strigosum*	AF487668	AF545556	AF487657
G.J.S. 00–108	*T. stromaticum*	AY937436	EU341807	DQ083013
DAOM 178713A	*T. tomentosum*	AY750882	AF545557	DQ085432
G.J.S. 01–287	*T. virens*	AY750894	EU341804	DQ083023
CBS 101526	*T. viride*	AY376053	EU248599	X93979
CBS 438.95	*T. viride*	DQ307522	EU341806	DQ315438
CBS 333.72	*T. viridescens*	DQ307523	EU341802	DQ315441
G.J.S. 74–83	*H. lutea*	EU338287	EU338327	EU330950
G.J.S. 85–26	*H. lutea*	EU338288	EU338328	EU330951
DIS 219C	*Trichoderma sp.*	EU338332	EU338329	EU330954

PCR amplifications were performed in a solution that contained: 2.5µl of 10X PCR Buffer (New England Biolab, Ipswich, Mass.) with $MgCl_2$ to a final concentration of 1.5 mM, 0.2 mM dNTPs, 0.2 µM of forward and reverse primers, 1.25 units of Taq Polymerase (New England Biolab), and 10–50 ng of genomic DNA and double-distilled water for a total volume to 25µl per reaction. The reactions were placed in a PTC-200 MJ Research thermocycler (Waltham, Mass.) using a touchdown program (Don et al. 1991). The touchdown PCR was initiated with a 2-min denaturation at 94°C followed by an initial 15 cycles of PCR amplification. The annealing temperature in the first amplification cycle was 65°C, which was reduced 1°C per cycle over the next 15 cycles. An additional 35 cycles were performed, each consisting of 30-s denaturation at 94°C, 30-s annealing at 48°C and 1-min extension at 72°C concluding with 10-min extension at 72°C. The resulting products were purified with ExoSAP-it kit (USB, Cleveland, Ohio) using the manufacturer's protocol. The purified amplicons were sequenced directly using the BigDye Terminator v3.1 chemistry in a 16 capillary automated DNA sequencer (ABI 3100; Applied Biosystems, Foster City, Calif.). For each locus, both strands were sequenced using the primers used in producing the amplicons. In the case of *rpb2*, two additional internal primers RPB-432F (5'-ATGATCAACAGAGGYATGGA) and RPB-450R (5′-TCCATRCCTCTGTTTGATCAT) were used in sequencing reactions. Sequences were edited and assembled using Sequencher 4.1 (Gene Codes, Madison, Wis.). Clustal X 1.81 (Thompson et al. 1997) was used to align the sequences; the alignment was adjusted manually with McClade version 3.06 software (Maddison and Maddison 2005).

Sequence analyses To determine the phylogenetic position of the Brevicompactum clade, the sequence alignment of the three gene sections for 66 isolates of *Trichoderma* species (Table 2) was analysed with maximum parsimony (MP), neighbour-joining (NJ) and Bayesian inference (BI). The MP analysis was carried out with PAUP* version b10 (Swofford 2002) using a heuristic search, with a starting tree obtained via 1000 random stepwise addition sequences, tree-bisection-reconnection as the branch-swapping algorithm, and MULTREES on. PAUP* was also used to

construct NJ trees, with a distance set to Kimura 2-parameter model. Bootstrap values for MP and NJ trees were calculated from 1,000 replicates. Two members of *Trichoderma* sect. *Longibrachiatum* were used as outgroup species. GenBank accession numbers are given in Table 2.

MrBayes 3.0 b4 (Huelsenbeck 2000; Huelsenbeck et al. 2001) was used to reconstruct phylogenetic trees based on the Bayesian approach (Mau et al. 1999; Rannala and Yang 1996). The Bayesian analysis used a different model of evolution for each of the three loci (ITS, *tef1*, *rpb2*). The models of DNA substitution for *rpb2* and *tef1* were determined previously (Chaverri and Samuels 2003; Chaverri et al. 2005) with Modeltest 3.6 (Posada and Crandall 1998). The parameters estimated for ITS by Modeltest were: general time reversible (GTR + I + G, nst = 6) model with gamma distributions and invariable sites; base frequencies = 0.2082, 0.3400, 0.2485; rates (Rmat) = 1.7117, 2.4357, 2.0885, 0.6750, 4.7081; gamma shape = 0.7900; proportion invariable sites (pinvar) = 0.6037. Four chains and 5,000,000 Markov chain-Monte Carlo generations were run, and the current tree was saved to a file every 100 generations. Stability of likelihood scores was confirmed with the software TRACER version 1.2.1 (Rambaut and Drummond 2004), which traces the parameter against the generation number. Once stability was reached both in terms of likelihood scores and parameter estimation, the first 5,000 trees were discarded (as burn-in).

Topological incongruence for the trees based on individual genes was examined with a reciprocal 70% bootstrap (BP) or a 95% posterior probability (PP) threshold (Mason-Gamer and Kellogg 1996; Reeb et al. 2004) to determine whether the sequences from the three genes should be combined in a single analysis. Bootstrap values were generated with neighbour joining (NJ) with 1,000 replicates and a maximum likelihood distance. Posterior probabilities were calculated with Bayesian analysis in MrBayes. A conflict was assumed to be significant if two relationships for the same taxa, one being monophyletic and the other non-monophyletic, both with BP = 70% and PP = 95%, were observed on each ITS, *tef1* and *rpb2* majority-rule consensus trees. The three partitions could be combined if no significant conflicts were detected.

The culture G.J.S. 85-26 (*H. lutea*) was used as an outgroup because this species was shown to be the closest to the Brevicompactum clade.

Secondary metabolites

Peptaibiotics: HPLC-ESI-Ion-Trap-Mass spectroscopy

Cultivation of strains All subcultures and main cultures used for peptaibiomics were grown at room temperature (23–26°C) under ambient daylight on PDA (Difco, Becton Dickinson, Heidelberg, Germany). Peptaibiotics studied are listed in Tables 1, 3, 4, 5, 6 and 7.

Subcultures were grown on PDA for 4 days and used for inoculation of the main culture.

Extraction After 6 days cultivation, fungal cultures were extracted with a mixture of 1:1 (*v/v*) CH_2Cl_2:MeOH, evaporated in vacuo, and cleaned over Sep-Pak C_{18} cartridges as described by Krause et al. (2006).

HPLC-UV-ESI-Ion-Trap- MS measurements

Analyses were performed on an Agilent 1100 HPLC equipped with Kromasil KR100 column, (150 mm × 4.6 mm i. d., 3.5 µm) held at 35°C. The UV detector was set at 205 nm (Krause et al. 2006; Degenkolb et al. 2006a, b).

Online HPLC/ESI-MS was performed on a LCQ ion trap MS (Thermo Finnigan MAT, San José, Calif.) using Excalibur v.1.2 software. Sequence analysis was carried out in the positive ESI mode in centroid mode with an accuracy of ± *m/z* 0.5. A collision induced dissociation (CID) energy of 0% was used for scanning molecular masses ($[M+H]^+$/$[M+Na]^+$) and fragments resulting from cleavage of the extremely labile Aib-Pro bond. CID energies of 45% and 65% were used for generating series of characteristic "in-source"-fragment ions. The collision energy for MS^n experiments was set between 25 and 65 V, depending on the precursor ion, typically at 45 V. Fragment ion series were assigned in accordance with the previously used nomenclature. In cases where the isomeric amino acids Leu/Ile or Val/Iva (Iva, isovaline) could not be distinguished, the abbreviations Lxx and Vxx were used instead (Krause et al. 2006; Degenkolb et al. 2006a, b).

Detection of DL-Alaol by GC/MS measurements For GC/MS measurements, a GC-A17 coupled to a QP-5000 MS (Shimadzu, Kyoto) was used. The instrument was equipped with a Chirasil-L-Val (i.e., *N*-propionyl-L-valine-*tert*-butylamide polysiloxane) capillary column, 25 m × 0.25 mm i.d. (Varian-Chrompack, Darmstadt). Helium was used as the carrier gas. EI mass spectra were recorded at 70 eV. A subfraction of 1 mg of the methanolic extract, also used for HPLC and LC/MS measurements, was dried under a cold stream of N_2 and hydrolyzed in a ReactiVial with 6 N HCl at 110°C for 18 h. The chirality of Alaol was determined after derivatization of the dried hydrolysate with *N*-pentafluoropropionic anhydride (PFPAA) by GC/MS in SIM mode at *m/z* 190−191. *N*-pentafluoropropionyl derivatives of L-Alaol and DL-Alaol were used as standard samples (Küsters and Portmann 1994). The following

Table 3 Peptaibiotic patterns of strains from the *Trichoderma brevicompactum* complex (excluding *Hypocra rodmanii*)

Species	Strain	Peptaibiotics: molecular weight					
		ALM	TCP	TCT-A	TCT-B	TBV	TFR
Trichoderma brevicompactum	CBS 109720 = IBT 40866 = DAOM 231232 (ex-type)	1964 [**1**][a] 1,978 [**2**, **3**], 1992 [**4**]	726 [5, 6]; 740 [**7**, 8]; 754 [**9**, 10]	1125 [19, **20**, **21**]; 1139 [22, **23**]	1210 [11, 12]; 1224 [13, **14**]; 1238 [15, **16**]; 1252 [17, **18**]	n.d.	n.d.
	CBS 119569 = IBT 40839	1964 [**1**], 1,978 [**2**, **3**], 1992 [**4**]	726 [5]; 740 [**7**, **8**] 754 [9, 10]	1153 [24, 25]; 1167 [26, **27**, 28]; 1181 [29, 30]	n.d.	n.d.	n.d.
	CBS 119570 = IBT 40840	1964 [**1**], 1,978 [**2**, **3**], 1992 [**4**]	726 [5]; 740 [7, **8**]; 754 [9, 10]	1153 [24, 25]; 1167 [26, **27**, 28]; 1181 [29, 30]	n.d.	n.d.	n.d.
	CBS 112444 = IBT 40861	1964 [**1**], 1,978 [**2**, **3**], 1992 [**4**]	740 [**7**, **8**]; 754 [9, 10]	1111 [80, 81]; 1125 [**20**, **21**]; 1139 [**23**]; 1153 [82]	1210 [11]; 1,224 [13, 14]; 1238 [**16**, 83, 84, 85]; 1252 [**17**, **18**]	n.d.	1207 [**64**]
	CBS 112446 = IBT 40862	1963 [**76**], 1964 [**1**], 1977 [77], 1978 [**2**, **3**], 1992 [**4**]	740 [7, **8**]; 754 [9, 10]	1111 [80, 81]; 1125 [20, 21]; 1139 [**23**]	1210 [11]; 1224 [13, 14]; 1238 [**16**, 83, 84, 85]; 1252 [**17**, **18**]	n.d.	1207 [**64**]; 1193 [**66**, **67**, 68]
	CBS 112447	1963 [**76**], 1964 [**1**], 1977 [**77**], 1978 [**2**, **3**], 1992 [**4**]	740 [7, 8]; 754 [9, 10]	1111 [80, 81]; 1125 [20, 21]; 1139 [**23**]; 1153 [25]	1210 [11]; 1224 [13, 14]; 1238 [**16**, 83, 84, 85]; 1252 [17, 18]	n.d.	1207 [**64**], 1193 [**66**, **67**, 68]
	CBS 121154 = G.J.S. 05–355	1963 [**76**]; 1977 [77]; 1977 [78]; 1991 [79]; 1950 [31]; 1964 [**1**], 1978 [**2**, **3**], 1992 [**4**]	726 [5]; 740 [7, 8]; 754 [9, 10]	1125 [86, 87, 88, 89]; 1139 [**90**]; 1153 [**91**]	n.d.	1127 [52]; 1141 [53, 54]; 1157 [61, 62] (in trace amounts)	1207 [**64**]; 1193 [**66**, 68]
Trichoderma turrialbense	CBS 112445 = IBT 40868 (ex-type)	1950 [31]; 1964 [**1**], 1978 [**2**, **3**], 1992 [**4**]	726 [5, 6]; 740 [7, 8]; 754 [9, **10**]	1139 [22, **23**]; 1153 [24, **25**]; 1167 [26, **27**, 28]	1224 [13, 14]; 1238 [**15**, **16**]; 1252 [17, 18]; 1266 [**33**, **34**, 35, 36]; 1280 [37, 38]	n.d.	n.d.
	CBS 122554 = BBA 72294	1963 [**76**]; 1977 [77]; 1977 [78]; 1991 [79]; 1964 [**1**], 1978 [**2**, **3**], 1992 [**4**]	740 [7, **8**]; 754 [9, 10]	1097 [92]; 1111 [93]; 1125 [**94**]	1196 [95]; 1210 [96, 97]; 1224 [**98**, **99**, **100**, **101**]; 1238 [102, 103]	1127 [52]; 1,141 [53]; 1157 [62]	1207 [64]
Trichoderma arundinaceum	CBS 119575 = IBT 40842 = G. J.S. 05–180 (ex-type)	1963 [**76**]; 1977 [**77**]; 1977 [**78**]; 1991 [79]; 1964 [**1**], 1978 [**2**, **3**], 1992 [**4**]	740 [7, 8]; 776 [9, 10]; 770 [**39**]	n.d.	n.d.	1127 [50-52]; 1143 [58, 59]; 1141 [**53**, **54**]; 1157 [60, 61, **62**]	1207 [64]
	CBS 119576 = ATCC 90237 = IBT 9471	1964 [**1**], 1978 [**2**, **3**], 1992 [**4**, 63]	726 [5, 6]; 740 [7, 8]; 756 [**10**]; 770 [39, 41]; 784 [42, 43]	n.d.	1238 [15, 16]; 1252 [17, 18]; 1266 [33–36] (in trace amounts)	1099 [44–47]; 1113 [48, 49]; 1127 [50–52]; 1141 [53, 54]; 1129 [55–57]; 1143 [58, 59]; 1157 [60–62]	1207 [64]

Table 3 (continued)

Species	Strain	Peptaibiotics: molecular weight					
		ALM	TCP	TCT-A	TCT-B	TBV	TFR
	NRRL 3199	1964 [**1**], 1978 [**2**, **3**], 1992 [**4**]	740 [70]; 754 [71, 72]; 756 [73]; 770 [74, 75]	n.d.	n.d.	1099 [44–47]; 1113 [48, 49]; 1127 [50–52]; 1141 [53, 54]; 1129 [55–57]; 1143 [58, 59]; 1157 [60–62]	1207 [64]; 1193 [66–69]
	CBS 119577 = IBT 40863	1964 [**1**], 1978 [**2**, **3**], 1992 [**4**]	726 [5, 6]; 740 [7, 8]; 754 [9, 10]	n.d.	n.d.	1099 [44–47]; 1113 [48, 49]; 1127 [50–52]; 1141 [53, 54]; 1129 [55–57]; 1143 [58, 59]; 1157 [60–62]	1207 [64]
	CBS 121153 = G.J.S. 90–2	1963 [**76**]; 1977 [77]; 1977 [**78**]; 1991 [79]; 1964 [**1**], 1978 [**2**, **3**], 1992 [**4**]	754 [10]; 770 [**39**, **41**]	n.d.	n.d.	1113 [48, 49]; 1127 [52]; 1141 [53, 54]; 1129 [57]; 1143 [58, 59]; 1157 [60–62]	1207 [64]
Trichoderma protrudens	CBS 121320 = DIS 119f (ex-type)	1963 [**76**]; 1977 [77]; 1977 [**78**]; 1991 [79]; 1964 [**1**], 1978 [**2**, **3**], 1992 [**4**]	754 [10]; 770 [**39**, **41**]	n.d.	n.d.	1113 [48, 49]; 1127 [52]; 1141 [53, 54]; 1129 [**57**]; 1143 [**58**, **59**]; 1157 [60, **61**, **62**]; 1171 [104–106]	1207 [**64**]; 1193 [65, 66, 68]

[a] Consecutive numbering for recurrent sequences from [1]–[75] is adopted from Degenkolb et al. (2006a); new compounds are listed in ascending order from [76] on. Numbers in bold indicate major compounds in the HPLC elution profiles produced by an individual strain
ALM F30/F50 Alamethicin F30/F50, *TCP* trichocompactin, *TBV* trichobrevin A and B, *TCT-A* trichocryptin A, *TCT-B* trichocryptin B, *TFR* trichoferin, *n.d.* not detected. For ALMs, TCPs, and TFRs, the predominant $[M+H]^+$ ions are listed; for TCTs and TBVs, the predominant $[M+Na]^+$ ions

modified temperature program was used for GC separation: injector and interface temperature, 250°C each; initial temperature, 65°C, 4 min hold; 3°C/min until reaching 100°C, 3.5 min hold; 40°C/min until reaching 190°C, 5 min hold. The pressure program was as follows: initial pressure 3.2 kPa, 4 min hold; 0.2 kPa/ min until reaching 7 kPa, 3 min hold. The split ratio was set 30:1.

Peptaibiotics: MALDI-TOF analysis

Growth conditions Cultures were routinely grown at 25°C on malt extract (Oxoid, Wesel, Germany) agar plates and prepared on day 6 in the sporulation phase. Peptaibiotics from MALDI-TOF MS analysis are summarized in Table 1.

Extraction and preparation of mycelium for MALDI-TOF analysis A few µg of mycelium were directly spotted onto target wells of a 100-position sample plate and immediately mixed with 1 µl of matrix solution [10 mg/ml dihydroxybenzoic acid (DHB, from Anagnostec, Golm, Germany) in acetonitrile / methanol / water (1: 1:1, *v/v*) and 0.3% trifluoroacetic acid]. The sample matrix mixture was allowed to air-dry prior to analysis.

MS analysis by MALDI-TOF MS

Low-molecular mass peptides were measured on a VOYAGER DE-PRO - time of flight mass spectrometer from Applied Biosystems using a nitrogen laser beam ($\lambda = 337$ nm), with the MS in the delayed extraction mode, allowing the determination of monoisotopic mass values. A low mass gate of 800 Da improved the measurement by filtering out the most intensive matrix ions. The mass spectrometer was used in the positive ion detection and reflector mode.

Table 4 Sequences of new 11- and 12-residue peptaibiotics of different strains from the *Trichoderma brevicompactum* complex (excluding *Hypocrea rodmanii*)

Peptaibiotic[a]	Molecular weight ([M+Na]$^+$)		Residue											
			1	2	3	4	5	6	7	8	9	10	11	12
TCT-A_VIa [80]	1111	Ac	Vxx	Aib	Pro	Vxx	Aib	Pro	Aib	Vxx	Aib	Pro	Lxxol	
TCT-A_VIb [81]	1111	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Aib	Aib	Aib	Pro	Lxxol	
TCT-A_IIIc [82]	1153	Ac	Lxx	Aib	Pro	Lxx	Aib	Pro	Lxx	Aib	Aib	Pro	Lxxol	
TCT-B_IIIc [83]	1238	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol
TCT-B_IIId [84]	1238	Ac	Vxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol
TCT-B_IIIe [85]	1238	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Aib	Vxx	Aib	Pro	Lxxol
TCT-A_Ic [86]	1125	Ac	Vxx	Aib	Pro	Lxx	Aib	Pro	Lxx	Aib	Aib	Pro	Vxxol	
TCT-A_Id [87]	1125	Ac	Lxx	Aib	Pro	Lxx	Aib	Pro	Lxx	Aib	Aib	Pro	Aibol	
TCT-A_Ie [88]	1125	Ac	Vxx	Aib	Pro	Lxx	Aib	Pro	Lxx	Aib	Aib	Pro	Vxxol	
TCT-A_1f [89]	1125	Ac	Lxx	Aib	Pro	Lxx	Aib	Pro	Vxx	Aib	Aib	Pro	Vxxol	
TCT-A_IIc [90]	1139	Ac	Lxx	Aib	Pro	Lxx	Aib	Pro	Lxx	Aib	Aib	Pro	Vxxol	
TCT-A_IIIc [91]	1153	Ac	Lxx	Aib	Pro	Lxx	Aib	Pro	Lxx	Aib	Aib	Pro	Lxxol	
TCT-C_Ia [92]	1097	Ac	Vxx	Aib	Pro	Vxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Alaol	
TCT-C_IIa [93]	1111	Ac	Vxx	Aib	Pro	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol	
TCT-C_IIIa [94]	1125	Ac	Lxx	Aib	Pro	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol	
TCT-D_Ia [95]	1196	Ac	Vxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Alaol
TCT-D_IIa [96]	1210	Ac	Vxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol
TCT-D_IIb [97]	1210	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Alaol
TCT-D_IIIa [98]	1224	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol
TCT-D_IIIb [99]	1224	Ac	Vxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol
TCT-D_IIIc [100]	1224	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol
TCT-D_IIId [101]	1224	Ac	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol
TCT-D_IVa [102]	1238	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol
TCT-D_IVb [103]	1238	Ac	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol
TBV-B_IVa [104]	1171	Ac	Aib	Ser	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	
TBV-B_IVb [105]	1171	Ac	Aib	Ser	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	
TBV-B_IVc [106]	1171	Ac	Aib	Ser	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol	

[a] The *N*-terminal sequences AcVxx-Aib and AcLxx-Aib were tentatively assigned from sequence homologies with those trichocryptins/trichobrevins previously reported by Degenkolb et al (2006a)

Characterization by MALDI-TOF MS analysis was performed on the same MALDI instrument in linear delayed extraction mode using an acceleration voltage of 20 kV and a low mass gate of 1,500 Da. Spectra for individual specimens were compiled, averaging results from at least 100 shots taken across the width of the specimen for *m/z* 2,000–20,000.

Mycotoxin and secondary metabolite profiling

Cultivation and extraction Liquid cultivations were performed in 25-ml Blue cap bottles containing 4 ml potato-dextrose broth (Difco) for 10 days in darkness. Cultures were extracted over night using 15 ml ethyl acetate, which was then evaporated in vacuo and redissolved in 500µl acetonitrile /water (2:1, v/v) and filtered through a 0.45-µm PTFE syringe filter (Nielsen et al. 2005). Plate cultures were grown on oatmeal agar and PDA (Difco) for 10 days in darkness at 25°C, and a 0.6-cm^2 agar culture was cut out and transferred to a 2-ml vial along with the 10-cm^2 culture mat which was scraped off using a scalpel. The sample was ultrasonicated with 0.75 ml acetonitrile for 1 h, and filtered through a 0.45-µm PTFE syringe filter.

HPLC-DAD-ESI-TOF-MS Analysis was performed on an Agilent 1100 system equipped with a photo diode array detector (DAD), and a Luna C_{18} II column (Phenomenex, Torrance, Calif.) and coupled to a LCT orthogonal time-of-flight MS (Waters-Micromass, Manchester, UK), with a Z-spray ESI source and a LockSpray probe (Nielsen et al. 2005).

Samples were analyzed in positive ESI$^+$ and ESI$^-$ using a water-acetonitrile gradient system starting from either 5% acetonitrile, which was increased linearly to 100% in 23 min holding for 5 min, or 15% acetonitrile, which was increased linearly to 100% in 20 min holding for 5 min (Nielsen et al. 2005). In both ESI$^+$ and ESI$^-$ two scan functions (1 s each) were used: the first with a potential

Table 5 Sequences of new 7-residue hypocompactins (HCP), of 14-residue hypocrodicins (HRC), and 19-residue trichokonins (TKO) produced by *Hypocrea rodmanii*

Peptaibiotic	Residue																		
	(MW)[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
HCP-I [107]	738	[125][b]	Gly	Ala	Lxx	Aib	Gly	Lxx	Lxxol											
HCP-II [108]	752	[125][b]	Gly	Ala	Lxx	Vxx	Gly	Lxx	Lxxol											
HCP-III [109]	766	[125][b]	[182]c	Vxx	Lxx	Aib	Gly	Lxx	Lxxol											
HCP-IV [110]	780	[125][b]	[182][c]	Vxx	Lxx	Vxx	Gly	Lxx	Lxxol											
HCP-V [111]	814			[329][d]	Lxx	Aib	Gly	Lxx	Lxxol											
HCP-VI [112]	828			[329][d]	Lxx	Vxx	Gly	Lxx	Lxxol											
HRC-A [113]	1437	Ac	Aib	Gln	Vxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol				
HRC-B [114]	1451	Ac	Aib	Gln	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol				
HRC-C [115]	1465	Ac	Aib	Gln	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol				
HRC-D [116]	1380	Ac	Aib	Gly	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol				
HRC-E [117]	1394	Ac	Aib	Ala	Lxx	Aib	Pro	Vxx	Vxx	Aib	Pro	Aib	Lxx	Aib	Pro	Lxxol				
TKO-V [118]	1866	Ac	Aib	Ala	Aib	Ala	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
TKO [119]	1880	Ac	Aib	Aib	Ala	Ala	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol

[a] Molecular weight (MW) refers to the $[M+H]^+$ ions in the case of HCPs and TKOs, but to $[M+Na]^+$ in the case of HRCs

[b] Tentatively assigned as C_8 fatty acid residue (*n*-octenoyl or homologue), according to structural homologies. See text for details

[c] Tentatively assigned as Gly residue

[d] Only partial sequences can be given because of low abundance of *N*-terminal fragment ions

difference of 14 V between the skimmers scanning *m/z* 100 to 900, the second with 40 V between the skimmers scanning *m/z* 100–2,200.

Data analysis was performed as described previously (Nielsen et al. 2005). Unknown peaks were matched against an internal reference standard database (~730 compounds) as well as the 33,557 compounds in Antibase 2007 (Laatsch 2007).

Results

For ease of discussion, the new names that will be introduced below are used in this and the "Discussion" sections.

Phylogeny

To position *T. brevicompactum* and its relatives within *Trichoderma*, we sequenced parts of three genes *tef1*, *rpb2,* and ITS (Fig. 1). The alignment of three loci included a total of 2,369 characters in the analyses (848 for *rpb2*, 837 for *tef1*, 684 for ITS), including insertions and deletions. Ambiguously-aligned regions were manually excluded from the analyses (356 characters). In the maximum parsimony analyses, the consistency and homoplasy indices for the combined dataset were, respectively, 0.333 and 0.667. Of the included characters, *rpb2* provided the most parsimony informative characters (34%), followed by ITS (20.8%), and *tef1* (19.4%); *tef1* provided the least informative characters, in part because most of the ambiguously-aligned characters were in the large intron of *tef1*.

The reciprocal 70% BP and 95% PP thresholds for individual loci show that the topologies of the three genes are congruent (results not shown) and therefore the partitions were combined in a single tree (Fig. 1). Figure 1 represents a Bayesian phylogram with the best log likelihood (LnL = −22,426.97). Bootstrap results from the MP and NJ analyses are indicated in Fig. 2 but trees are not shown. The results of all BI, MP, and NJ analyses show high BP and PP values for the clade that includes *T. brevicompactum, T. protrudens, T. turrialbense, T. arundinaceum*, and *H. rodmanii*, hereafter the Brevicompactum clade. These five species received high bootstrap support in parsimony analysis and high posterior probabilities in Bayesian analysis.

Results of the phylogenetic analyses reveal high BP and PP for previously described clades/lineages (Kindermann et al. 1998; Chaverri and Samuels 2003; Samuels 2006; International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy: http://www.isth.info/biodiversity/index.php). The Brevicompactum clade is distinct from other known lineages and thus represents a separate lineage

Table 6 Structural variations of peptaibiotics from the *Trichoderma brevicompactum* complex (excluding *H. rodmanii*)

Peptaibiotic	Residue																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ALM F30	Ac	Aib	Pro	Aib	Ala	Aib	Ala	Gln Glu	Aib	Aib Vxx Lxx	Aib	Gly	Vxx Lxx	Aib	Pro	Vxx	Aib	Aib	Glu	Gln	Pheol
ALM F50	Ac	Aib	Pro	Aib	Ala Aib	Aib	Ala Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
TCP	Oc	Gly	Ala	Lxx	Aib Vxx	Gly Ala Ser	Vxx Lxx	Lxxol													
TBV	Ac	Aib	Ala Ser	Aib Vxx	Aib Vxx Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Alaol Aibol Vxxol Lxxol									
TCT-A	Ac	Vxx Lxx	Aib	Pro	Vxx Lxx	Aib	Pro	Aib Vxx Lxx	Aib Vxx Lxx	Aib	Pro	Lxxol									
TCT-B	Ac	Vxx Lxx	Aib	Pro	Vxx	Vxx Lxx	Aib	Pro	Aib Lxx	Aib Vxx Lxx	Aib	Pro	Lxxol								
TCT-C	Ac	Vxx Lxx	Aib	Pro	Vxx	Aib	Pro	Lxx	Vxx Lxx	Aib	Pro	Alaol									
TCT-D	Ac	Vxx Lxx	Aib	Pro	Vxx	Vxx Lxx	Aib	Pro	Lxx	Vxx Lxx	Aib	Pro	Alaol								
TFR	MDA	Pro	AHMOD Desmethyl-AHMOD	Ala	Aib	Aib Vxx	Aib Vxx Lxx	Gly Ala Aib	Aib	Aib	AAE AMAE										

ALM Alamethicin (F30: acidic, F50: neutral), *TCP* trichocompactin, *TBV* trichobrevin, *TCT* trichocryptin (subfamilies A–D), *TFR* trichoferin, *Oc* n-octanoy,; *MDA* 2-methyldecanoic acid, *AHMOD* 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid, *AMAE* 2-[(2′-aminopropyl)-methylamino]-ethanol, *AAE* 2-(2′-aminopropyl)amino-ethanol

Table 7 Peptaibiotics, mycotoxins and hydrophobins

Number	Pseudomolecular ion ($[M+H]^+$or $[M+Na]^+$) of peptaibiotics and hydrophobins[a]
I. ALAMETHECINS (ALM)	
1	1964
2	1978
3	1978
4	1992
31	1950
76	1963
77	1977
78	1977
79	1991
II. TRICHOCOMPACTIN (TCP)	
5	726
6	726
7	740
8	740
9	754
10	754
40	756
39	770
41	770
42	784
43	784
70	740
71	754
72	754
73	756
74	770
75	770
III. TRI0CHOCRYPTIN-A (TCT-A)	
19	1125
20	1125
21	1125
22	1139
23	1139
24	1153
25	1153
26	1167
27	1167
28	1167
29	1181
30	1181
80	1111
81	1111
82	1153
86	1125
87	1125
88	1125
89	1125
90	1139
91	1153
IV. TRI0CHOCRYPTIN-B (TCT-B)	
11	1210
12	1210
13	1224
14	1224
15	1238
16	1238
17	1252
18	1252
33	1266
34	1266
35	1266
36	1266
83	1238
84	1238
85	1238
V. TRICHOCRYPTIN-C (11-residue peptaibols with C-terminal Alaol) (TCT-C)	
92	1097
93	1111
94	1125
VI. TRICHOCRYPTIN-D (12-residue peptaibols with C-terminal Alaol) TCT-D	
95	1196
96	1210
97	1210
98	1224
99	1224
100	1224
101	1224
102	1238
103	1238
VII. TRICHOBREVIN (TBV)	
44	1099
45	1099
46	1099
47	1099
48	1113
49	1113
50	1127
51	1127
52	1127
53	1141
54	1141
55	1129
56	1129
57	1129
58	1143
59	1143
60	1157
61	1157
62	1157
104	1171
105	1171
106	1171
VIII. TRICHOFERIN (TFR)	
64	1207
65	1193
66	1193
67	1193

Table 7 (continued)

Number	Pseudomolecular ion ([M+H]$^+$or [M+Na]$^+$) of peptaibiotics and hydrophobins[a]
68	1193
69	1193
IX. HYPOCOMPACTIN (HPC)	
107	738
108	752
109	766
110	780
111	714
112	728
X. HYPORODICIN (HRC)	
113	1437
114	1451
115	1465
116	1380
117	1394
XI. TRICHOKONIN ((TKO)	
118	1866
119	1880
HYDROPHOBIN BIOMARKERS (HPH)	
300	6658
301	6879
302	6943
303	7118
304	7289
305	7319
306	8755
307	9499
308	9567
TRICHOTHECENES	
400	Trichodermin TDERMIN)
401	Harzianum A (HARZ_A)

Consecutive numbering from 1 to 75 according to Degenkolb et al. 2006a
[a] For details, see text

within *Trichoderma*/*Hypocrea*, as was suggested by Kraus et al. (2004) for the single species *T. brevicompactum*.

Because of low bootstrap support (Fig. 1), the Brevicompactum clade is in an unresolved polytomy with *H. victoriensis* and the Viride, Minutisporum, Megalocitrina, and Lutea Clades. However, based on branch length the closest relative to the Brevicompactum clade is the Lutea clade (Fig. 1).

For convenience, we included only representative cultures of members of the Brevicompactum clade in Fig. 1. In order to clarify the interrelationships of members of the Brevicompactum clade, we analyzed all 28 isolates that we know belong in that clade in our subsequent analyses (Fig. 2a–d) (the 29th, CBS 113214, was received too late to include in the phylogenetic analysis). We adopted the phylogenetic species recognition of Dettman et al. (2003). Briefly a clade is recognized as an independent evolutionary lineage if it satisfies either of two criteria: (1) genealogical concordance: the clade is present in the majority of the single-locus trees; or (2) genealogical nonconcordance: the clade is well supported in at least one single-locus tree and is not contradicted in any other single-locus tree at the same level of support. We sequenced three unlinked loci: *tef1* (Fig. 2b), *rpb2* (Fig. 2c), and ITS (Fig. 2c). The properties of the data set for each gene and the combined multi locus sequence (MLS; Fig. 2a) are given in Table 2. In this second analysis, the sequences varied strikingly in their variability; *tef1* had the highest number of parsimony informative characters (144 out of 596 or 24%) followed by *rpb2* (14.6%) and ITS (2.2%).

Figure 2a represents a Bayesian inference majority-rule consensus tree based on MLS. This tree shows a strongly supported Brevicompactum clade (9) that is divided into two sister subclades: *Hypocrea rodmanii* (7) and the Brevicompactum complex (8), each of which was highly supported in the combined data set and in the individual gene trees based on *tef1* and *rpb2* (Fig. 2b,c). The *T. brevicompactum* complex was divided further into a polytomy comprising three main clades: 1 (*T. brevicompactum*, *T. turrialbense*), 2 (*T. arundinaceum*), and the single culture CBS 121320 (*T. protrudens*). Subclades 1 and 2 were also highly supported in the individual gene trees (Fig. 2b,c). The *T. brevicompactum* clade (3) again showed two supported subclades (4, 5); the ex-type culture of *T. brevicompactum* (CBS 109720) is in subclade 4. Subclade 4 did not have support in the *rbp2* tree (Fig. 2c) but subclade 5 received strong support in both of the individual gene trees (Fig. 2b,c). The two members of subclade 5 (CBS 112446, CBS 112447) differed by only one allele despite their widely divergent geographic origins (India and Mexico). There was no support for taxonomic separation of clade 3 from morphology, secondary metabolites or biogeography.

The two isolates of node 6 (*T. turrialbense*) originated from the same area in Costa Rica. This clade is highly supported in MLS, *tef1* and *rpb2*. Despite a strong sister relationship with *T. brevicompactum* s. str. (clade 1), it differs from *T. brevicompactum* in producing harzianum A and in subtle morphological characters (Table 8).

The other large clade (clade 2, *T. arundinaceum*) consisted of 8 isolates. This clade had high support in the MLS, *tef1* and *rpb2* trees. Despite their diverse geographic origins, their sequences were highly homogenous. Such a high homogeneity among isolates of one species having such diverse origin is uncommon in *Trichoderma*.

Trichoderma protrudens, which was found as an endophyte in sapwood of *Theobroma cacao* in India, had strong sister relationship to clades 1 and 2 in MLS (Fig. 2a) and the single gene trees (Fig. 2b,c). The ITS sequence for this isolate differed from *T. brevicompactum* and *T. turrialbense* by a 1-bp insertion.

The three isolates of *Hypocrea rodmanii* (clade 7) were collected in the Mid Atlantic states (Maryland and Virginia)

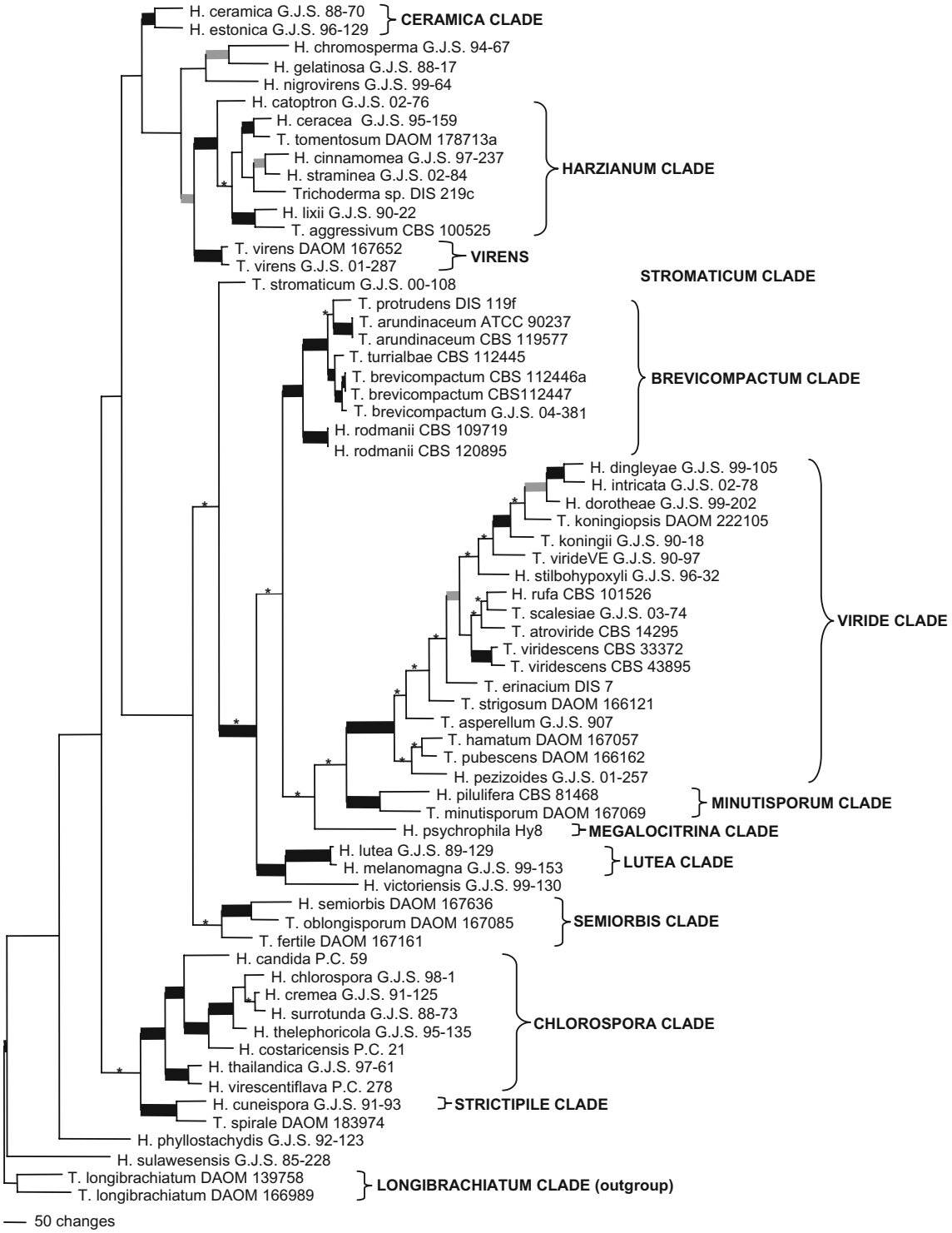


Fig. 1 Bayesian phylogram of combined ITS, *tef1*, and *rpb2* sequence data. The tree presented had the best log likelihood (LnL=−22,426.97) in the Bayesian analysis. *Thick black lines* represent nodes with posterior probability >90%, and Parsimony and Neighbour-Joining bootstraps >70%. *Thick gray lines* represent nodes with posterior probability >90%, Neighbor-Joining bootstrap >70%, and Parsimony bootstrap <70%. Nodes with *asterisks* (*) represent clades with posterior probabilities >90%. Clade/lineage names based in part on: Samuels 2006; Chaverri and Samuels 2003; and ISTH (http://www.isth.info/biodiversity/index.php)

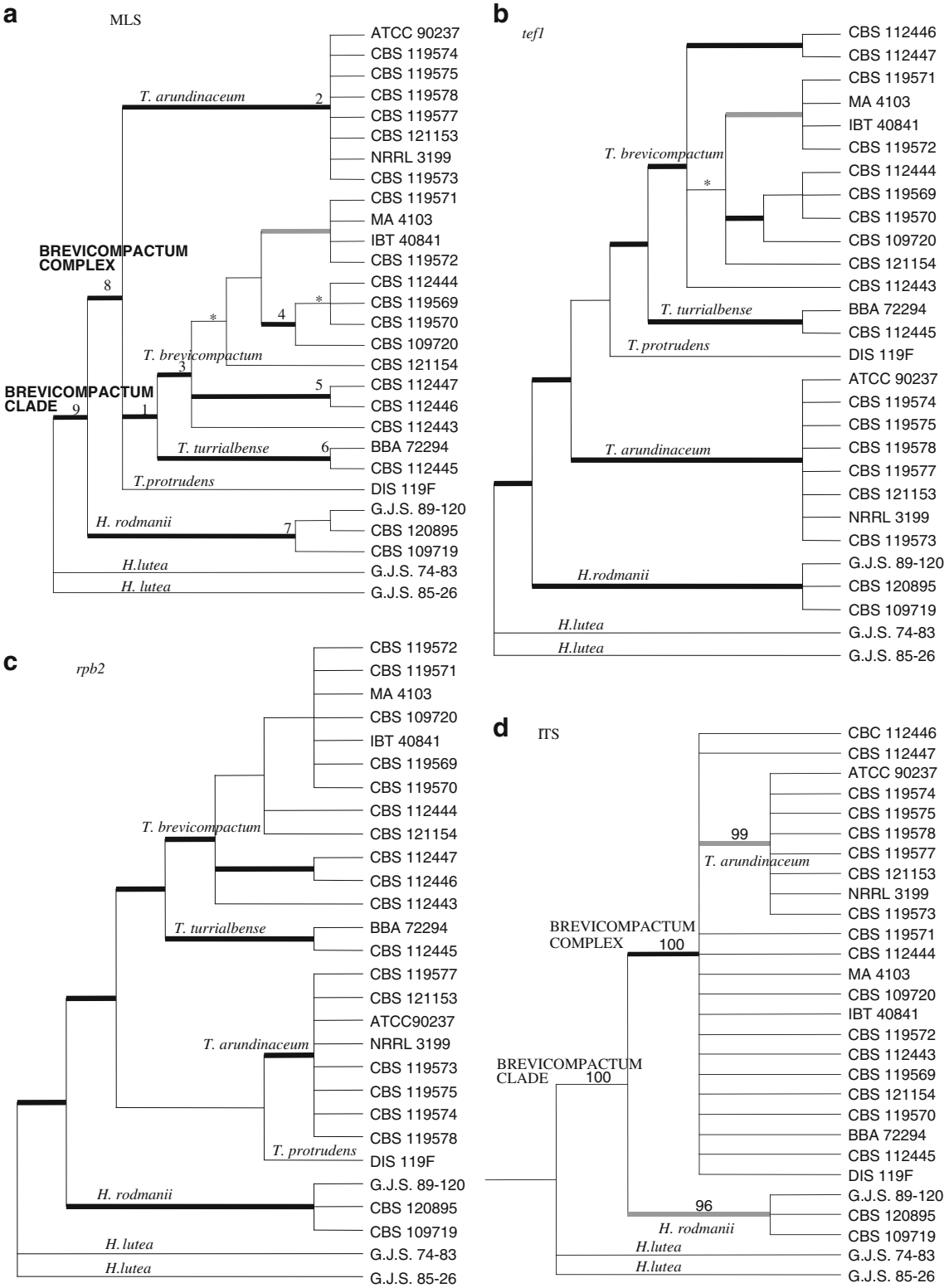


Fig. 2 Majority-rule (50%) consensus tree resulting from Bayesian analysis of **a** combined 3-gene, **b** *tef1,* **c** *rpb2* and **d** ITS dataset. Branches with *thick black lines* represent clades with posterior probability (PP) ≥ 0.95 and bootstrap Maximum parsimony (BS-MP) and Bootstrap Neighbour-Joining (BS-NJ) values ≥ 70%. *Thick gray lines* represent clades with PP ≥ 0.95 and BS-MP or BS-NJ or both < 70%. *Asterisks* (*) represent clades with PP ≥0.90. *Numbers* on nodes correspond with node numbers used in the text

Table 8 Continuous characters of morphology and colony

Character		Taxa				
		Trichoderma arundinaceum	*Trichoderma brevicompactum*	*Trichoderma protrudens*	*Hypocrea rodmanii*	*Trichoderma turrialbense*
Dominant distribution		Cosmopolitan, temperate and tropical	Cosmopolitan, temperate and tropical	India: Kerala	USA: MD, VA	Costa Rica
Conidia length (μm)		(2.2)2.7–3.5(5.0)	(2.2)2.7–3.0(3.7)	(2.5)2.7–3.2(3.5)	(1.7)2.0–2.5(2.7)	(2.2)2.5–3.0(3.2)
95% CI		3.1–3.2	2.9–3.0	2.9–3.0	2.2–2.3	2.7–2.8
		n=270	*n*=330.	*n*=30	*n*=60	
Width (μm)		(1.7)2.5–3.0(3.5)	(2.0)2.2–2.7(3.0)	(2.2)2.5–2.7(3.0)	(1.7)2.0–2.2(2.5)	(2.0)2.2–2.7(3.0)
95% CI		2.6–2.7	2.5–2.6	2.5–2.7	2.0–2.1	2.5–2.6
		n =270	*n*=330	*n*=30	*n*=60	*n*=60
L/W		(0.9−)1.0−1.4(−1.7)	(0.9)1.1–1.3(1.4)	(1.0)1.0–1.3(1.5)	(0.9)1.0–1.2(1.4)	(0.8)1.0–1.1(1.5)
95% CI		1.16–1.20	1.16–1.19	1.1–1.2	1.07–1.12	1.06–1.10
		n = 270	*n*=330	*n*=30	*n*=60	*n*=60
Phialides length (μm)		(3.7)4.5–7.0(12.7)	(3.7)4.7–6.7(15.0)	(4.0)5.5–9.0(12.0)	(3.0)4.0–5.5(7.0)	(3.7)4.5–6.7(12.0)
95% CI		5.5–5.7	5.6–5.9	6.7–8.0	4.5–4.9	5.3–5.9
		n=270	*n*=330	*n*=30	*n*=60	*n*=60
Max. width (μm)		(2.0)3.0–4.0(4.5)	(2.5)3.0–3.7(4.5)	(2.2)2.5–3.0(3.2)	(2.5)2.7–3.2(3.7)	(2.5)2.7–3.7(4.2)
95% CI		3.4–3.5	3.3–3.4	2.7–3.0	2.9–3.1	3.1–3.3
		n=270	*n*=330	*n*=30	*n*=60	*n*=60
Base (μm)		(1.2)2.0–3.0(3.7)	(1.3)2.0–2.7(3.7)	(1.5)1.7–2.7(3.2)	(1.0)1.5–2.2(2.5)	(1.5)2.0–2.7(3.7)
95% CI		2.5–2.6	2.4–2.5	2.1–2.3	1.7–2.0	2.3–2.5
		n=270	*n*=330	*n*=60	*n*=30	*n*=60
Length/width		(1.1)1.2–2.2(5.2)	(1.1)1.3–2.2(5.1)	(1.4)1.8–3.4(5.0)	(1.0)1.3–1.7(2.4)	(1.2)1.3–2.3(4.3)
95% CI		1.6–1.8	1.7–1.8	2.4–2.9	1.5–1.7	1.7–1.9
		n=270	*n*=330	*n*=30	*n*=60	*n*=60
Width of supporting cell (μm)		(2.0)3.0–4.2(6.0)	(1.7)2.7–4.0(5.7)	(2.2)2.5–3.0(3.2)	(2.2)3.0–4.0(4.5)	(2.0)2.5–3.5(4.2)
95% CI		3.5–3.6	3.3–3.5	2.6–2.8	3.3–3.5	3.0–3.3
		n=270	*n*=330	*n*=30	*n*=60	*n*=60
Ratio phialide length/width of supporting cell		(0.8)1.1–2.3(6.5)	(1.0)1.3–2.3(4.8)	(1.5)2.0–3.5(6.5)	(0.8)1.1–1.7(2.5)	(0.9)1,2–2.6(6.0)
95% CI		1.6–1.7	1.7–1.8	2.5−3.0	1.4–1.5	1.7–2.1
		n=270	*n*=330	*n*=30	*n*=60	*n*=60
Ratio phialide width/width of supporting cell		(0.5)0.8–1.2(1.6)	(0.6)0.8–1.2(1.7)	(0.7)0.9–1.3(1.4)	(0.6)0.7–1.1(1.4)	(0.6)0.9–1.3(1.8)
95% CI		0.97–1.01	1.0–1.1	1.0–1.1	0.9–1.0	1.0–1.1
		n=270	*n*=330	*n*=30	*n* - 60	*n*=60
Colony radius PDA 72 H (mm)						
15°C	Range	2–6	0–6	1–3	3–7	0–5
	Mean	5	2		6	3
	SD	1	2		2	2
	n	7	11	1	2	2
	CI	4–5	2–3		5–8	1–5
20°C	Range	9–37	14–39	17–28	29–33	21–36
	Mean	32	30		31	25
	SD	7	7		2	6
	n	7	11		2	2
	CI	29–35	27–33	20–36	29–32	
25°C	Range	32–48	30–50	30–38	31–40	35–45
	Mean	41	42		35	40
	SD	4	5		4	4
	n	7	11	1	2	2
	CI	39–45	40–44		32–39	40–44

Table 8 (continued)

Character		Taxa				
		Trichoderma arundinaceum	*Trichoderma brevicompactum*	*Trichoderma protrudens*	*Hypocrea rodmanii*	*Trichoderma turrialbense*
Dominant distribution		Cosmopolitan, temperate and tropical	Cosmopolitan, temperate and tropical	India: Kerala	USA: MD, VA	Costa Rica
30°C	Range	40–69	36–60	52–58	35–42	44–63
	Mean	54	52		37	56
	SD	7	6		3	7
	n	7	11	1	2	2
	CI	51–57	50–54		36–42	50–54
37°C	Range	0–18	0–30	23−45	0	3–38
	Mean	8	7			11
	SD	7	10			15
	n	7	11	1	2	2
	CI	6–12	4–10			4–10
Colony radius SNA 72 H (mm)						
15°C	Range	1–8	0–5	5–7	2–4	2–5
	Mean	4	1.5		3	2
	SD	2	2		1	1.5
	n	7	11	1	2	3
	CI	–5	1–2		2–4	13–21
20°C	Range	0–39	12–34	19–29	10–19	21–38
	Mean	30	24		17	26
	SD	5	5		4	7
	n	7	11	1	2	2
	CI	27–32	22–26			20–35
	CI					
25°C	Range	32–48	19–48	30–41	13–28	38–50
	Mean	41	35		22	44
	SD	4	11		5	4
	n	7	1	1	2	2
	CI	39–43	33–37		16–27	40–48
30°C	Range	40–55	20–56	39–47	7–26	54–59
	Mean	47	41		19	56
	SD	5	9		7	2
	n	7	11	1	2	2
		45–50	38–45		11–26	54–59
37°C	Range	0–22	0–25	16–27	0	1–45
	Mean	7	5			14
	SD	5	7			19
	n	7	11	1	2	2
	CI	5–9	2–7			0–25
Temperature of first appearance of green conidia on PDA grown in intermittent light						
		20−25°C	25(−30)°C	30°C	20−25°C	20−25°C

and formed a highly supported clade (7) in MLS, *tef1* and *rpb2* that was sister to the Brevicompactum complex. In the ITS tree (Fig. 2d), this clade received strong support in Bayesian analysis, but its bootstrap support was low (Fig. 2d).

In brief, phylogenetically, *T. brevicompactum* (Fig. 2a, clade 3), *T. arundinaceum* (Fig. 2a, clade 2), *T. turrialbense* (Fig. 2a, clade 6), *T. protrudens* (Fig. 2a) and *H. rodmanii* (Fig. 2a, clade 7) have support in the majority (2/3) of the three individual gene trees and thus meet the genealogical concordance criteria of Dettman et al. (2003) for recognizing species. *Trichoderma protrudens* was consistently independent of all other lineages in all of the analyses. ITS sequences were able to discriminate unequivocally *H. rodmanii* and *T. arundinaceum*; but *T. brevicompactum*, *T. turrialbense,* and *T. protrudens* were indistinguishable by ITS. The present work confirms the reidentification as *T.*

arundinaceum of three *Trichoderma/Hypocrea* strains that have been reported in the peptaibiotic or mycotoxin literature. Culture NRRL 3199 is cited as *T. viride* in U.S. Patent 3833723 (Coats et al. 1974) for production of alamethicin and has become the standard source for alamethicins (e.g., Kirschbaum et al. 2003). Culture ATCC 90237 was reported in the literature, as *T. harzianum,* as the original source of harzianum A (Corley et al. 1994). Culture CBS 113214, a soil isolate, was reported by Lee et al. (2005, as F000527) as a likely new *Hypocrea* sp. that produces harzianum A.

Peptaibiotics

Peptaibiotics: HPLC-ESI-Ion-Trap-MS

General Remarks The patterns of peptaibiotics produced by *T. brevicompactum* CBS 109720, CBS 119569, CBS 119570, *T. arundinaceum* CBS 119576, CBS 119577, NRRL 3199 and *T. turrialbense* CBS 112445 (as *T.* cf. *brevicompactum*) were investigated earlier (Degenkolb et al. 2006a). To maintain conformity and to prevent confusion about the identity of recurrent sequences mentioned throughout the text and in tables, we adopt the consecutive numbering of peptaibiotics from **1** to **75** previously introduced by Degenkolb et al. (2006a). Consequently, numbering of additional, mostly new sequences proceeds consecutively from substance **76–119**. The peptaibiotics pattern of the above seven strains is shown in Table 3. New sequences of peptaibiotics detected in the *Trichoderma* strains (excluding *T./H. rodmanii*) are listed in Table 4, sequences of peptaibiotics from *T./H. rodmanii* in Table 5, and general sequences ("building schemes") of peptaibiotics found in the Brevicompactum clade are listed in Table 6. All strains of *T. brevicompactum*, *T. arundinaceum*, *T. turrialbense*, and *T. protrudens* listed in Table 1 produced a number of 20-residue peptaibols, alamethicins (ALMs), as major components. The alamethicins, which are the most thoroughly investigated peptaibols, were recently reviewed (Leitgeb et al. 2007).

Peptaibiomics of Trichoderma brevicompactum Some intraspecific variation among the ALM profiles was observed. ALMs F30 [**1–4**] were produced by four strains, whereas the remaining three strains produced a mixture of the acidic ALMs F30 and the neutral ALMs F50/5, F50/6a, F50/7, and F50/8b [**76–79**]. It appears, however, that in cases where both subgroups of ALMs were produced, ALMs F30 dominated over ALMs F50. These results confirm the observation of Kirschbaum et al. (2003) who described a time-dependent formation of ALM subgroups by *T. arundinaceum* NRRL 3199. The latter produced the neutral ALM F50 (Gln^{18}-Gln^{19}) at the beginning of the fermentation; after about 5 d the ALM F50 concentration decreased whereas the concentration of the acidic ALM F30 (Glu^{18}-Gln^{19}) increased.

The second most abundant group were the 11-residue trichocryptins A [**19–30**, **80**, **81**, **86–91**]. They represent deletion sequences of the 12-residue trichocryptins B [**11–18**, **83–85**], lacking the amino acid residue in position 5, which is either Lxx or Vxx. Again, there is intraspecific variation in the pattern of trichocryptins produced. The trichocryptins A are found in every investigated strain but chain length varied, depending on the amino acids in the exchange position 1, 4, 7, and 8. The trichocryptins B display the same building scheme as trichcryptins A but carry an additional Vxx residue in position 4. Variable amino acids are therefore located in position 1, 5, 8, and 9.

Trichoferins (TFR) are a group of minor, 10-residue lipoaminopeptides produced by four of the seven strains. The major component, TFR A [**64**], is produced by each of the four trichoferin-positive strains, but there is some intraspecific variation in the pattern of the four minor homologues, TFR C-E [**66–68**]. Notably, trace amounts of trichobrevins [**52–54**, **61**, **62**] were found in CBS 121154 but not in any of the other six strains. The pattern of peptaibiotics produced by that strain is less complex than in any other *T. brevicompactum* strain investigated.

Peptaibiomics of Trichoderma turrialbense Trichoderma turrialbense is distinguished from *T. brevicompactum* mainly by the production of different trichothecenes as pointed out above. Like *T. brevicompactum*, *T. turrialbense* produces ALMs F30 [**1–4**] as the major group of peptaibiotics, although larger amounts of ALMs F50 [**76–79**] are also present in CBS 122554 but not in CBS 112445. Trichocryptins A [**23–28**] and B [**13–18**, **33–38**] were found in CBS 112445, being the only strain to produce major amounts of trichocryptins B, which display $[M+Na]^+$ ions at *m/z* 1266 [**33–36**] and 1280 [**37**, **38**]. In contrast, *T. turrialbense* CBS 122554 produced four 11-residue trichocryptins C [**92–95**] and eight 12-residue trichocryptins D [**96–103**], the former representing deletion sequences of the latter. As shown in Table 6, both of these novel subgroups exhibit high structural homology with trichocryptins A and B, but they carry an L-alaninol (Alaol) residue at their *C*-terminus. This is the first report to unambiguously prove the presence of L-Alaol as a *C*-terminal constituent of peptaibiotics. Small amounts of trichobrevins A [**52**, **53**] and B [**62**] and trichoferin A [**64**] were also detected in strain CBS 122554. From its pattern of peptaibiotics, the new species *T. turrialbense* is intermediate between *T. brevicompactum* and *T. arundinaceum*.

Peptaibiomics of Trichoderma arundinaceum This includes strain NRRL 3199, a patent strain originally reported as "*T.*

viride" and generally considered to be the classical commercial source of ALMs (Kirschbaum et al. 2003; Leitgeb et al. 2007). The 12-residue trichobrevins (TBV, **44–62**) were recognized as the second most abundant group of peptaibiotics of *T. arundinaceum*. Depending on the strain investigated, TBV B-IIIb [**61**] and B-IIIc [**62**], or A-IVa [**53**] and A-IV-B [**54**] were observed as the predominant compounds. Thus, the common presence of both alamethicins and trichobrevins as the two major groups of peptaibiotics was recognized as the most important feature distinguishing *T. brevicompactum*/*T. turrialbense* from *T. arundinaceum*/*T. protrudens*. Trace amounts of trichocryptins B were also detected in strain CBS 119576.

Peptaibiomics of Trichoderma protrudens The pattern of peptaibiotics produced by CBS 121320 is quantitatively identical to that of all five strains of *T. arundinaceum* studied. CBS 121320 produced three new, positionally isomeric minor compounds, the 11-residue trichobrevins B-IVa [**104–106**], in small, but in somewhat larger amounts than in *T. arundinaceum*, enabling us to sequence them.

Additional minor components, such as 7-residue trichocompactins III b (**10**), Va (**39**), and Vb (**41**), as well as four 10-residue lipoaminopeptides, trichoferins A (**64**), B (**65**), C (**66**), and E (**68**), were found.

Peptaibiomics of Hypocrea rodmanii Hypocrea rodmanii did not produce any of the peptaibiotics typical of the *Trichoderma* species treated above. The most conspicuous difference is the absence of alamethicins F30/F50, trichocryptins, trichobrevins, and trichoferins, which were the distinctive peptaibiotics of *T. brevicompactum*, *T. arundinaceum*, *T. turrialbense* and *T. protrudens*.

Both investigated strains of *Hypocrea rodmanii* produced six new 7-residue lipopeptaibols, named hypocompactins (HCP) I-VI [**107–112**]. Four of them, HCPs I-IV [**107–110**], exhibit homology with four 7-residue lipostrigocins A1-A4 from *T.* cf. *strigosum* CBS 119777 (Degenkolb et al. 2006b) and the 7-residue trichocompactins produced by *T. brevicompactum*, *T. turrialbense*, *T. arundinaceum*, and *T. protrudens*. We assume that the *N*-terminal Gly of hypocompactins I-IV has been blocked by a C_8 mono-unsaturated, branched or non-branched fatty acid, probably *n*-octenoyl. This is based on LC/high-resolution CID-MS data (K.F. Nielsen and T. Degenkolb, unpublished data) and structural homologies with the lipostrigocins, trichogin GA IV from *T. longibrachiatum* M 3431 (Auvin-Guette et al. 1992), and trikoningin KB I from *T. koningii* 903589 (Auvin-Guette et al. 1993), all carrying an *N*-terminal *n*-octanoyl-Aib residue. This assumption is further supported by the presence of a *cis*-4-decenoyl residue bound to Gly in the trichodecenins I and II. The producer of the latter two lipopeptaibols was reported as *T. viride* (Fujita et al. 1994), but neither details of taxonomic identification nor a strain accession number were given. Hypocompactins V and VI [**111**, **112**] could only be sequenced partially because of the very low intensities of the *N*-terminal *b*-type fragment ions. The hypothesis of *N*-terminal *n*-octenoyl-Gly in HCP I-IV leads to the hypothesis that *n*-octanoyl-Gly (Oc-Gly) might be present in the all TCPs, as supported by LC/high-resolution CID-MS data (K.F. Nielsen and T. Degenkolb, unpublished data).

Hypocrea rodmanii CBS 120895 and CBS 109719 produced compounds [**113**–**117**] representing five new 14-residue peptaibols, hyporodicins (HRC) A–E. All of them carry the *C*-terminal octapeptide Vxx-Aib-Pro-Aib-Lxx-Aib-Pro-Lxxol. This *C*-terminus has also been identified in some of the trichobrevins and trichocryptins; and it was first reported for the 14-residue harzianins HC-I and HC-VI from *T. harzianum*, strains M-90361 and M-903603, with Vxx = Val and Lxx = Leu (Rebuffat et al. 1995). The close relationship of the hyporodicins [**113**–**117**], with harzianins HC is further supported by the structure of the *N*-terminal pentapeptide Ac-Aib-Gln-Lxx-Aib-Pro found in [**113**–**115**]: the same sequence with Lxx = Leu has been reported previously for harzianins HC-X, HC-XIII, and HC-XV (Rebuffat et al. 1995).

Two 19-residue peptaibols, the compounds [**118**] and [**119**], were produced. Compound [**118**] exhibited the same fragmentation pattern as trichokonin V (TKO-V) from a fungicolous strain of *T. koningii* that was isolated from a fruiting body of *Ganoderma lucidum* (Huang et al. 1995). Consequently, it could be identical with trichokonin V, or represent a positional isomer. The second trichokonin-like homologue carries a Vxx residue instead of Aib in position 16 of the peptide chain. Thus, compound [**119**] might represent a deletion sequence of trichokonin VII (Huang et al. 1995), as it lacks the Ala residue of the former in position 6. Peptaibols with the same *C*-terminal sequences were described from *T. koningii* LCP984209 (Landreau et al. 2002), a marine strain that was isolated as a contaminant from the cockle *Cerastoderma edule* (Sallenave et al. 1999).

Typing of strains by intact cell MALDI-TOF mass spectrometry (ICMS) and hydrophobins

Sporulating mycelium of strains grown on PDA was subjected on day 6 to intact cell mass spectrometry (ICMS). Patterns in the high mass metabolite range of 1,000 to 2,000 Da and in the low mass proteome region of 4,000 to 12,000 Da were recorded (Tables 1 and 7). The low mass protein region provides molecular ions of hydrophobins, small hydrophobic proteins that are excreted and proteolytically processed. Each spectrum consisted of 3–11 masses

of different intensities, and the mass data were analysed by UPGMA cluster analysis (Pearson Correlation, not shown). The data are grouped into 3 main clusters, *Trichoderma/Hypocrea rodmanii* (CBS 120895), *T.arundinaceum/T. protrudens* (NRRL 3199, CBS 119575, CBS 119576/CBS 121320), and *T. turrialbense* (CBS 122554). A total of 58 different masses were observed. None of the masses recorded for *H. rodmanii* and *T. protrudens* (CBS 121320) were shared by other strains. This supports the conclusion that these isolates represent individual species. *T. arundinaceum* and *T. protrudens* have been grouped by UPGMA cluster analysis or use of the commercial program SARAMIS in one cluster, since the 4 dominant masses and their spacings are fairly similar (CBS 121320 with dominating peaks at m/z 5566, 7188/7249 and 9623, *T. arundinaceum* with corresponding masses of m/z 5605, 7172/7222 and 9650). All *T. arundinaceum* strains share the masses *m/z* 5603, 8648 and 9652, which were not detected in any other strains. These masses can be considered as biomarkers for this species.

More complex is the situation in the *T. brevicompactum/T. turrialbense* cluster. Some of the masses are found in both subclusters, as reflected by the cluster analysis. Inspection of the mass spectra, however, reveals that two of the dominating masses, respectively, in each subcluster are not shared by the other (*m/z* 7092 and 9552 in *T. turrialbense*, *m/z* 6946 and 9572 in *T. brevicompactum*), while two significant masses of the Brevicompactum subcluster are shared as minor peaks in the Turrialbense subcluster (*m/z* 7112, and 8753), and one significant peak (*m/z* 9307) of the Turrialbense subcluster is shared by the Brevicompactum subcluster. Both subclusters share additional minor peaks (*m/z* 7293 and 8815). Analysis of the spectra with the program SARAMIS (Anagnostec) developed for ICMS identification of microbes locates the Turrialbense subcluster within a Brevicompactum cluster. The data suggest that certain hydrophobins may be shared in both subclusters, while others are not. Because hydrophobins are small proteins directly encoded by DNA, identical proteins indicate largely identical gene sequences. Mass data alone do not permit recognition of a species, as we cannot deduce any information on the respective genetic background. Only sequence information on the respective hydrophobins will permit a clear evaluation (Fig. 3).

Mycotoxin and secondary metabolite profilies

Two trichothecene mycotoxins were detected by LC-HRMS in members of the Brevicompactum complex (Table 1), trichodermin and harzianum A. The latter compound was produced by all members of *T. turrialbense, T. protrudens*, and *T. arundinaceum*, whereas *T. brevicompactum* produced trichodermin as previously described (Nielsen et al. 2005). Production of these were 30–100 times higher on PDA than oatmeal agar (OAT).

Besides tricothecenes, several isocoumarins (polyketides) including diaportinol and diaportinic acid (matching authentic reference standards) were detected in shake cultures of *T. turrialbense, T. protrudens*, *T. arundinaceum,* and *T. brevicompactum*.

Trichoderma arundinaceum and *T. protrudens* produced large amounts of an undescribed compound (no likely matches in Antibase 2007), with a $[M-H]^-$ ion of *m/z* 535.1627 and UV max of 200 nm. As this compound did not ionize in ESI^+, it cannot be assigned as a peptide. Notably, it was also observed in *T. brevicompactum* but in 100- to 1,000-fold lower amounts.

All species except *H. rodmanii* produced a unique metabolite with an assumed elementary composition of $C_{10}H_{16}O_4$ ($[M-H]^-$ *m/z* 199.0967 calculated 199.0872 Da, deviation −1.7 ppm) and UV-max 214 nm (no likely matches in Antibase 2007), in ESI^- it displayed a significant loss of CO_2 and showed a $[M-2H + Na]^-$ ion strongly indicating that the molecule contained a carboxylic acid moiety.

Neither trichothecenes nor isocoumarins were detected in *H. rodmanii* (CBS 120895 and CBS 109719), and the only metabolites detected in ESI^+, ESI^-, and UV/VIS were the peptides, which are discussed under Peptaibiomics.

Putative pathways of trichothecene biosynthesis are shown in Fig. 4.

Biogeography

Isolates of *T. brevicompactum* and *T. arundinaceum* are sympatric and probably cosmopolitan. The two known cultures of *T. turrialbense* were collected in Costa Rica, and the only known culture of *T. protrudens* was isolated as an endophyte of the trunk of one *Theobroma cacao* tree in India. *Hypocrea rodmanii* originates from the Mid Atlantic region of the eastern U.S.A. (Maryland, Virginia).

Multidimensional Scaling (MDS)

MDS, a parameter-free data reduction procedure, was performed to detect any grouping of the strains that could be determined by the values of the variables used. Three different analyses were run (Fig. 6).

The first included only morphological data and is presented in Fig. 6a. The stress of the final configuration is 0.1818, which indicates only a moderate fit of the data to the model. While the Brevicompactum and Arundinaceum clades cannot be distinguished based on available morphological and cultural characters, CBS 109719 and CBS 120895 (*H. rodmanii*) form a distinct group, and also CBS 121320 (*T. protrudens*) is clearly distinguishable from all other strains.

The two other analyses were carried out on a subset of cultures for which all metabolite data were available.

When the combined morphological and metabolite characters are used (Fig. 6b), the stress of the final configuration is 0.134 and the MDS performed on the metabolite data alone (Fig. 6c) yields a stress of 0.144, both values slightly better than that obtained with the morphological data alone, but still indicating only a moderate fit of the data to the model. The results of both analyses are very similar and thus confirm the modest contribution of morphological data to the separation of the clades. The scatterplot produced by the MDS with both morphological and metabolite data (Fig. 6b) reveals that isolates tend to group together according to their taxonomy. The major groups (*H. rodmanii*, *T. brevicompactum*, *T. arundinaceum*, and *T. protrudens*) are confirmed, although the separation is not complete. *Hypocrea rodmanii* is separated from all other strains and *Trichoderma protrudens* clusters closer to *T. arundinaceum,* thus reinforcing its phylogenetic similarity of the two species. The two *T. turrialbense* cultures, on the other hand, are widely separated from each other and cluster with *T. brevicompactum* and *T. arundinaceum*. The difference between the two strains of *T. turrialbense* may be explained by differences in trichocryptins C and D. The isolate CBS 121154, *T. brevicompactum* from Cameroon, occupies an intermediate position between *T. arundinaceum*, *T. brevicompactum*, and isolate CBS 122554 of *T. turrialbense* because of its production of trichobrevins [**61**] and [**62**], and trichocompactins [**86–91**].

Species delimitation

The cultures of *H. rodmanii* were derived from isolated single ascospores. Two cultures (CBS 120897, CBS 122581) were not included in the phylogenetic analysis but are undoubtedly representative of this species. Macro-

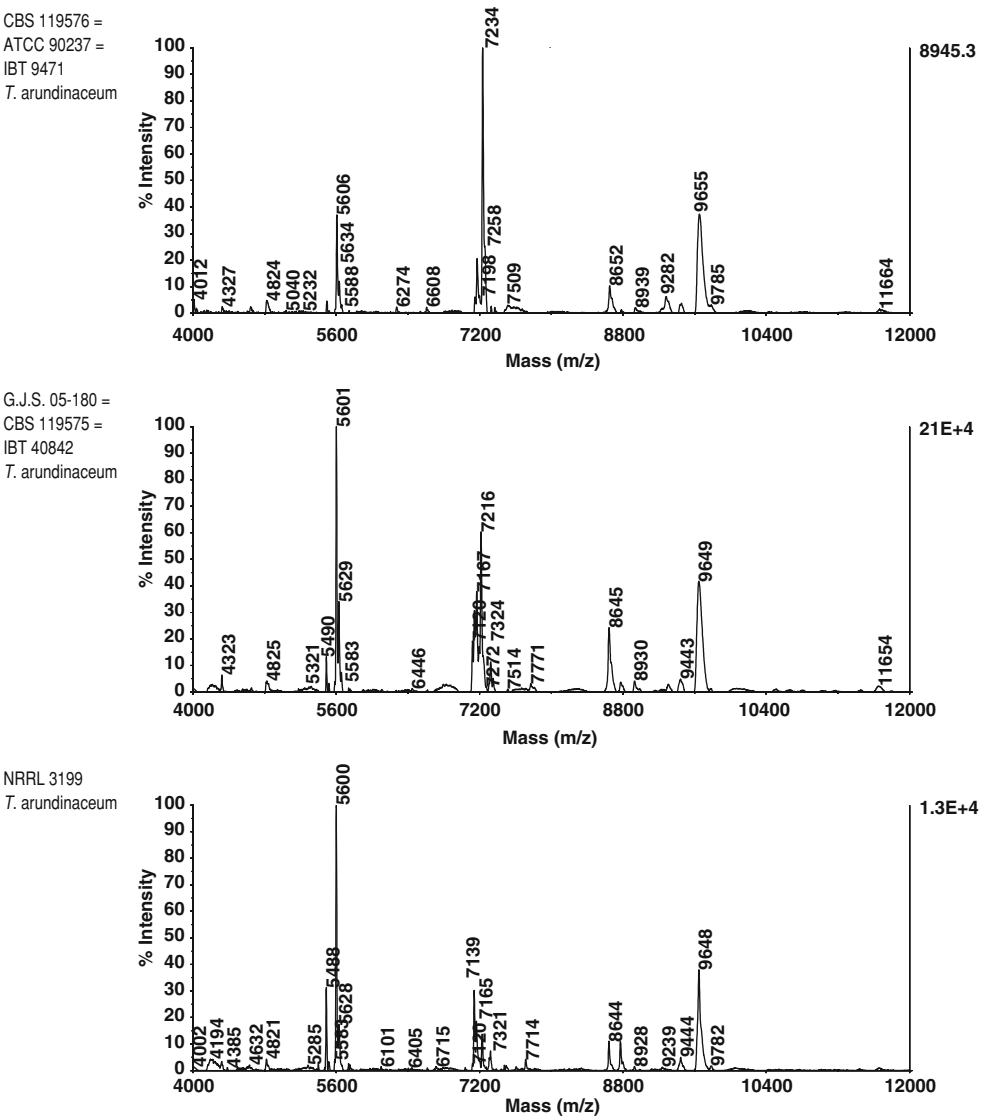


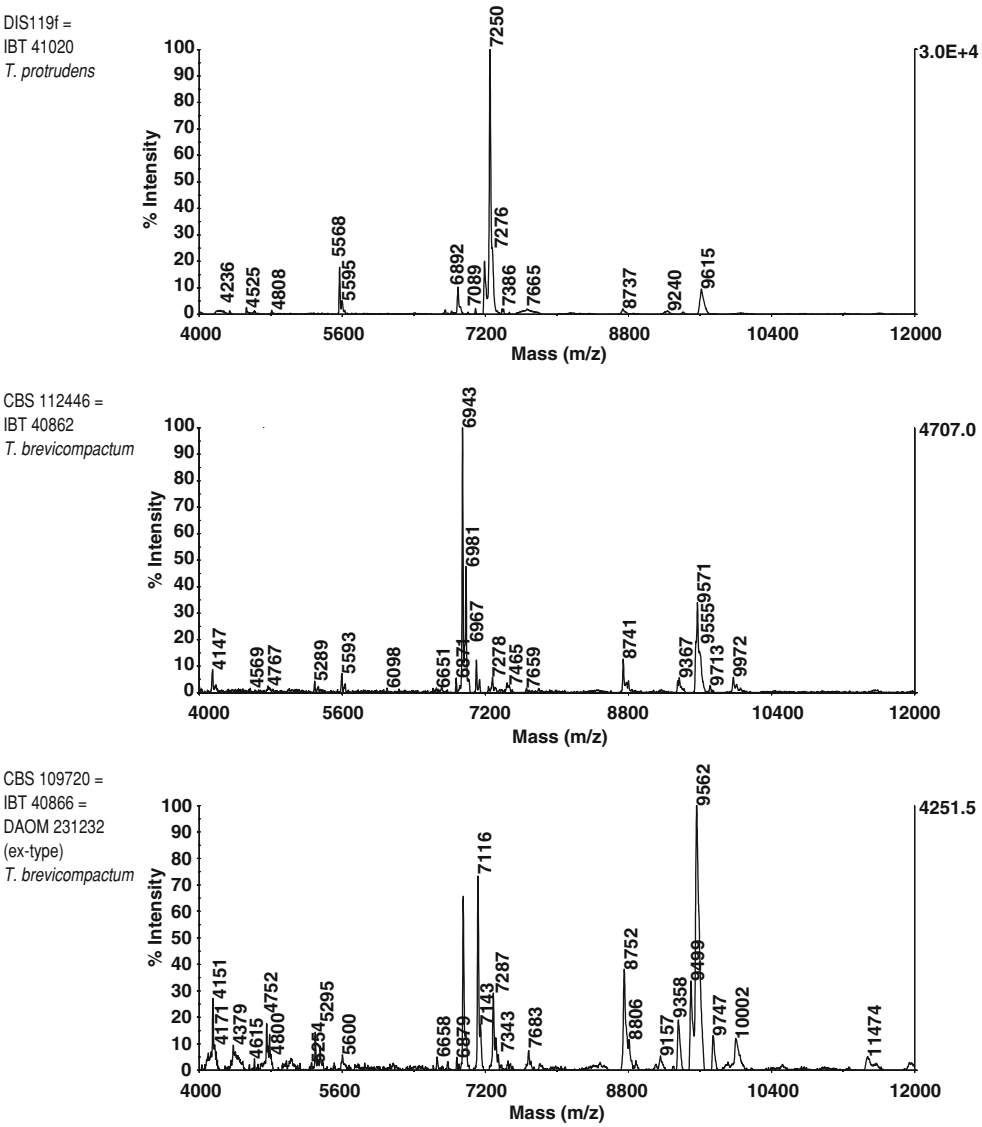
Fig. 3 Intact cell MALDI-TOF mass spectra showing the fingerprint region. One of duplicate or triplicate spectra is shown

and microanatomy of stromata (Figs. 7j-o and 11a-e) are unremarkable in the genus. Ascospores are typical of *Hypocrea* in being hyaline and finely spinulose; the part-ascospores are dimorphic (Fig. 11h, i). There is very little of the stroma morphology or anatomy to distinguish it from other species that have luteous stromata, such as *H. lutea*, which has hyaline ascospores, or *H. straminea* (Chaverri and Samuels 2003), a species that has green ascospores. It is the anamorph that distinguishes this species. Teleomorphs are not known for other members of the Brevicompactum clade.

Two apomorphies combine to characterize the Brevicompactum clade: subglobose conidia and the formation of long white, unbranched or little to frequently branched, conidiophores that project conspicuously from the surface of the pustule (Figs. 8b, 10b, 12c and 13b). These may be sterile over a considerable part of their length but bear one to a few phialides at the tip (e.g., Figs. 8c, 9b, c and 13c). Conidia arise from near their base in a more or less slimy mass; thus, pustules of conidia often appear papillate or lanose. Typical pustules on SNA and CMD are very compact and hemispherical, and free conidiophores within the pustule are not visible when viewed with a stereo microscope. On CMD there is a tendency for pustules to be more extensive, flat and more loosely organized. The papillate aspect is slightly less well developed in *T. turrialbense.* The original description of *T. brevicompactum* (Kraus et al. 2004) described the long extensions as developing in old cultures, but in our experience they are conspicuous elements from an early stage on SNA and CMD. There is a tendency for the projecting conidiophores of *T. brevicompactum, T. turrialbense* and *T. protrudens* to be more branched than in *T. arundinaceum*.

In addition to conidiophores with long extensions, all of the species discussed here produce completely fertile conidiophores that do not have long extensions (e.g., Figs. 8j, 9g

Fig. 3 (continued)



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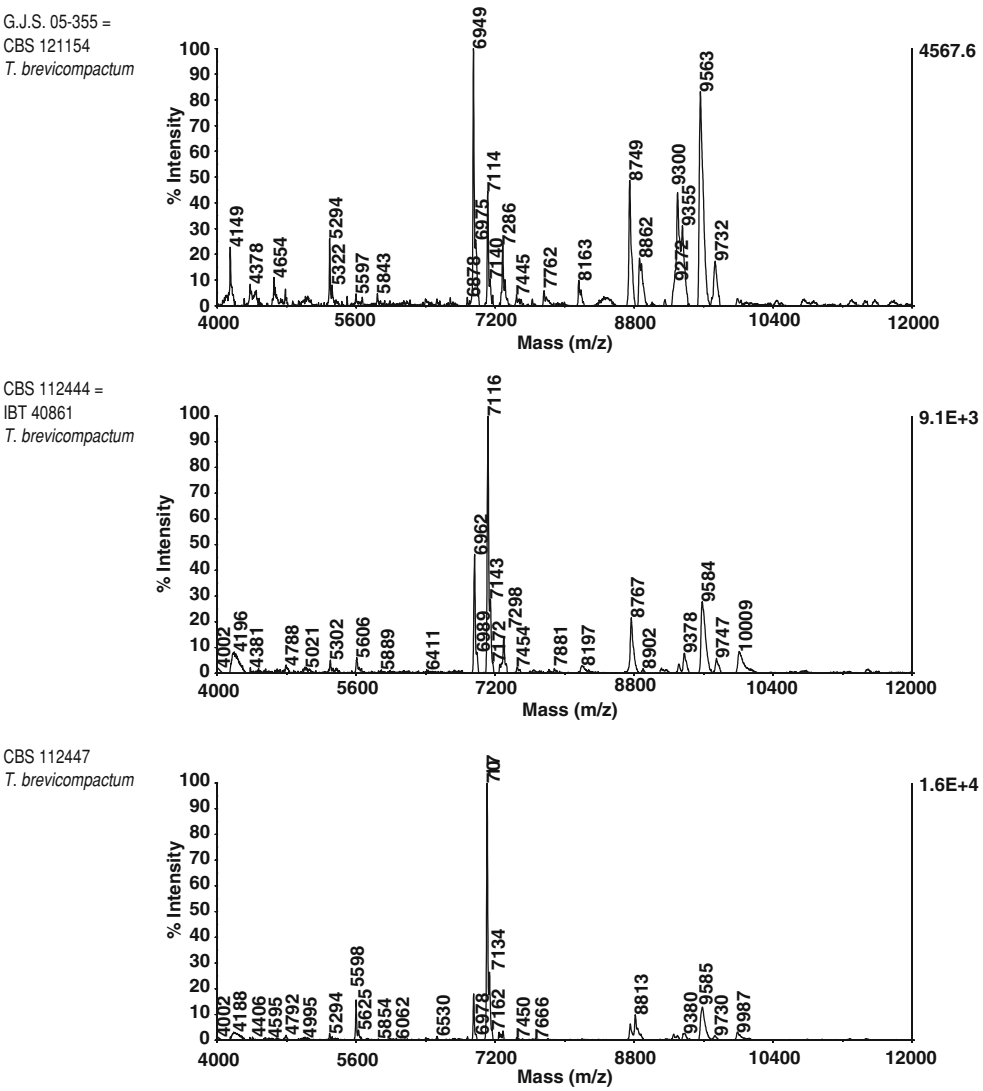


and 10h). These conidiophores resemble *T. harzianum* (Bissett 1991).

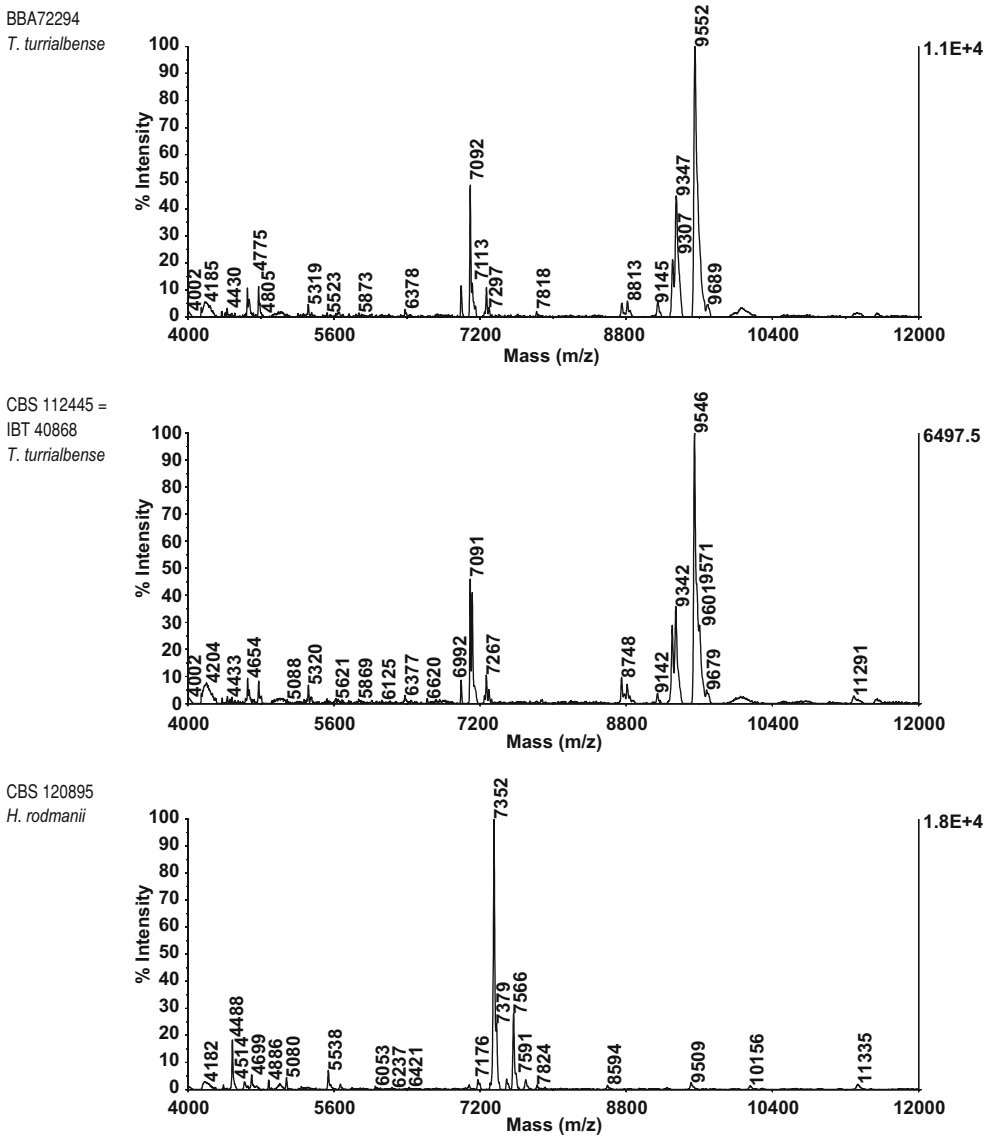
Hypocrea rodmanii is separated from the Brevicompactum complex by its slower rate of growth, smaller conidia and phialides, the production of trichokonins, hyporodicins, and hypocompactins. Unlike all members of the Brevicompactum clade, it does not produce alamethicins or trichothecene-type toxins. The small green, globose conidia combine with projecting, partially sterile conidiophores to distinguish this species from all known *Hypocrea* and *Trichoderma* species.

Members of the Brevicompactum complex are not easily separated on the basis of phenotype (Fig. 6; continuous characters are summarized in Table 8).

Trichoderma protrudens is distinguished by its longer phialides, its high growth rate at 37°C, production of harzianum A, and its unique pattern of hydrophobins. *Trichoderma turrialbense* is very closely related to *T. brevicompactum*; we distinguish these species mainly on the basis of production of trichodermin by *T. brevicompactum* and harzianum A by *T. turrialbense. Trichoderma arundinaceum* is distinguished from *T. brevicompactum* by the production of harzianum A and by the lack of a synanamorph in CMD cultures of the former (see below). Overall, the phialides of *T. protrudens* are longer and narrower than in all other species treated, with a consequently larger L/W ratio. The cells that produce phialides are narrowest in *T. protrudens*, 2.5–3.0 µm, as compared to 3–4 µm in the other species. The phialides of *H. rodmanii* are shortest.

No microscopic characters reliably and practically separate *T. brevicompactum, T. turrialbense* and *T. arundinaceum*. Small, but consistent differences in conidial sizes, especially when seen at the 95% confidence intervals and growth rates can be seen in Table 8 and Fig. 5. There is a very slight tendency for conidia of *T. arundinaceum* to be longer and wider than in the other species. *Trichoderma arundinaceum* and *T. brevicompactum* have a larger l/w ratio while the

Fig. 3 (continued)



l/w ratio of conidia of *H. rodmanii* and *T. turrialbense* is smaller; the l/w ratio of conidia of *T. prodrudens* is intermediate between the two groups (Fig. 5). *Trichoderma turrialbense* differs from both *T. brevicompactum* and *T. arundinaceum* in having a slightly faster rate of growth at 25 and 30°C on SNA. The formation of, especially, different peptaibiotics in the respective species (trichocryptins in *T. brevicompactum/T. turrialbense* vs trichobrevins in *T. arundinaceum/T. protrudens*), supports a distinction between *T. brevicompactum* and *T. arundinaceum.*

Chemotaxonomic data separate three clusters, *T. protrudens* (a), *T. arundinaceum* (b) and *T. brevicompactum* (c1)/ *T. turrialbense* (c2) due to the presence of one unique non-ribosomal peptide synthtase (NRPS) gene for trichoferin, and high-level expression of trichobrevins. All three groups may produce alamethicins. Trichocryptins A are characteristic for (c1). Major amounts of trichobrevins are found only in (a) and (b), whereas CBS 112154 is the only isolate in (c) to produce traces of trichobrevins. Trichocryptins B are found in (c) only. The structural data of the compounds does not reflect their phylogenetic difference. A comparison of a single domain of all respective alamethicin synthetases would provide this information.

It is thus not possible to clearly differentiate clusters *T. protrudens* from *T. arundinaceum*, or *T. turrialbense* from *T. brevicompactum* by their metabolite profiles, as there is just a single compound, either harzianum A or an unknown peptaibol besides 4 or 5 others, and it might well be that, e.g., new strains of *T. protrudens* producing trichodermin will be discovered. On the other hand, it is very clearly possible to separate *T. protrudens/T. arundinaceum* (a, b) from *T. brevicompactum/T. turrialbense* (c) due to different

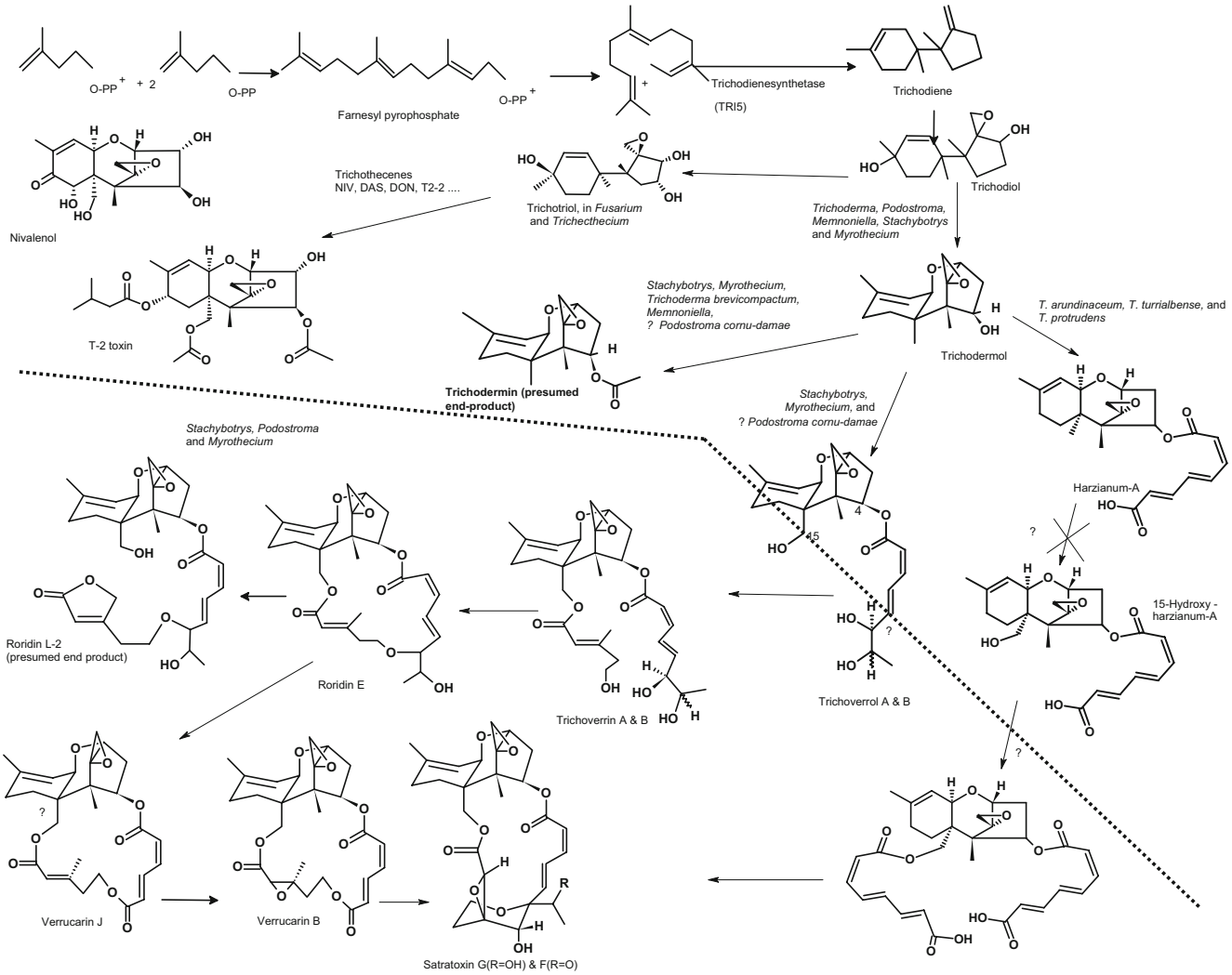


Fig. 4 Overview of putative pathways of trichothecene biosynthesis. Trichodiol is an important branching point with one path leading to trichotriol (T-2 toxin of *Fusarium*), and one leading to trichodermol. Trichodermol is the point of divergence for the simple trichothecene-type toxins trichodermin and harzianum A of *Trichoderma*/*Hypocrea*, and for the macrocyclic trichothecenes of *Myrothecium* and *Stachybotrys*. Because *Trichoderma* lacks the enzyme needed to hydroxylate the 15-position, the second anchor required to form the macrocyclic ring is missing, and either harzianum A or trichodermin are the end products. The identity of the fungus reported as *Podostroma cornu-damae* cannot be confirmed; see the discussion in the text. Simple trichothecene-type toxins are shown above the *dotted line*, and macrocyclic trichothecenes are below the *line*

pattern of trichobrevins (low concentration in c) and trichocryptins B (high concentration in c, trace amounts in *T. arundinaceum* CBS 119576).

The pattern of peptaibiotics of the two strains of *T. turrialbense* is similar but not identical. Both strains produce alamethicins as the major group of peptaibiotics. Trichobrevins are present in only small amounts, if at all, a fact that clearly distinguishes these two strains from *T. arundinaceum*. The major difference between CBS 112445 and CBS 122554 is the presence of 11- and 12-residue trichocryptins C and D in the latter. Trichocryptins C and D terminate in L-alaninol (Alaol), whereas we find Vxxol or Lxxol in trichocryptins A and B. So far, CBS 122554 is the only strain in which we have unambiguously found *C*-terminal Alaol and also the first report of Alaol as a *C*-terminal constituent of peptaibiotics. We did not find Alaol at the *C*-terminus of CBS 112445 trichocryptins. However, that difference is more interesting from a biochemical point of view. The building scheme of all trichcryptins A, B, C, and D is the same, and differences may be explained by positional isomerism.

Discussion

Analyses of partial sequences of *rpb2* indicate that *Trichoderma brevicompactum* represents a separate lineage in *Trichoderma*/*Hypocrea*, which we refer to here as the Brevicompactum clade. Kraus et al. (2004) suggested the

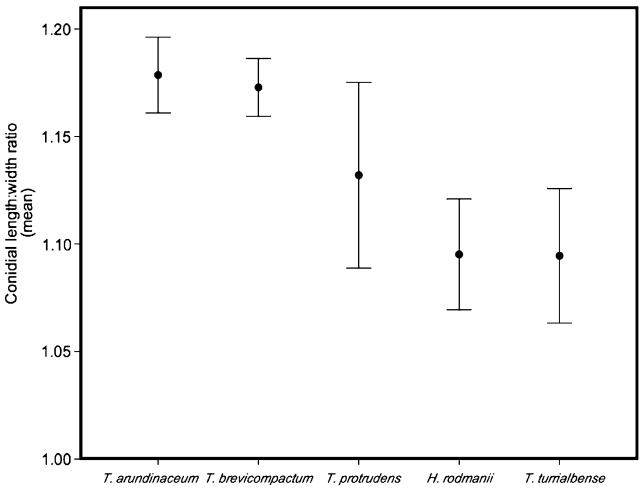


Fig. 5 95% confidence intervals of conidium length/width ratios

phylogenetic distinctiveness of the single species, *T. brevicompactum.* As can be seen from Fig. 1, the Lutea clade is the closest relative of the Brevicompactum clade when branch lengths are considered; this confirms the findings of Kraus et al. (2004).

Additional collections emphasize the two lineages that were seen in the original description of *T. brevicompactum* (Kraus et al. 2004). However, increased sampling has added one new *Hypocrea*, an endophyte in a trunk of cacao in India, and additional *Trichoderma* cultures from diverse geographic sources. The combination of sequences of *tef1, rbp2* and ITS with phenotype data derived from micromorphology, colony morphology and secondary metabolites has led us to recognize five species, of which four are new. Members of the Brevicompactum clade are notable for their subglobose conidia. The only three other species that have smooth, subglobose conidia are *T. harzianum* and *T. aggressivum* (Harzianum clade) and *T. atroviride* (Viride clade); these species are not closely related to each other or to *T. brevicompactum*.

The pattern of peptaibiotics produced by *H. rodmanii* does not identify it as a close relative of *T. brevicompactum* (see Fig. 6c), and it does not produce the trichothecenes or other metabolites that are unique to the Brevicompactum complex. Thus, the apparent relationship of *H. rodmanii* to other members of the clade could be a sampling artefact. With the discovery of additional taxa, the relationship between *H. rodmanii* and members of the Brevicompactum compex may change.

Kraus et al. (2004) cannot be criticized for having recognized only one species. Although they found that *T.* brevicompactum could be distinguished from *H. lutea*, *T. virens* and *T. harzianum* on the basis of carbohydrate utilization, they found virtually no difference among their isolates of *T. brevicompactum.* Our own examination of the morphological and cultural characters of an expanded panel of isolates and analysis using multidimensional scaling revealed only subtle differences. However, the anomalous production of trichothecene-type toxins among the cultures identified by Kraus et al. (2004) as *T. brevicompactum* led us to search for additional phenotypic data from metabolites (peptaibiotics) and physical phenotype.

As studies of species rich genera such as *Trichoderma* expand and include increasing numbers of cultures, more phylogenetic diversity is revealed than can be accounted for by phenotype. In the current work, very small differences in characters such as rate of growth or length/width ratio of conidia are apomorphic for species and combine with metabolic characters in defining species. However 'small' these differences are, they are representative of the species diversity and are taxonomically significant, a point that was made by Hawksworth (2001) with regard to the true diversity of species of fungi.

Although no teleomorph has been discovered for any of the members of the Brevicompactum clade, except for *H. rodmanii*, the moderate diversity among isolates of *T. brevicompactum* suggest recombination. Part of the diversity of *T. brevicompactum* may be due to geographic diversity, as exemplified by CBS 121154 (Cameroon) and CBS 112443 (Papua New Guinea), whereas the remaining cultures are from more or less contiguous continental areas where one might expect genetic interchange. In contrast, *T. arundinaceum* is noteworthy for its apparent lack of phylogenetic diversity despite having essentially the same geographic distribution as most of the *T. brevicompactum* cultures, which suggests that *T. arundinaceum* is clonal. Despite strong morphological similarity and overlapping ranges, the phylogenetic analysis indicates considerable phylogenetic distance with no genetic interchange between the two species.

Kraus et al. (2004) were surprised that *T. brevicompactum* could be closely related to *H. lutea* because of the conspicuous differences in the respective conidiophores, 'pachybasium-like' in *T. brevicompactum* and 'gliocladium-like' in *H. lutea* and its sister species, *H. melanomagna*. However, the morphology of the gliocladium-like anamorphs of *H. lutea* (*Gliocladium viride)*, and *H. melanomagna* is so unusual in the genus as to give no clues about the relationships of these species (see Domsch et al. 2007). No anamorph in *Hypocrea* and no species of *Trichoderma* is strictly similar to *G. viride* in anything other than wet, green pigment and phylogeny. However, gliocladium- or verticillium-like anamorphs occur in unrelated positions of *Trichoderma* either as the primary anamorphs (*T. virens*, *H. gelatinosa*, *T. crassum, H. nigrovirens*) or as synanamorphs of more typical *Trichoderma* species that produce primary conidia in pustules (Chaverri and Samuels 2003). Kraus et al. (2004) did not describe gliocladium- or verticillium-like

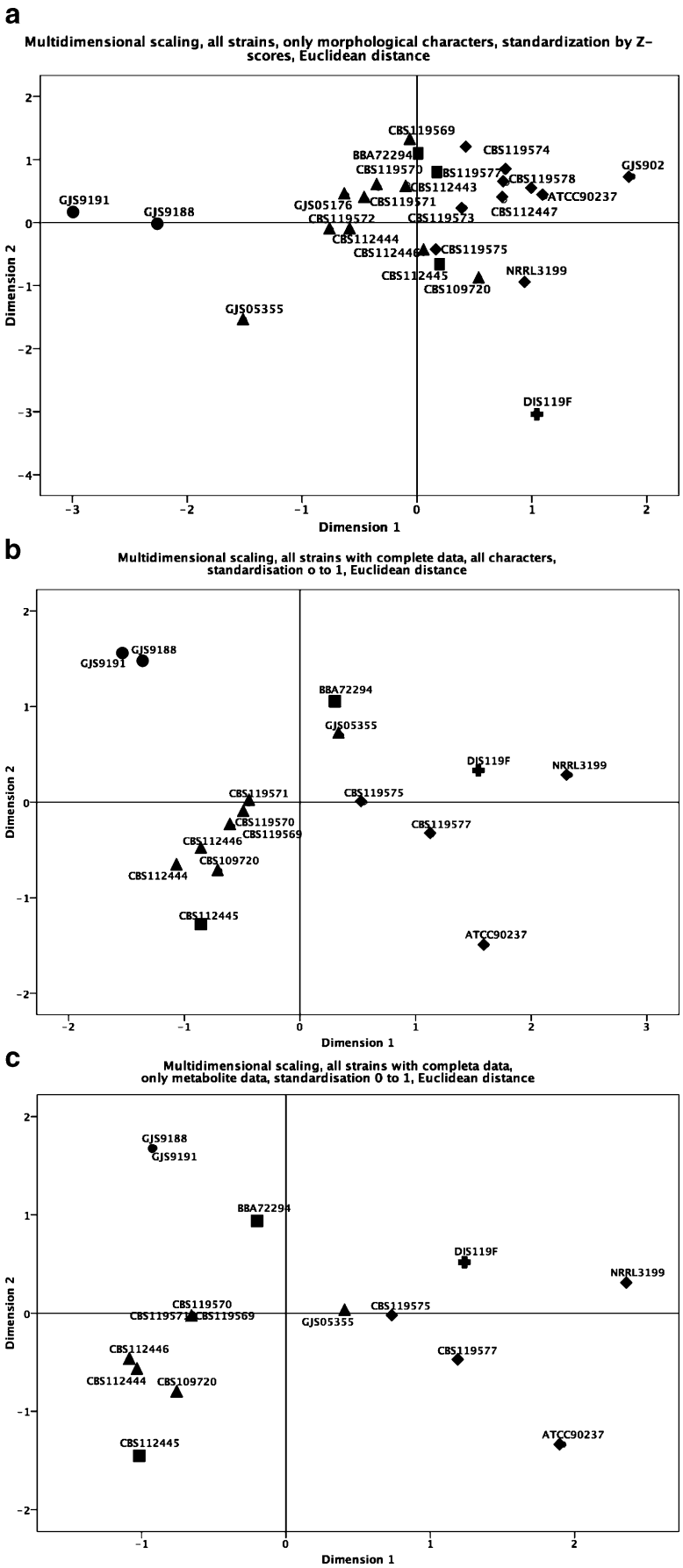


Fig. 6 Results of multidimensional scaling. **a** Morphological characters alone. **b** Combined morphological and metabolic characters. **c** Metabolic characters alone. The following strains lacked metabolic data and thus were excluded from B and C: G.J.S. 05–182, G.J.S. 05–183, G.J.S. 05–184, CBS 112443, G.J.S. 05–178, G.J.S. 05–174, G.J.S. 04–380, G.J.S. 05–176. ♦=*T. arundinaceum,* ▲=*T. brevicompactum,*✚=*T. protrusum,* ●=*H. rodmanii,* ■=*T. turrialbense*

conidiophores for *T. brevicompactum*, but the formation of these conidiophores may depend upon the medium that is used. We did not observe them on PDA and only rarely on SNA whereas on CMD the synanamorph is abundantly formed in all but *T. arundinaceum*. Possibly in *H. lutea* and *T. virens,* where the primary conidiophore is gliocladium-like, the 'true' *Trichoderma* morph has been lost leaving only the synanamorph.

The culture CBS 121320, *T. protrudens,* an endophyte from the trunk of a cacao (*Theobroma cacao*) tree, occupied a unique lineage within the Brevicompactum complex in the phylogenetic analysis (Figs. 1 and 2a–c). In other cases, endophytic *Trichoderma* species are represented by single cultures that occupy unique lineages, sometimes basal to more complex species (e.g., DIS 328gi, G.J.S. 04–40 basal to *T. viride*; Jaklitsch et al. 2006; Hanada et al. 2008) or in the case of *T. koningiopsis* endophytic isolates formed solitary internal lineages or endophyte-pure internal lineages (Samuels et al. 2006). The endophytic habit seems to have limited the ability of the strains living within plant tissue to exchange genetic material with 'freeliving' strains. Perhaps living as an endophyte has compromised their ability to survive in what could be a much more demanding environment outside of the host plant.

The Brevicompactum clade is characterized by the production of simple trichothecene-type mycotoxins. With the exception of *H. rodmanii*, all members of the clade produce either trichodermin or harzianum A. Various species of *Trichoderma* are reported to produce trichothecenes (Nielsen et al. 2005). Corley et al. (1994) characterized harzianum A from a culture that was identified as *T. harzianum* ATCC 90237, which we have re-identified here as *T. arundinaceum.* Lee et al. (2005) reported production of harzianum A by a strain (F000527 = CBS 113214) isolated directly from a soil sample collected in Daejeon, Korea, and identified by DNA sequences as a *Hypocrea* sp. Recently, a new trichothecene homologue, harzianum B, was isolated from the same strain (Jin et al. 2007). We received this strain too late to include in our phylogenetic analysis, but we have identified it also as *T. arundinaceum.* The culture NRRL 3199, a patent strain of unknown provenance identified as *T. viride,* was also shown to produce harzianum A and was re-identified here as *T. arundinaceum*.

A few studies have claimed production of simple trichothecene-type toxins by species of *Trichoderma* (Adams and Hanson 1972; Bamburg and Strong 1969; Watts et al. 1988; Cvetnić and Pepelnjak 1997; see also Sivasithamparam and Ghisalberti 1998 for a review), but in most of the studies, nonspecific analytical methods such as TLC and HPLC were used and no details of taxonomic identification were given. Of these reports, we have only been able to examine the *T. polysporum* strain (IMI 40624) reported by Adams and Hanson (1972). Although we can confirm that it is correctly identified, it did not produce trichothecenes on any of the numerous media that we tested. In contrast to these simple trichothecenes, macrocyclic trichothecenes have been reported from a rare, deadly poisonous, East Asian species, *Podostroma cornu-damae* (Saikawa et al. 2001). However, we cannot confirm the identity of the specimen used by these authors as apparently neither a voucher specimen nor culture were deposited. The Japanese cultures, NBRC 9005 (*P. cornu-damae*) and NBRC 9523 (*P. giganteum*), both of unknown provenances, produce macrocyclic trichothecenes (K.F. Nielsen, unpublished data). The respective ITS and *tef1* of these two cultures are identical, suggesting that there has been contamination of one culture by the other. The sequences place the cultures in the Viride clade (Fig. 1) (K.F. Nielsen and G.J. Samuels, unpublished data). We cannot confirm the production of simple, non-macrocyclic trichothecene mycotoxins, including T-2, by any species of *Trichoderma*/*Hypocrea* outside of the Brevicompactum clade, and the phylogenetic results presented here show that this clade is phylogenetically distant from any species that are used in biological control. In a survey of *Trichoderma* species from all known clades, we have not encountered macrocyclic trichothecenes using a LC-MS approach (K.F. Nielsen, unpublished data.). Despite the production of alamethicins by members of the *T. brevicompactum* clade (Degenkolb et al. 2006a), which could enhance their abilities as biocontrol agents (Corley et al. 1994), the production of trichothecene-type toxins could limit their practical application in integrated crop management schemes.

Recently, Favilla et al. (2006) and Poirier et al. (2007) claimed that the 20-residue peptaibols alamethicin and paracelsin, the 16-residue antiamoebin and other 11-residue trichobrachins (Mohamed-Benkada et al. 2006; Ruiz et al. 2007) were highly toxic in three in vitro invertebrate models, viz. *Crassostrea gigas*, *Artemia salina* and *Daphnia magna*. An alternative explanation of the toxicitiy reported by Favilla et al. (2006) and Poirier et al. (2007) is that the batch of the alamethicin standard that they used (Sigma-Aldrich; product number A-4665) was contaminated with the trichothecene harzianum A. In our own work, we have found that this particular Sigma alamethicin contains harzianum A; and this group of trichothecene-type toxins is highly toxic to *Artemia salina* (K.F. Nielsen, unpublished results). Notably, it took 40 years to recognize that *Trichoderma "viride"* NRRL 3199, which now has been re-identified here as T. *arundinaceum*, also produces harzianum A. Thus, alamethicin samples and standards tested in toxicity assays prior to 2005 could very likely have given false positive results based upon contamination of the samples by trichothecene-type mycotoxins (Degenkolb et al. 2008a). There are no good GLP studies of the toxicity of trichodermin and harzianum A. Trichodermin inhibits the

chain elongation of protein synthesis by binding to the peptidyltransferase (Gilly et al. 1985), whereas the much more potent T-2 toxin from *Fusarium* inhibits initiation of protein synthesis (Liao et al. 1976). There are contradictory results regarding the toxicity of harzianum A: Corley et al. (1994) observed no cytotoxicity in hamster kidney cells whereas Lee et al. (2005) reported this simple trichothecene to be highly toxic to several cell lines.

Harzianum A is especially interesting when considered in light of production of the highly toxic (>10 times more than T-2 toxin) macrocyclic trichothecenes by other hypocrealean fungi, including *Stachybotrys*, *Myrothecium*, and *Podostroma cornu-damae* (syn. *Hypocrea cornu-damae*) noted above (Saikawa et al. 2001). Harzianum A may be a missing link between the simple (trichodermin, T-2 toxin, etc.) and macrocyclic trichothecenes: the missing hydroxyl group on the 15-carbon atom in harzianum A would provide the possibility of attaching an additional octa-(*2Z,4E,6E*)-trienedioic acid (or a related compound) on the C-15. The two arms can then condense to a form a macrocyclic ring (Fig. 4).

Production of isocoumarins by all strains of the Brevicompactum clade, except *H. rodmanii,* (including diaportinol and diaportinic acid) is interesting because this requires the expression of genes for polyketides. However, production of these metabolites is not restricted to the Brevicompactum complex as it is detected throughout *Trichoderma* (K.F. Nielsen, unpublished data) as well as in, e.g., *Penicillium nalgiovense*. Quantitatively, isocoumarins seem to vary considerably from time to time during long-term growth, especially in potato-dextrose broth.

Peptaibiotics are characteristic linear or cyclie α-aminoisobutyrate-containing peptides that are produced mainly by species of *Trichoderma*/*Hypocrea* (for a review, see Degenkolb et al. 2003, 2007, 2008a, b). The Brevicompactum clade is a rich source of new peptaibiotics. Degenkolb et al. (2006a) discovered that 69 of the 75 peptaibiotics produced by species that we describe here as *T. brevicompactum*, *T. arundinaceum* and *T. turrialbense* were new to science. All peptaibiotics consecutively assigned as compounds [**80**]–[**117**] in this study also represent new compounds. All members of the Brevicompactum complex produced the 7-residue trichocompactins (TCP) or 7-residue hypocompactins (HCP). Similar 7- or 8-residue peptaibiotics, mostly lipopeptaibols, were also found in *T. aggressivum* f. *europaeum* CBS 100526, *H. dichromospora* CBS 337.69 (Krause et al. 2006), and *T.* cf. *strigosum* (Degenkolb et al. 2006b); they represent deletion sequences of 10-, 11- or, 12-residue lipopeptaibols. This subgroup of peptaibiotics is biosynthesized by species from different sections and clades of *Trichoderma*; thus its chemotaxonomic relevance is low (Degenkolb et al. 2006b).

Alamethicins and alamethicin-like peptaibols are not restricted to the Brevicompactum complex. They have been found throughout *Trichoderma*, with very similar atroviridins (Oh et al. 2002) and polysporins (New et al. 1996). The respective synthetases can be classified by specificities of their adenylate domains in positions 6, 9, 12, 17 and 20. Most analyses have been carried out with *T. arundinaceum* NRRL 3199, and the alamethicins of the F30/50 type (Kirschbaum et al. 2003) have previously been found in strains CBS 109720, IBT 40839, IBT 40840, CBS 112445, ATCC 90237, and IBT 40863 (Degenkolb et al. 2006a). These represent a special type of synthetase with module 6 preferring Ala against Aib, module 9 strictly Val, module 12 strictly Leu, module 17 preferring Aib against Vxx, and module 20 strictly Pheol.

Hydrophobins are small hydrophobic proteins presumably ubiquitous in filamentous fungi. They are usually secreted and processed, being components of the outer surfaces of walls of hyphae and conidia. Besides their roles in cell wall structure they may mediate interactions between the fungus and the environment such as surface recognition during pathogenic interaction with plants, insects or other fungi, but also in symbiosis. A hydrophobin gene found in a biocontrol strain of *T. asperellum* (T203, Viride clade) enabled the root attachment and colonization that are steps in initiation of a resistance reaction in host the plant to a parasite (Viterbo and Chet 2006). The size of hydrophobins ranges from approximately 75 to 400 amino acid residues containing eight positionally conserved cysteine residues; they can be divided into two classes according to their hydropathy profiles and spacing between the conserved cysteins (Linder et al. 2005). The number of hydrophobin genes detected in fungal genomes may exceed 10, and expression varies with physiological conditions. We here investigated sporulating mycelium under conditions previously applied for class II hydrophobins (Neuhof et al. 2007a). The unique hydrophobin patterns of *H. rodmanii, T. protrudens and T. arundinaceum* identify these as discrete species, while *T. brevicompactum and T. turrialbense* show partially overlapping sets of protein masses. For a review of Class II hydrophobin gene families in *Trichoderma*, see Kubicek et al. (2008).

In a recent intact cell mass spectroscopy (ICMS) study of 32 strains of 29 different species of *Trichoderma/Hypocrea*, hydrophobin patterns specific both at the species and isolate (subspecies) level were observed (Neuhof et al. 2007b). Two to four marker masses of hydrophobins were evaluated for each species. In 21 cases, single masses are shared within experimental errors by two species. A comparison of *H. atroviridis/T. atroviride* mycelium and conidia showed two identical masses. An evaluation of 3

strains of *H. jecorina/T. reesei* revealed three identical masses in one case besides three differing masses, and five differing masses in another case. These differing masses have been attributed in two cases to different post-translational processing of the hydrophobins Hfb1 and Hfb2.

In the case of *T. brevicompactum and T. turrialbense*, the two dominating mass peaks differ, supporting individual species. However, two significant masses of the Brevicompactum group and one mass of the Turrialbense group have also been detected as minor mass peaks in each group respectively. Two additional minor mass peaks are shared as well. These apparent similarities need to be resolved at the sequence level.

Taxonomy

For continuous characters, see Table 8.

1. *Trichoderma arundinaceum* Zafari, Gräf. & Samuels, sp. nov. Figures 7a–c and 8.

Trichodermati brevicompacto G. F. Kraus, C. P. Kubicek & W. Gams simile sed harzianum A produens et in agaro dicto SNA magis celeriter crescens. Conidia subglobosa vel ovoidea, (2.2−)2.7–3.5(−5.0) × (1.7−)2.5–3.0(−3.5) µm.

Teleomorph None known.

Characteristics in culture Optimum temperature for growth on PDA and SNA 30°C. Barely growing at 37°C on PDA or SNA. Colonies grown on PDA sometimes sporulating at 37°C in intermittent light after 72–96 h. On PDA after 5 days at 25°C under intermittent producing conidia in 3 or 4 conspicuous concentric rings, colony margin deeply scalloped. Conidia on PDA yellowish green (K&W 28–30 D-F 8). No pigment diffusing through the agar; no distinctive odour. Colonies grown on SNA in intermittent light forming conidia within 72 (96) h at (25) 30°C. On SNA after 1 week under intermittent light conidia forming abundantly around the colony margin in a broad band, most typically as a continuous lawn, sometimes as discrete, 1.5 mm diam, flat pustules, yellow rarely noted in developing conidia, conidia similar in color to conidia formed on PDA and CMD. Colonies grown on CMD at 25°C under light (12 h cool white fluorescent/12 h darkness) >9 cm diam within one week, conidia green, forming in pustules mainly around the colony margin and to a lesser extent in concentric rings behind the colony margin, similar to the pustules formed on SNA. Pustules formed on CMD and SNA flat, ca. 1 mm diam, remaining discrete or becoming confluent, dense, spiky or papillate from projecting, terminally fertile conidiophores and wet masses of conidia at the base of the projecting conidiophores. Conidiophores on CMD and SNA comprising a broad, more or less conspicuous central axis with lateral and often terminal fertile branches; fertile primary branches profuse, separated by short internodes, typically paired, arising at or near right angles with respect to the main axis, typically increasing in length with distance from the tip; primary branches typically more than 2 cells in length; secondary branches arising at right angles from primary branches, often comprising a single, broad cell; phialides arising in clusters of 3–5 at tips of primary and secondary branches; often a single branch of a conidiophore extending beyond the surface of the pustule, branched or unbranched, straight, smooth, septate, bearing a verticil of a few lageniform phialides at the tip. Phialides slightly enlarged in the middle and lageniform when arising on widely spaced fertile branches or shorter and wider, ampulliform, when arising from crowded fertile branches. Conidia subglobose to ovoidal, (2.2−)2.7–3.5(−5.0) × (1.7−)2.5–3.0(−3.5) µm, smooth, green. Synanamorph not observed on CMD. Chlamydospores not observed on CMD after 1 week at 25°C, intermittent light.

Etymology From Latin '*arundo*,' a reed or cane or anything made of a reed or cane such as a fishing rod, in reference to the long conidiophores that project from the pustules.

Habitat Soil.

Known distribution Iran, Namibia, United States (Miss.).

Holotype Iran: Hamadan, from soil (BPI 878405 ex CBS 119575; ex-type culture CBS 119575).

Additional cultures see Table 1.

2. *Trichoderma brevicompactum* G.F. Kraus, C.P. Kubicek & W. Gams, Mycologia 96: 1063. 2004. Figures 7d–f and 9

Telemorph None known.

Characteristics in culture Optimum temperature for growth on PDA and SNA 30°C. Slowly growing at 37°C, sometimes sporulating on PDA and SNA after 96 h at 37°C. On PDA after 1 week at 25°C under intermittent light producing conidia in several closely spaced, broad, concentric rings; colony margin entire. Conidia on PDA yellowish green (K&W 28–30 D-F 8). No pigment diffusing through the agar; no distinctive odor. Colonies grown on SNA in intermittent light forming conidia within

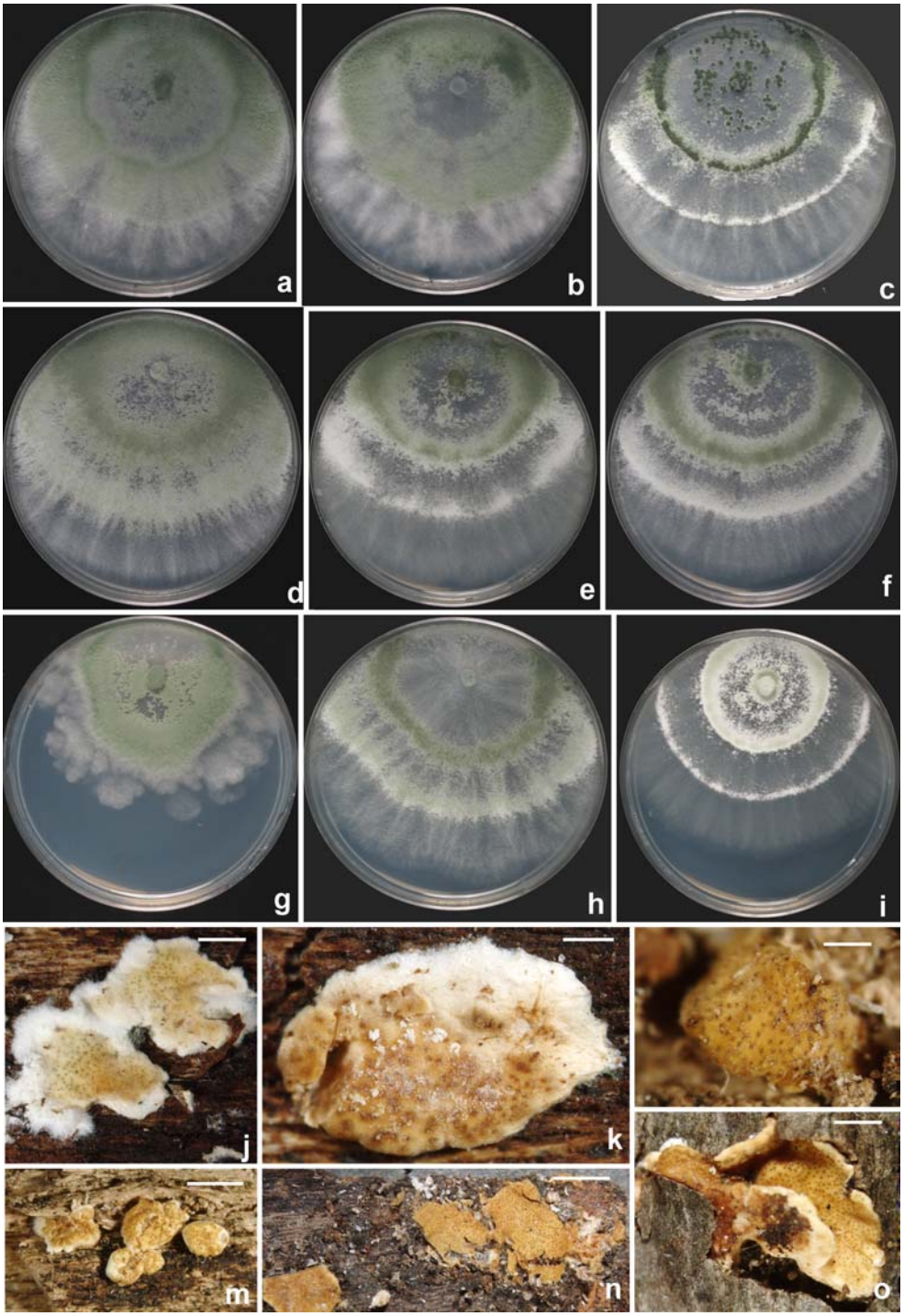


Fig. 7 **a–i** Cultures of *Trichoderma* species and *Hypocrea rodmanii* on PDA 96 h, intermittent light, in 9-cm-diam Petri dishes. **a–c** *T. arundinaceum*. **a** ATCC 90237. **b** NRRL 3199. **c** CBS 119578. **d–f** *T. brevicompactum*. **d** CBS 112444. **e** G.J.S. 04–380. **f** IBT 40841. **g** *T. protrudens*. CBS 121320. **h** *T. turrialbense* CBS 112445. **i** *H. rodmanii* CBS 120895. **j–o** *H. rodmanii*, immature (**j**) and mature (**k–o**) stromata. **j, k** from CBS 122581; **l** from CBS 120897; **m** from G.J.S. 91–90; **n** from CBS 122582; **o** from CBS 1208979. *Scale bars*: **j, k, l, o**=0.5 mm; **m, n**=1 mm

72–96 h at (25) 30°C. On SNA after 1 week under intermittent light conidia forming abundantly around the colony margin and in obscure concentric rings between the margin and the inoculum, rings formed of confluent pustules, complete or broken, conidia at first yellow then similar in color to conidia formed on PDA and CMD grown on SNA in intermittent light forming conidia within 72–96 h at (25) 30°C. On SNA after 1 week under intermittent light conidia forming mainly around the colony margin and to a lesser extent in concentric rings behind the colony margin, similar to the pustules formed on SNA. Pustules formed on CMD and SNA flat, to 2 mm diam, remaining discrete or becoming confluent, tending to be loosely organized, conidial masses appearing to be moist; numerous conspicuous white, usually

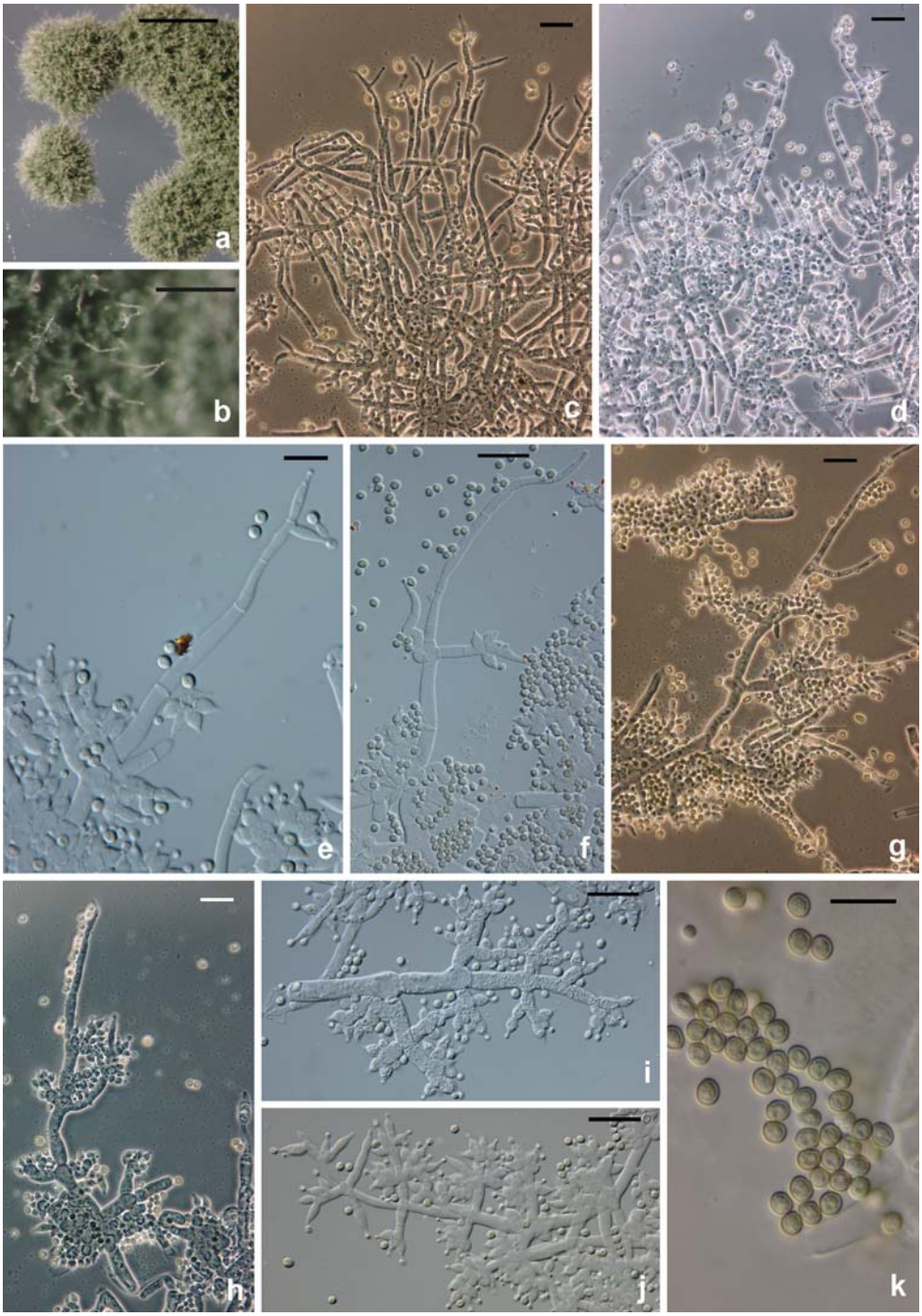


Fig. 8 *Trichoderma arundinaceum*. **a, b** Pustules. Protruding conidiophores seen in **b**. **a** on SNA, **b** on CMD. **c**–**j** Conidiophores. **c, e, f, g, j** from SNA; **d, h, i** from CMD. **k** Conidia, from SNA. **c** = CBS 121153, **d** = CBS 119573, **e, f, j, k** = CBS 119575, **i** = ATCC 90237. *Scale bars*: **a** = 1 mm, **b**=250 μm, **c**–**k**=10 μm

branched, terminally fertile conidiophores protruding from easch pustule. Conidiophores on CMD and SNA comprising a more or less distinct, broad central axis with fertile branches; fertile branches profuse, separated by short internodes, typically paired, arising at or near right angles with respect to the main axis, typically increasing in length with distance from the tip; primary branches often comprising a single, broad cell producing phialides in a dense terminal cluster, or primary branches multicellular and narrower, producing phialides at the tip and rebranching to produce secondary branches terminating in phialides; often a single branch of a conidiophore extending beyond the surface of the pustule, straight, septate, smooth, unbranched or sparingly branched with long internodes between branches, each branch terminating in a verticil of 1–3 phialides or the terminal phialides held in an appressed penicillus of 3–5 on a broad cell.

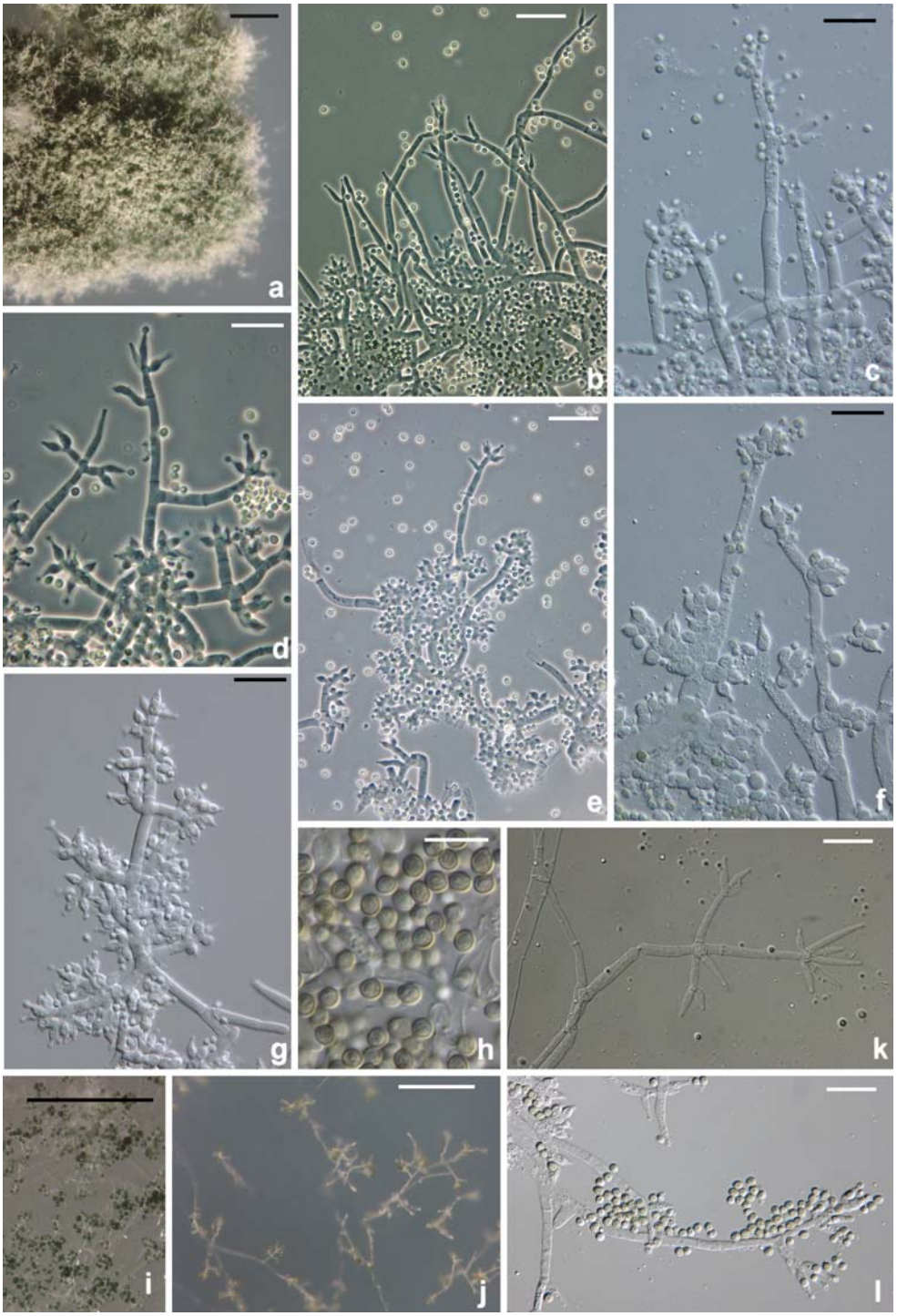


Fig. 9 *Trichoderma brevicompactum*. **a** Pustule on SNA. **b–g** Conidiophores taken from pustules. b, d from SNA: c, e-g from CMD. h. Conidia from SNA. **i-l**. Synanamorph on CMD. **a, e** from G.J.S. 04–380, **b** from CBS 109720, **c** from CBS 119570; **d** from CBS 119572, **f** from CBS 119569, **g, j, k** from CBS 112446, **h** from CBS 121154, **i** from CBS 112443, **l** from CBS 112447. *Scale bars*: **a**=0.5 mm, **b–h, k, l**=10 um; **i**=1 mm, **j**=150 μm

Phialides slightly enlarged in the middle and lageniform when arising on widely spaced fertile branches; shorter and wider, ampulliform, when arising from crowded fertile branches. Conidia subglobose, (2.2−)2.7–3.0(−3.7) × (2.0−)2.2–2.7 (−3.0) μm smooth, yellowish green in mass. Synanamorph forming on CMD, verticillium- or gliocladium-like, single conidiophores arising from agar surface and the scant aerial hyphae, conidia held in wet, green heads. Chlamydospores produced only in few cultures on CMD after 1 week at 25°C under intermittent light, terminal, subglobose, (5.7) 8.0–11.0 (13.5) × (5.0) 6.5–9.2 (10.2) μm.

Habitat Soil.

Known distribution St. Vincent and the Grenadines, Caribbean Region (Union Island), India, Iran, Papua-New

Guinea, Peru, United States (N.Y., Wis.), México, Costa Rica, Iran, Colombia. Probably cosmopolitan.

Holotype UNITED STATES, New York, Geneva, New York State Agricultural Experimental Station, isolated from soil in a sunflower field, 20 June 2000, *S. Petzolt & G.E. Harman* (DAOM 231232! Ex-type culture CBS 109720).

Additional cultures examined See Table 1.

3. *Trichoderma protrudens* Samuels & Chaverri, sp. nov. Figures 7g and 10.

Trichodermati arundinaceo Zafari, Gräfenhan & Samuels simile sed phialides longiores et angustiores et in agaro dicto PDA temperatura 37°C magis celeriter crescens. Conidia subglobosa vel ovoidea, (2.5−)2.7–3.2 (−3.5) × (2.2−)2.5–2.7(−3.0) µm.

Teleomorph None known.

Characteristics in culture Optimum temperature for growth on PDA and SNA 30°C; some growth visible at 35°C after 72 h on PDA and SNA. Colonies grown on PDA in intermittent light forming conidia within 72 h at 30°C, on

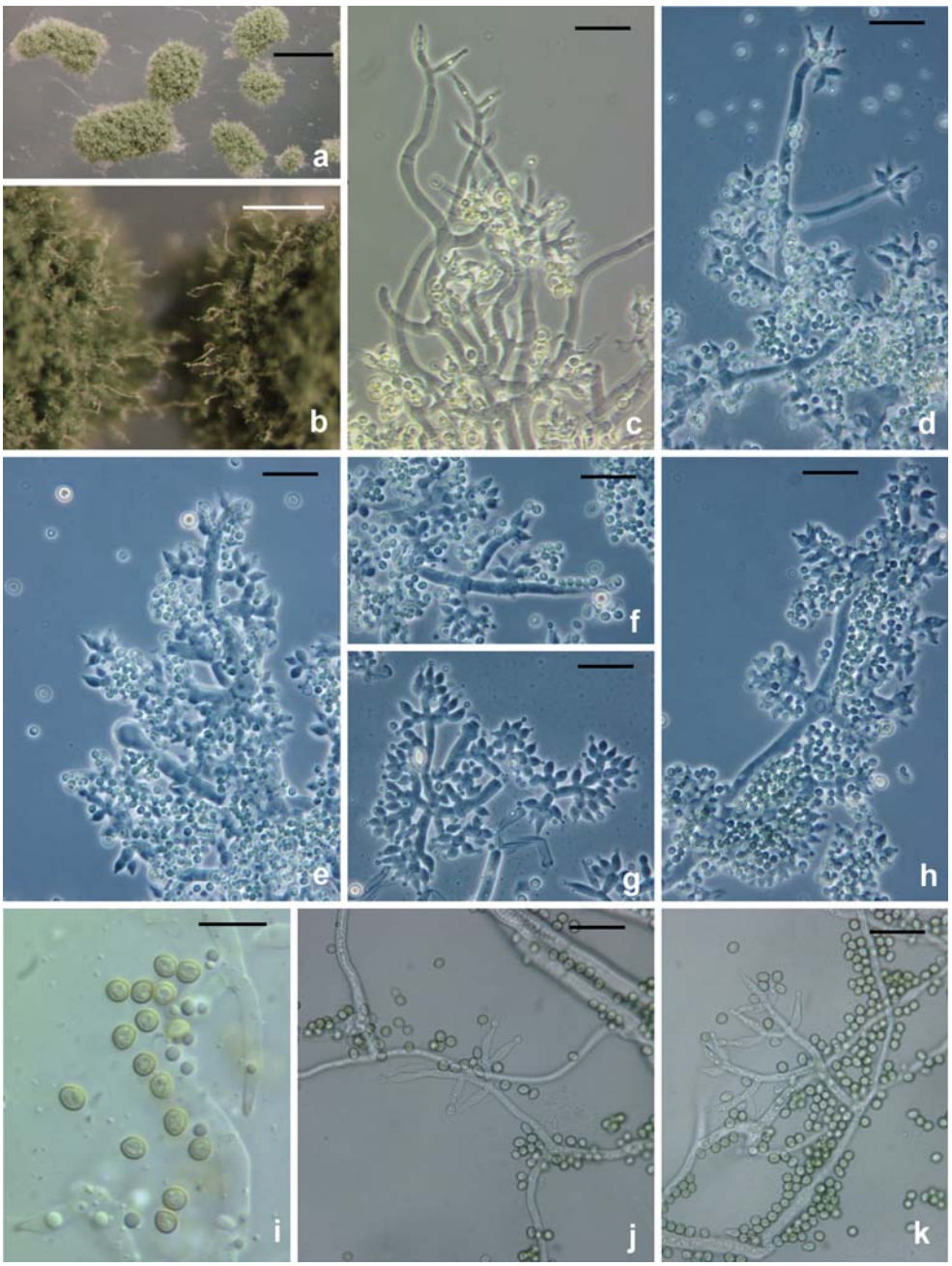


Fig. 10 *Trichoderma protrudens*. **a, b** Pustules. Protruding conidiophores seen in **b**. **a, b** from SNA. **c**–**h** Conidiophores. **c** from CMD; **d, e, f** from SNA; **g, h** from CMD. **i** Conidia, from CMD. **j, k** Synanamorph on CMD. All from CBS 121320. *Scale bars*: **a**=1 mm, **b**=150 µm, **c**–**k**=10 µm

SNA within 96 h at 30°C; sporulating after 72 h in intermittent light on PDA, not sporulating on SNA at 37°C. On PDA after 1 week at 25°C under intermittent light producing conidia in 2 or 3 rather conspicuous concentric rings of coalescing, flat pustules; colony margin scalloped. Conidia on PDA grayish green to dark green (K&W 27 C-F 7). No pigment diffusing through the agar; no distinctive odor. On SNA and CMD after 1 week under intermittent light conidia forming abundantly in the aerial mycelium around the margin of the colony and in discrete, 1–2 mm diam, pustules; pustules more abundant on SNA than on CMD, pustules on SNA formed behind the margin, on CMD in a marginal band; conidia yellowish green (K&W 29 F 8). Pustules on CMD and SNA pulvinate to hemispherical, very compact, conidial masses appearing to be moist; conspicuous long, white, terminally fertile conidiophores arising from each pustule. Conidiophores on CMD and SNA comprising a rather distinct, broad central axis with lateral and often terminal fertile branches; fertile branches profuse, separated by short internodes, typically paired, arising at or near right angles with respect to the main axis, typically increasing in length with distance from the tip; primary branches often comprising a single, broad, cell producing phialides in a dense terminal cluster, or primary branches multicellular and narrower, producing phialides at the tip and rebranching to produce secondary branches with another cluster of phialides; often a single branch of a conidiophore extending beyond the surface of the pustule, straight, septate, smooth, unbranched or sparingly branched, with long internodes between branches, each branch terminating in a verticil of a few phialides. Phialides slightly enlarged in the middle and lageniform when arising on widely spaced fertile branches or shorter and wider, ampulliform, when arising from crowded fertile branches. Synanamorph abundant in the aerial mycelium, verticillium-like, conidia held in wet, green heads. Conidia subglobose to ovoidal, (2.5−)2.7–3.2(−3.5) × (2.2−)2.5–2.7 (−3.0) μm, smooth, deep green in mass. Chlamydospores not observed.

Etymology '*protrudens*' refers to the protruding conidiophores that arise from pustules.

Habitat Isolated as an endophyte from trunk of *Theobroma cacao*.

Known distribution India (Kerala), known only from the type collection.

Holotype INDIA, Kerala, Kannara, Plantation Crops Research Institute, isolated from trunk of 8–10-m-tall tree of *Theobroma cacao,* 5 Nov 1999, *H. C. Evans* CBS 121320 (BPI 878378; ex-type culture CBS 121320).

4. *Hypocrea rodmanii* Samuels & Chaverri, sp. nov. Figures 7j–o, 11 and 12

Stromata lutea, KOH-, 2–3 × 1–2 mm. Ascosporae hyalinae, spinulosae. Pars distalis ascosporarum subglobosa vel globosa, (3.0−)3.2−4.0(−4.5) × (2.7−)3.2–3.7(−4.2) μm; pars proximalis cuneiformis vel oblonga, (3.0−)4.5−4.5 (−7.0) × (2.2−)2.7−3.2(−3.7) μm. Anamorphosis *T. brevicompacto* G. F. Kraus, C. P. Kubicek & W. Gams similis. Conidia subglobosa, (1.7−)2.0–2.5(−2.7) × (2.0−)2.2–2.7(−3.0) μm.

Stromata superficial, sometimes forming below bark, at first semi-effused, light yellow (K&W 4A4-6) with darker ostiola and a white margin, becoming pulvinate, 2–3 × 1–2 mm, ca. 1.5 mm high, darker yellow (4B7–8, 5B6–7), not reacting to KOH, broadly attached with edges slightly free, surface plane, perithecial elevations not evident, ostiola appearing as darker, viscid dots. Cells of the stroma surface in face view angular, ca. 4.5 × 3.7 μm, thin-walled. Surface region of stroma 15–20 μm wide, not pigmented, cells in section *textura epidermoidea*, 2.5–4.5 × 2.5–3.0 μm, walls slightly thickened. Tissue below stroma surface region of intertwined, ca. 2.5 μm wide, thin-walled hyphae. Perithecia elliptic in section, 215–270 μm tall, 150–250 μm diam, ostiolar canal 60–70 μm long (n = 3), perithecial apex not protruding through the stroma surface, cells not distinct from cells of the surrounding stroma surface. Tissue below perithecia *textura epidermoidea*, 8–24 × 6–10 μm, thin-walled. Asci cylindrical, (60) 70–87 (110) × (3.0) 4.0–5.5 (6.5) μm, apex with a shallow ring, 8-spored. Part-ascospores hyaline, finely spinulose, dimorphic; distal part-ascospores subglobose to globose, (3.0) 3.2–4.0 (4.5) × (2.7) 3.2–3.7 (4.2) μm; proximal part-ascospores wedge-shaped to oblong, (3.0) 4.5–4.5 (7.0) × (2.2) 2.7–3.2 (3.7) μm.

Characteristics in culture and anamorph Optimum temperature for growth on PDA 25–30°C and on SNA 20–30°C, not growing at 37°C. Colonies grown on PDA in intermittent light forming conidia within 48 h at 30°C; after 4 days in intermittent light conidial production in 3 or 4 conspicuous, narrow concentric rings of densely aggregated conidia. Conidia on PDA grayish green (K&W 27–28B-C4-6). No pigment diffusing through the agar; no distinctive odor. Conidia not forming on SNA within one week in intermittent light. Colonies grown on CMD at 25°C intermittent light >9 cm diam within one week, conidia forming in a broad ring around the margin in confluent grayish green, cottony pustules; long, sterile hairs arising from the pustules, sometimes hairs producing one phialide at the tip. Conidiophores on CMD comprising a ca. 5-μm-wide main axis from which fertile branches arise near the base; the terminal part of the conidiophore (to 100 μm) septate, straight, thin-walled; apex blunt, sterile or bearing a single,

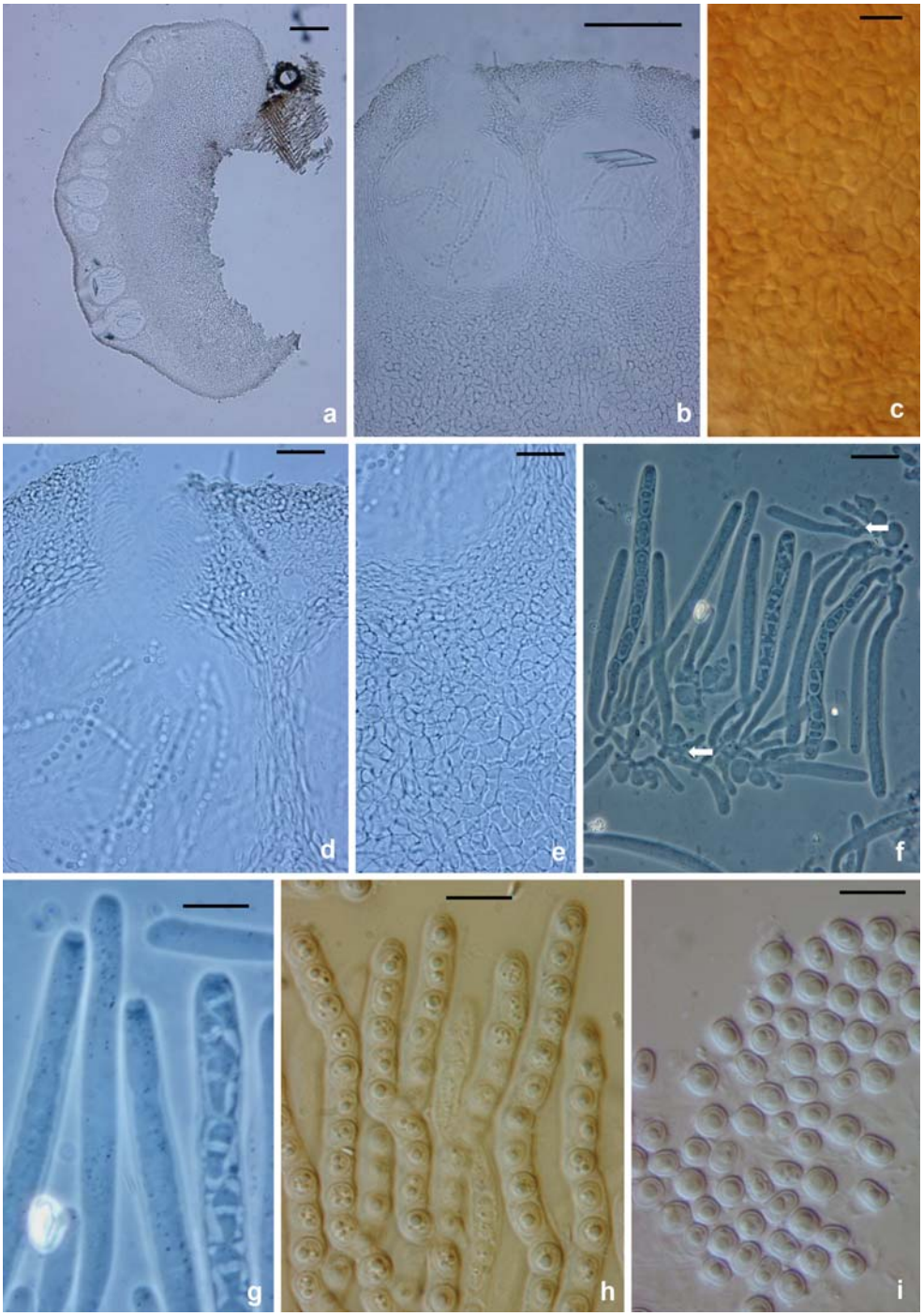


Fig. 11 *Hypocrea rodmanii*, teleomorph. **a** Longitudinal section through a stroma. **b** Median longitudinal section through two perithecia. **c** Cells of the stroma surface in face view. **d** Median longitudinal section through a perithecium showing details of the stroma surface and ostiolar region. **e** Cells of the stroma below perithecia. **f** Young asci showing croziers (*arrows*) and developing ascospores. **g, h** Asci. Apical rings visible in **g**. **i** Discharged ascospores. **a, b, d, e, i** from G.J.S. 91–90; **f, g** from G.J.S. 91–89; **c, h** from G.J.S. 91–88. *Scale bars*: **a, b**=100 μm; **c–f, g-i**=10 μm

terminal phialide. Branches arising at right angles to the main axis at or near 90°, often consisting of one or a few broad cells, or branches longer and of narrower cells; fertile branches often paired, mostly progressively longer with distance from the tip, often rebranching to produce secondary branches; secondary branches typically comprising a single, broad cell with a terminal cluster of phialides. Conidiophores often lacking a sterile elongation, internodes between secondary branches short and phialides densely disposed on short secondary branches. Phialides held in divergent heads of 3–5, ampulliform. Conidia subglobose, (1.7−)2.0–2.5(−2.7) × (2.0−)2.2–2.7(−3.0) μm, smooth, grayish green in mass. Synanamorph not observed. Chlamydospores not observed after 1 week on CMD under intermittent light.

Etymology Hypocrea rodmanii is named in honour of Dr. James E. Rodman, U.S. National Science Foundation, in

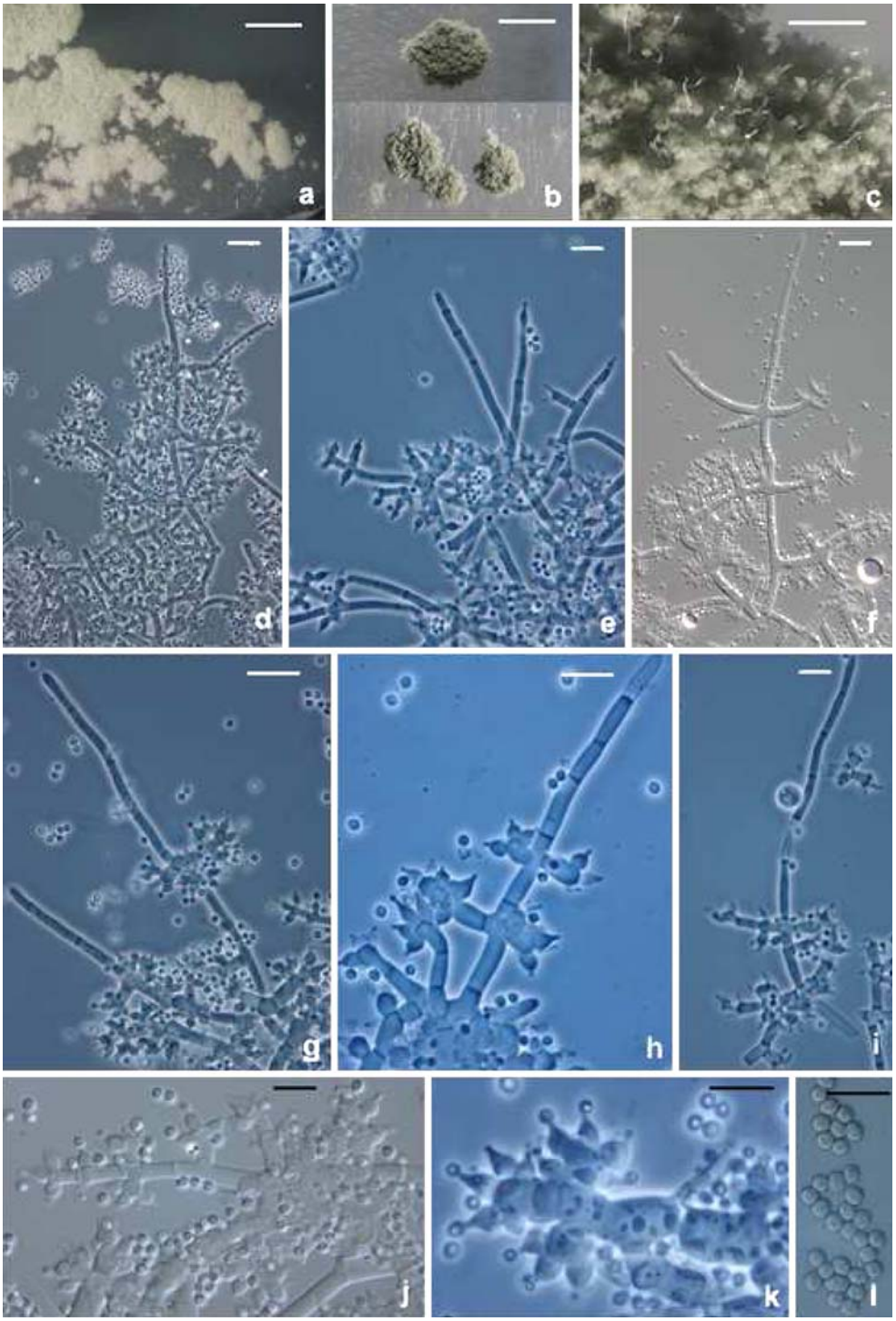


Fig. 12 *Hypocrea rodmanii*, *Trichoderma* anamorph. **a**–**c** Pustules from CMD. **d**–**k** Conidiophores, detail of phialides shown in **k**. **d, e, g–k** from CMD, **f** from SNA. **l** Conidia, from CMD. **a, c, f** from G.J.S. 91–89; **b, d, g, j** from G.J.S. 91–88; e, h, i, k, l from G.J.S. 91–91. *Scale bars*: a, b=1 μm, **c**=150 μm, **d–l**=10 μm

recognition of vision and tenacity in developing the NSF program Partnerships Enhancing Expertise in Taxonomy (PEET), which supported the authors of this species as, respectively, mentor and student.

Habitat On decorticated, rotten wood, often below flaking bark.

Known distribution United States (Maryland, Virginia).

Holotype UNITED STATES, Virginia, Giles County, Cascades Recreation Site, 4 mi N of Pembroke, along Little Stony Creek, 37°02′N, 80°35′W, elev. 838 m, 18 Sep 1991, on branchlets, *G.J. Samuels, C.T. Rogerson, S.M. Huhndorf, S. Rehner & M. Williams* (BPI 1112859, ex-type culture CBS 120895).

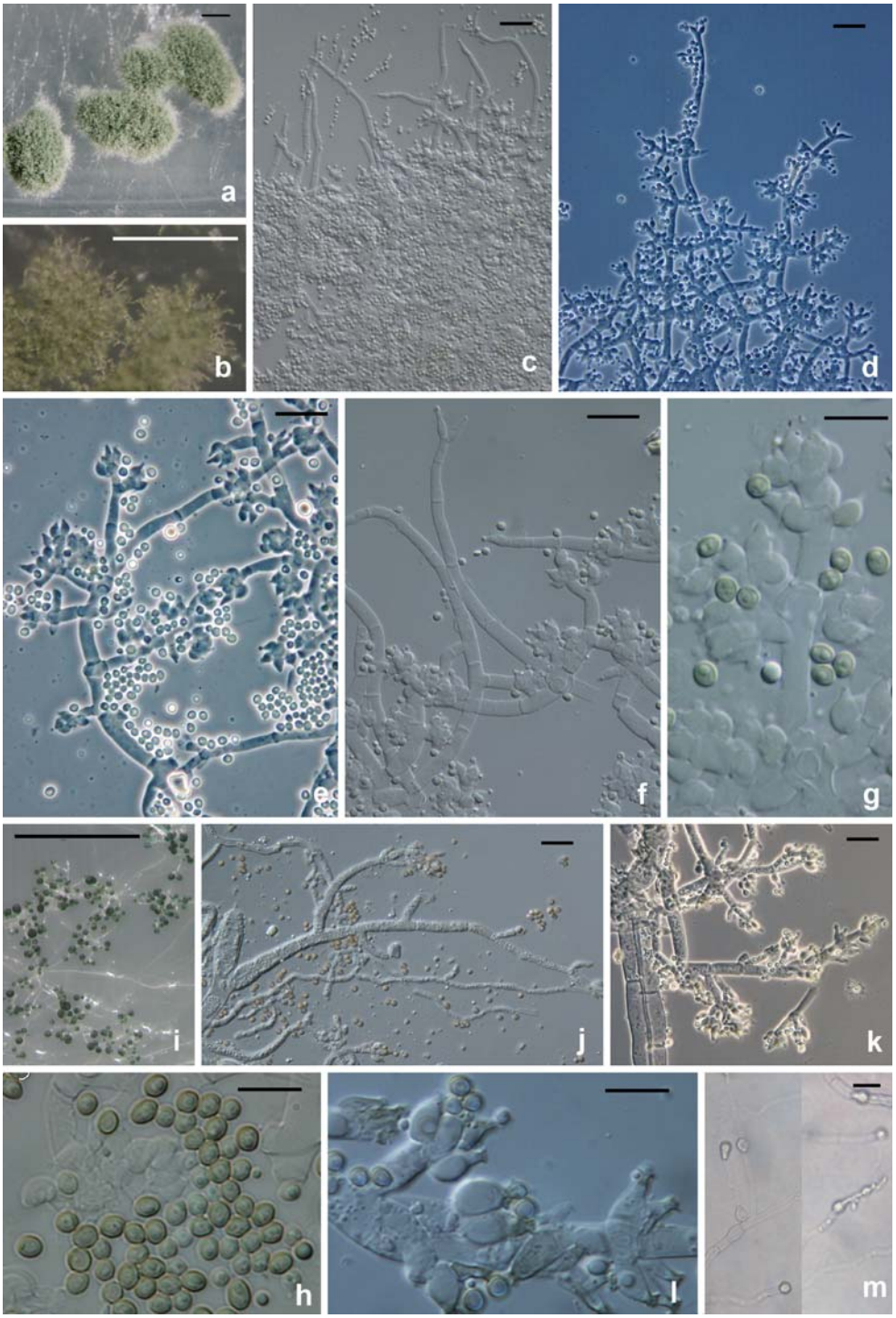


Fig. 13 *Trichoderma turrialbense.* **a, b** Pustules, protruding conidiophores visible in **b**. **a** from CMD, **b** from SNA. **c–g** Conidiophores. **c, d, e, f** from SNA; **g** from CMD. **h** Conidia from SNA. **i–l** Synanamorph on CMD. Details of phialides seen in **l**. **m** Chlamydospores from CMD. **a c, d, f, k, l** = GJS 07–74, **b, e, g, h, j, m** = CBS 112445. *Scale bars*: **a, b**=0.5 mm, **c–m**=10 μm

Additional specimens examined Three specimens with the same collecting data as the holotype (G.J.S. 91-90 = BPI 1112861; BPI 1112862, culture G.J.S. 91-91 = CBS 109719; BPI 1112860, culture G.J.S. 91-89 = CBS 120897; BPI 1112861). Maryland, Garrett County, 5 mi N of Barton, Little Savage River Ravine, on decorticated wood, 23 Sep 1989, *G.J. Samuels (89–120), C.T. Rogerson, W.R. Buck, R.C. Harris* (NY = CBS 122582); same collecting data, *G.J. Samuels (89–116)* (NY = CBS 122581).

Comments The form and pigmentation of the stroma of *H. rodmanii* suggest *H. lutea*, but the anamorph readily distinguishes this species from other known species.

5. *Trichoderma turrialbense* Samuels, Degenkolb, K.F. Nielsen & Gräf. sp. nov. Figures 7h and 13.

Trichodermati brevicompacto G. F. Kraus, C. P. Kubicek & W. Gams simile sed harzianum A produens. Conidia subglobosa, (2.2−)2.5–3.0(−3.2) × (2.0−)2.2–2.7 (−3.0) µm.

Teleomorph None known.

Characteristics in culture Optimum temperature for growth on PDA and SNA 30°C; very little growth at 37°C after 72 h. On PDA forming conidia under intermittent light within 72 h at 30°C, sometimes also sporulating on PDA at 37°C in intermittent light after 96 h. On PDA at 25°C after 5 days under intermittent light producing conidia in broad concentric rings, the oldest conidia in the center and the youngest at the edge of the colony; colony margin even. Conidia on PDA yellowish green (K&W 28–30 D-E 8). No pigment diffusing through the agar; no distinctive odor. Colonies grown on SNA under intermittent light forming conidia only sporadically after 72 h at 30°C. On CMD and SNA after 1 week at 25°C under 12 h cool white fluorescent light/12 h darkness conidia forming abundantly around the colony periphery in more or less conspicuous, compact flat pustules 0.5–1 mm diam, yellow green ('deep green,' K&W 29 E-F 8). Pustules compact or loosely arranged, conidiophores more or less extensively branched, often with long internodes between branches, with a conspicuous main axis; often producing one or a few divergent phialides from the tip; branches arising at right angles to the main axis, short, progressively longer with distance from the tip. Secondary branches arising at right angles from the primary branches, typically unicellular, each terminating in 2−5 divergent or convergent (gliocladium-like) phialides. Phialides ampulliform, shorter and broader when crowded. Conidia subglobose, (2.2−)2.5–3.0(−3.2) × (2.0−)2.2–2.7 (−3.0) µm, smooth, green, appearing to be held in globose wet heads. On CMD and SNA conidiophores of synanamorph arising from surface of agar and from aerial mycelium in abundance, 70–180 µm long, 5–14 µm wide at the base, lateral branches arising at an angle of < 90° with respect to the main axis, terminating in an appressed head of phialides or lower branches producing secondary branches with an appressed head of 2–5 phialides. Phialides ampulliform, 6–7 µm long, 3.0–3.5 µm wide at the widest point, collarette often flared. Chlamydospores not observed on CMD.

Etymology '*turrialbense'* refers to the town of Turrialba, Costa Rica, the only place where this species is known to occur.

Holotype Costa Rica, Turrialba, La Montaña, isolated from soil in a maize field, date not known, S. Danielsen 017, comm. M. Lübeck (BPI 878379, ex-type culture CBS 112445).

Additonal culture examined Costa Rica, Turrialba, isolated from banana roots, date unknown, A. zum Felde S14 comm. R. Sikora (BPI 878380; live culture BBA 72294= CBS 122554).

Comments The culture CBS 112445 was included in the original description of *T. brevicompactum,* where it was reported under the number '4105.' The main distinction between *T. turrialbense* and *T. brevicompactum* is that the main toxin of the former is harzianum A whereas the main toxin of the latter is trichodermin. As can be seen from Table 8, there are slight differences in conidial length, length/width of conidia, width of phialides and cells from which phialides arise. The peptaibiotics pattern of *T. turrialbense* is similar to that of *T. brevicompactum*.

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REVIEW

Natural Cyclopeptaibiotics and Related Cyclic Tetrapeptides: Structural Diversity and Future Prospects

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Linearity is not considered a prerequisite anymore, and extension of the current definition of 'peptaibiotics' to cyclic, Aib-containing peptides is proposed. Sequences and bioactivities, together with ecophysiological importance of cyclopeptaibiotics and related cyclic tetrapeptides, and their fungal-taxonomic relationships, are discussed.

Introduction. – Peptaibiotics constitute a constantly growing family of peptide antibiotics [1–3]. Currently, more than 800 individual sequences of peptaibiotics, produced by members of *ca.* 20 fungal genera, are reported in the literature. Peptaibiotics are defined as linear polypeptide antibiotics which *i*) have a molecular weight between 500 and 2,200 Dalton, thus containing 5–21 amino acid residues; *ii*) show a high content of α-aminoisobutyric acid (Aib); *iii*) are characterized by the presence of other non-proteinogenic amino acids and/or lipoamino acids; *iv*) possess an acylated N-terminus, and *v*) have a C-terminal residue that, in most of them, consists of a free or acetylated amide-bonded 1,2-amino alcohol, but might also be an amine, amide, free amino acid, 2,5-dioxopiperazine, or sugar alcohol. The majority of Aib-containing peptides carries a C-terminal residue representing a 2-amino alcohol, and this subgroup is, therefore, referred to as *peptaibols*. In strongly lipophilic peptaibols, the N-terminus is acylated by octanoic, decanoic, or (*Z*)-dec-4-enoic acid, and these are named *lipopeptaibols* [4]. In the third subfamily of *lipoaminopeptides* (also named aminolipopeptides) the N-terminus is substituted by unbranched, α- or γ-methyl branched, saturated or unsaturated C_4–C_{15} fatty acids. An L-proline-, *trans*-4-hydroxy-L-proline, or *cis*-4-methyl-L-proline residue is found in position 1 of the peptide chain, and, in most cases, it is followed by a lipoamino acid residue in position 2. To our present knowledge, the compound, 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD), has only been recorded from this subfamily. A fourth subfamily comprises all other peptaibiotics that cannot be classified in any of the other three preceding

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subfamilies [1]. A review introducing nine subfamilies according to structural homologies of peptaibiotics has been published [5], but these need to be revised and updated, because more than half of the currently known sequences of peptaibiotics have been discovered since then [2][3][6].

Peptaibiotics are usually classified according to their main chain length, as long-chain (17–21 residues), medium-chain (11–16 residues), and short-chain (5–10 residues) sequences. Although not yet reported in literature, *Brückner* and co-workers [1] claimed the detection of very short chain (<5 residues) peptaibiotics that may represent drastically truncated sequences.

Cyclopeptaibiotics – A New Subfamily of Peptaibiotics. – We do not consider linearity as a necessary prerequisite anymore and propose to extend the above definition of 'peptaibiotics' to encompass also cyclic, Aib-containing peptides. Currently, this small subgroup comprises seven cyclic tetrapeptides all of them displaying the same building scheme (*Fig. 1*).

Sequences and bioactivities, ecophysiological and taxonomic importance of cyclopeptaibiotics and related cyclic peptides will be discussed below. Finally, this review is aimed at linking the remarkable chemical and biological properties of linear and cyclic Aib-containing peptides.

Chlamydocin. – Originally isolated from the soil fungus *Diheterospora* [7] (now *Pochonia*) *chlamydosporia* (Clavicipitaceae, Hypocreales; teleomorph *Metacordyceps* (syn. *Cordyceps*) *chlamydosporia* [8]) S 3440, chlamydocin (**1**) is regarded as the classical paradigm of a 'cyclopeptaibiotic'. The chlamydocin-producing species is known as an egg parasite of cyst nematodes such as *Heterodera schachtii* in beet or root-knot nematodes of the genus *Meloidogyne*. As this fungus is still found to be genetically heterogeneous [8], some biochemical heterogeneity can be expected that might entail the production of structurally variable, microheterogeneous mixtures of cyclic peptides by different strains. To the best of our knowledge, chlamydocin (**1**) is the first example of a cyclic, Aib-containing peptide reported in literature. Chlamydocin (**1**) was sequenced after trifluoroacetolysis and subsequent *Edman* degradation; the configuration of the amino acids was determined by treatment of the HCl hydrolysate with L- and D-amino acid oxidase. Notably, its proline residue was assigned the D-configuration. In this context, it should be pointed out again that isovaline (Iva) is frequently reported as D- or L-isomer in peptaibiotics, whereas all other chiral α-amino acids of linear peptaibiotics known so far have been reported to possess L-configuration. Both enantiomers of Iva may even be present in the same peptaibiotic [1].

The uncommon lipoamino acid of chlamydocin (**1**) was assigned as L-2-amino-9,10-epoxy-8-oxodecanoic acid (Aoe). A minor component, **2**, carrying L-2-amino-9,10-epoxy-8-hydroxydecanoic acid (Ahe) was also detected instead of Aoe in the crude extract of *Pochonia chlamydosporia* S 3440 [7].

Based on structural homology, it was demonstrated that chlamydocin (**1**) displayed unspecific phytotoxic bioactivity [9], which is in agreement with its cytostatic activity in rodents [10]. Stereoselective total syntheses of chlamydocin (**1**) and dihydrochlamydocin have been reported [11].

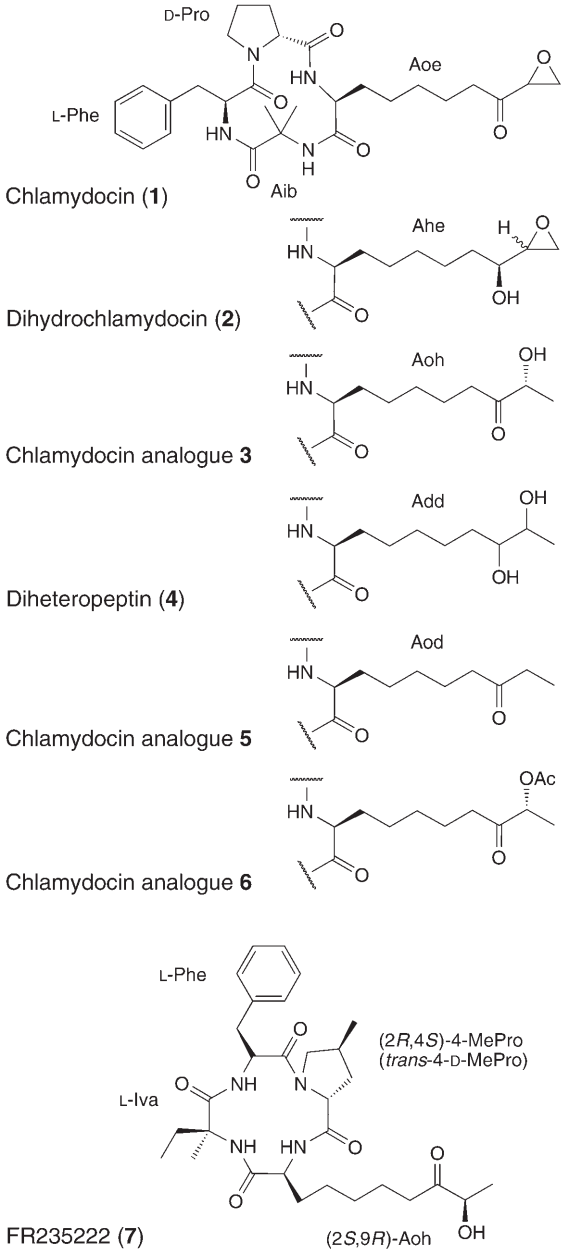


Fig. 1. *Structures of cyclotetrapeptaibiotics*

Chlamydocin Analogue from *Verticillium coccosporum*. – Twenty years after the first report on chlamydocin (**1**) had been published, a second cyclopeptaibiotic, **3**, was

isolated from a strain of '*Verticillium coccosporum*' (without accession number) obtained from the USDA-ARS collection of entomopathogenic fungi [12]. This isolate might be identical with *Pochonia suchlasporia* var. *catenata* CBS 789.85 – a strain that has originally been deposited as *Verticillium coccosporum*, subsequently preserved as *Verticillium suchlasporium* var. *catenatum*, and finally included in *Pochonia* [8]. The species *Pochonia suchlasporia* var. *catenata* is known as an egg parasite of the cereal cyst nematode *Heterodera avenae* and the Gypsy moth *Lymantria dispar*. The lipoamino acid of this analogue was assigned L-(9*S*)-2-amino-9-hydroxy-8-oxo-decanoic acid (Aoh). The analogue showed high phytotoxic activity against common Duckweed, *Lemna minor* [12].

Diheteropeptin. – In 1997, the isolation of diheteropeptin (**4**) from a *Diheterospora* sp. was reported [13]. The producing fungus was subsequently identified as *Diheterospora chlamydosporia* Q 58044 [14], thus representing the same species as the chlamydocin producer S 3440. Diheteropeptin (**4**) is distinguished from the above chlamydocin homologues by the presence of a (2*S*,8*R*,9*R*)-2-amino-8,9-dihydroxydecanoic acid (Add) residue [15]. The peptide inhibited histone deacetylase and mimicked transforming growth factor-β (TGF-β)-like activity in mink epithelial lung (M*v*1Lu) cells. It is known that TGF-β protects neuronal cells from damage caused by brain ischemia injury and *Alzheimer*'s disease. Thus, chlamydocin analogues were discussed as potential candidates for treatment of neuronal disorders [13][14]. An asymmetric total synthesis of diheteropeptin (**4**) has been elaborated [16].

Chlamydocin and Analogues from *Peniophora* cf. *nuda*. – Chlamydocin (**1**) and two new analogues, **5** and **6**, were recently isolated from a culture of *Peniophora* cf. *nuda* (Peniophoraceae, Russulales). The isolate was obtained from a soil sample collected in Tottori Prefecture, Japan [17]. The species is widely distributed and commonly associated with white rot of dead branches of woody angiosperms [18].

Electron microscopy revealed a dolipore-parenthesome septum, and sequencing of its 18S rRNA gene identified the fungus to be closely related to *Peniophora nuda* [17]. Notably, this is the first unequivocal report confirming the isolation of an Aib-containing peptide from a basidiomycete. Such a result is of chemotaxonomic importance, as all previous reports claiming the isolation of peptaibiotics from basidiomycetes [19–21] should be considered ambiguous, as they might have involved a mycoparasite of ascomycete affinity [1][22–24]. Most peptaibiotics reported to date were isolated from two families of the order Hypocreales – Hypocreaceae and Clavicipitaceae (*sensu lato*). Other Hypocrealean families have been positively screened for the presence of Aib and/or Iva, as recently reviewed [1], but, to the best of our knowledge, no basidiomycete taxa.

Two lipoamino acids, 2-amino-8-oxodecanoic acid (Aod) and its AcO derivative were found in the new chlamydocin analogues. Chlamydocin (**1**) and its two new analogues exhibited plant growth-retarding activity towards rice seedlings (*Oryza sativa* cv. 'Koshihikari'), a feature that is discussed to reduce lodging, permit higher nitrogen application rates, and increase yields. Plant growth-retarding activity was shown to be caused by decrease of the endogenous level of gibberellins (gibberellic acid 1 (GA_1)), along with a simultaneous increase of abscisic acid (ABA). Regulation of

these two plant hormones was postulated to be caused by inhibition of histone deacetylases (HDACs) that may differentially affect biosynthesis of GA_1 and ABA [25]. Similar plant growth retardation has previously reported for the HC-toxin (**12**; see the *Table* and *Fig. 3*) from *Cochliobolus carbonum*, the causing agent of Leaf Spot of Maize [26].

FR235222 – The First Iva-Containing Cyclotetrapeptaibiotic. – Isovaline (Iva) which is generally considered as the CH_2 homologue of Aib, has been reported from a number of linear peptaibiotics [1]. Notably, structure elucidation of the cyclotetrapeptide FR235222 (**7**), isolated from a culture of *Acremonium* sp. No. 27082 [27], revealed the presence of one L-Iva residue. The structure of FR235222 (**7**) as shown in *Fig. 1* was assigned cyclo(-(*2S,9R*)-2-amino-9-hydroxy-8-oxodecanoic acid-*trans*-4-D-MePro-L-Phe-L-Iva-) [28]. The producer was isolated from a soil sample in Aktia City, Japan, identified as *Acremonium* cf. *murorum* by its morphological characteristics, and finally deposited as *Acremonium* sp. FERRM BP-6539. The peptide displayed potent and selective inhibition of T-cell proliferation and lymphokine production. Furthermore, it exhibited potent inhibition of HDAC [29]. Total synthesis [30][31], NMR solution structure, and a binding model have recently been published [31].

Further Aib-Containing Cyclic Heptapeptides: Scytalidamides A and B. – Two cyclic heptapeptides (*Fig. 2*), scytalidamides A (**8**) and B (**9**) were reported from *Scytalidium* sp. CNC-310 [32]. The genus *Scytalidium*, however, is considered very heterogeneous with teleomorphs belonging to different ascomycetous genera.

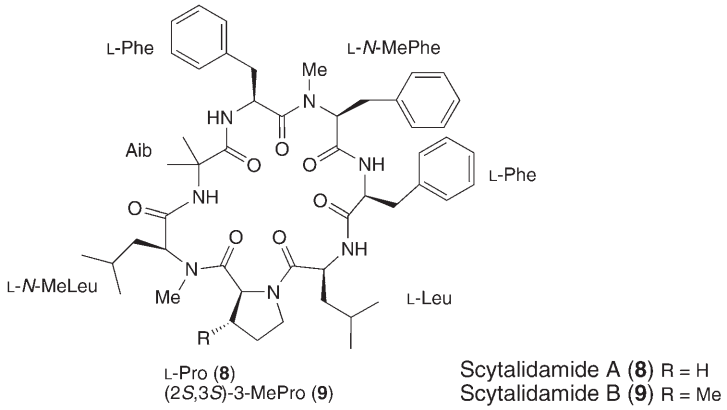


Fig. 2. *Structures of cycloheptapeptaibiotics, scytalidamides A* (**8**) *and B* (**9**)

The fungus was isolated from the surface of a sample of the green alga *Halimeda* sp. collected from a patch reef at a depth of 15 m from the northern end of Long Island, Bahamas. Thus, discovery of scytalidamides further extends the sources of Aib-containing cyclic peptides to marine habitats although the genus *Scytalidium* is generally known to be associated with terrestrial plants. Scytalidamides A and B (**8** and **9**, resp.) displayed moderate *in vitro* cytotoxicity towards HCT-116 human adenocarcinoma.

Both scytalidamides contain one Aib residue but display a building scheme different from the cyclopeptaibiotics and cyclic tetrapeptides mentioned above. The presence of further non-proteinogenic amino acids, however, supports a non-ribosomal biosynthesis: scytalidamide A (**8**) was assigned as cyclo(-L-Phe-L-*N*-MePhe-L-Phe-Aib-L-*N*-MeLeu-L-Pro-L-Leu-) and scytalidamide B (**9**) as cyclo(-L-Phe-L-*N*-MePhe-L-Phe-Aib-L-*N*-MeLeu-(*2S,3S*)-3-MePro-L-Leu-). For configuration analysis of the amino acids in the HCl hydrolysate, an advanced LC/MS method based on derivatization with L- or D-1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA or D-FDLA, a modified *Marfey*'s reagent) [33] was used. Preparative amounts of (*2S,3S*)-3-methylproline (MePro) were isolated and further characterized by NMR, HRFAB-MS, and LC/MS of its L- and D-FDLA derivatives. Total synthesis of scytalidamide A (**8**) has been reported in [34].

Aib-Free Cyclic Tetrapeptides. – A number of cyclic tetrapeptides lacking the Aib residue have been described. These Aib-free cyclic tetrapeptides containing D-Pro, D-pipecolic acid (Pip), and D-*O*-methyltyrosine (D-OMe-Tyr) are listed in the *Table*. The structures of the apicidins are shown in *Fig. 4*, those of the azumamides and microsporins are illustrated in *Fig. 5*, and those of the remaining Aib-free cyclic tetrapeptides are depicted in *Fig. 3*.

Notably, their producers are often known from highly competitive or specialized habitats. The producer of the phytotoxic peptides Cyl-1 (**10**) and Cyl-2 (**11**), *Cylindrocladium scoparium* [35], teleomorph *Calonectria morganii* [36], acts as a plant pathogen infecting a wide range of crop and ornamental plants [18][37], and the HC-toxin (**12**)-producing *Cochliobolus carbonum* is known as the causal agent of leaf spot of maize [9][41]. The two apicidin-producing *Fusarium* isolates were reported as endophytes from acacia and White Mangroves, respectively [46][49].

The configuration of the Phe residue of WF-3161 (**13**) was originally reported to be L [42], but subsequently corrected to D by 2D-NMR, and treatment of the HCl hydrolysate with D- and L-amino acid oxidase [43]. Finally, its total synthesis was described in [52].

Treatment with amino acid oxidase was also applied to the HCl hydrolysate of HC-toxin (**12**) [9] as well as Cyl-1 (**10**) [35], and Cyl-2 (**11**) [38], respectively.

For configuration analysis of the amino acids in the HCl hydrolysates of trapoxin A and B (**14** and **15**, resp.), HPLC on a chiral *Crown Pak CR* phase was used [44]. Crystalline trapoxin A (**14**) was investigated by X-ray-analysis [45]. Total synthesis of trapoxin B (**15**) and analogues has been reported in [53].

The configuration of the amino acids in the apicidins, **16**–**22**, was determined by 2D-NMR and amino acid oxidase [46][47].

The importance of D-amino acids has recently been compiled, including protocols for their analysis [54].

Can a Sponge Produce Cyclic Tetrapeptides? – Five cyclic tetrapeptides, azumamides A–E (**23**–**27**, resp.), were recently isolated from a frozen 2.2-kg sample of the marine sponge *Mycale izuensis*. They were shown to contain unusual lipoamino acids, namely (2*S*,3*R*)-3-amino-2-methylnon-5-enedioic acid 9-amide (Amnaa), or (2*S*,3*R*)-3-amino-2-methylnon-5-endioic acid (Amnda). Depending on the isomer,

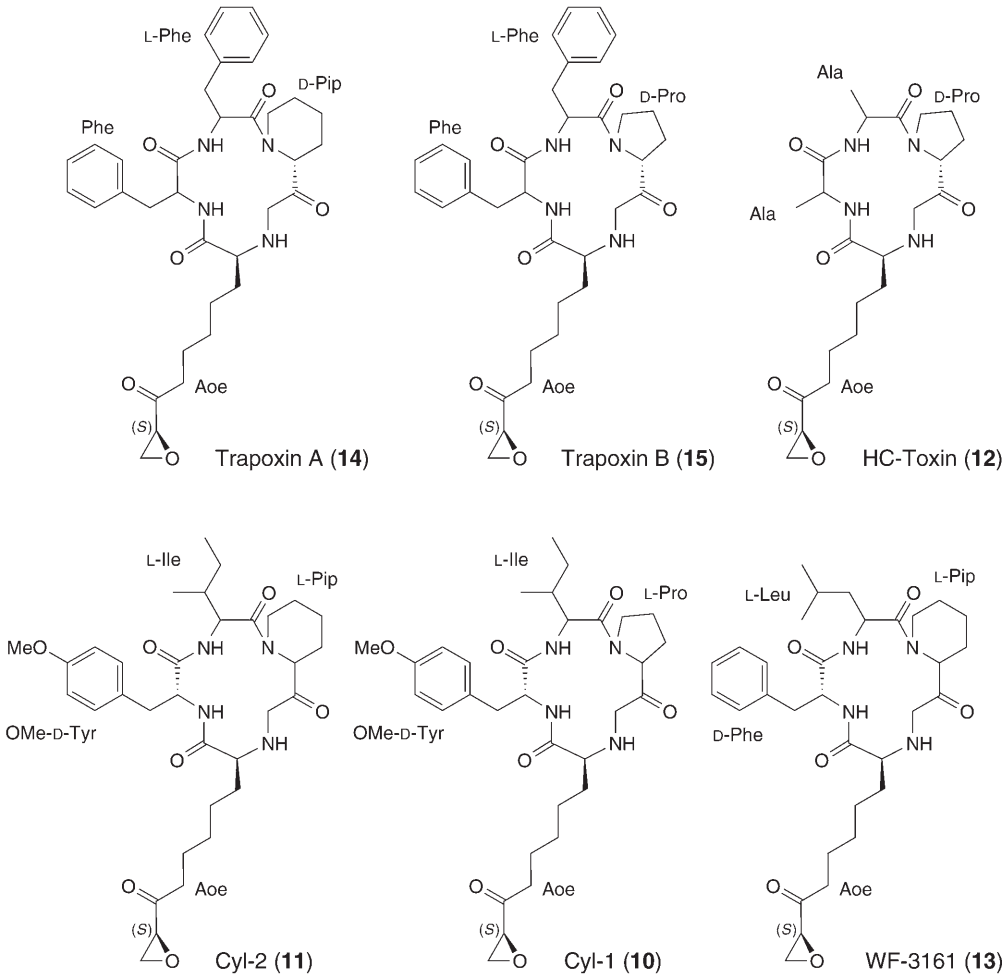


Fig. 3. *Structures of other, Aib-free cyclic tetrapeptides*

amino acid analysis revealed the presence of D-Val, D-Ala, D-Phe, and D-Tyr (*Fig. 5*). For amino acid analysis, *Marfey*'s reagent was used [50]. However, production of azumamides by a sponge, as reported, is highly questionable. Contrary to what has been stated in [50], we rather tend to assign the biosynthesis to a sponge-associated fungus whose presence has not been recognized in the material extracted.

This hypothesis is further supported by the recently reported isolation of microsporins A and B (**28** and **29**, resp.). These cyclic tetrapeptides were isolated from *Microsporum* cf. *gypseum* CNL-692. The fungus was found to be associated with the marine bryozoan *Bugula* sp. collected from the U.S. Virgin Islands [51]. The keratinophilic genus *Microsporum* (with teleomorphs in *Nannizzia*) has a worldwide distribution. Some species are known as geophilic saprotrophs, whereas others are

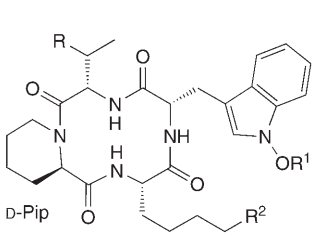
Table. *A Survey of Aib-Free Cyclic Tetrapeptides*

Cyclic tetrapeptide	Producing fungus		Geographic origin/Habitat	Sequence[a])
	Teleomorph	Anamorph (synonyms)		
Cyl-1 (**10**) [35]	*Calonectria morganii* [36] (Nectriaceae, Hypocreales)	*Cylindrocladium scoparium*	Pathogenic to a wide host range of crop plants, causing damping off, seedling root rot and seedling blight [18][37]	cyclo(-Aoe-Pro-Ile-OMe-D-Tyr-)
Cyl-2 (**11**) [37–40]				cyclo(-Aoe-Pip-Ile-OMe-D-Tyr-)
HC-toxin (**12**) [9][41]	*Cochliobolus carbonum*, race 1 (Pleosporaceae, Pleosporales)	*Bipolaris zeicola* (= *Drechslera zeicola* = *Helminthosporium carbonum*)	USA, from plants infected with leaf spot of maize (*Zea mays*)	cyclo(-Aoe-D-Pro-Ala-Ala-)
WF-3161 (**13**) [42][43]	*Petriella guttulata* No. 3161 (Microascaceae, Microascales)	*Sprothrix* sp., *Graphium* sp. (synanamorph)	Soil sample from Kamakura City, Kanagawa Prefecture, Japan.	cyclo(-L-Leu-L-Pip-L-Aoe-D-Phe-)
Trapoxins [44][45]	Not known	*Helicoma ambiens* RF-1023 (= FERM. BP-2751) (Tubeufiaceae, Pleosporales)	Not given	Trapoxin A (**14**) cyclo(-Aoe-D-Pip-Phe-Phe-) Trapoxin B (**15**) cyclo(-Aoe-D-Pro-Phe-Phe-)
Apicidins [46–48]	Not known	*Fusarium pallidoroseum* ATCC 74289 (= *Fusarium incarnatum?*)	Endophyte from branches of *Acacia* sp., Santa Rosa National Park, Guanacaste Province, Costa Rica [49]	Apicidin (**16**) cyclo(-Aod-D-Pip-Ile-OMe-Trp-) Apicidin A (**17**) cyclo(-Aod-D-Pip-Ile-Trp-) Apicidin B (**18**) cyclo(-Aod-D-Pro-Ile-OMe-Trp-)
		Fusarium sp. ATCC 74322 (Nectriaceae, Hypocreales)	Internal cortex of living roots of *Laguncularia racemosa*, Rincon River, Puntarenas Province, Costa Rica [49]	Apicidin C (**19**) cyclo(-Aod-D-Pip-Val-OMe-Trp-) Apicidin D_1 (**20**) cyclo(-Aoh-D-Pip-Ile-OMe-Trp-) Apicidin D_2 (**21**) cyclo(-Dod-D-Pip-Ile-OMe-Trp-) (Dod = 2-amino-8-dehydro-decanoic acid) Apicidin D_3 (**22**) cyclo(-Hdd-D-Pip-Ile-OMe-Trp-) (Hdd = 2-amino-8-deoxy-9-hydroxydecanoic acid)

Table (cont.)

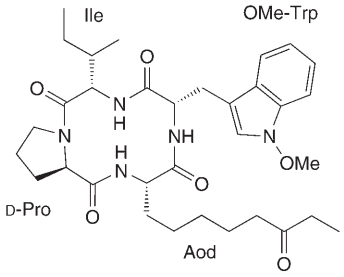
Cyclic tetrapeptide	Producing fungus		Geographic origin/Habitat	Sequence[a])
	Teleomorph	Anamorph (synonyms)		
Azumamides [50]	Azumamides were claimed to be isolated from the marine sponge *Mycale izuensis* (Porifera, Demospongiae, Mycalidae). This is highly questionable as all other cyclic peptides treated here were obtained from fungal sources. Thus, a sponge-associated fungus is predicted to be the producer.		Amakusa Islands, Southern Japan	Azumamide A (**23**) cyclo(-Amnaa-D-Phe-D-Ala-D-Val-) Azumamide B (**24**) cyclo(-Amnaa-D-Tyr-D-Ala-D-Val-) Azumamide C (**25**) cyclo(-Amnda-D-Phe-D-Ala-D-Val-) Azumamide D (**26**) cyclo(-Amnaa-D-Phe-D-Ala-D-Ala-) Azumamide E (**27**) cyclo(-Amnda-D-Phe-D-Ala-D-Val-)
Microsporins [51]	Not known	*Microsporium* cf. *gypseum*	US, Virgin Islands, associated with the marine bryozoan *Bugula* sp. (Cheilostomatida, Bugulidae)	Microsporin A (**28**) cyclo(-Aod-D-Pip-Phe-Ala-) Microsporin B (**29**) cyclo(-Ahd-D-Pip-Phe-Ala-)

[a]) Exchange positions are underlined. Abbreviations of constituents not explained in the table are given in the text.



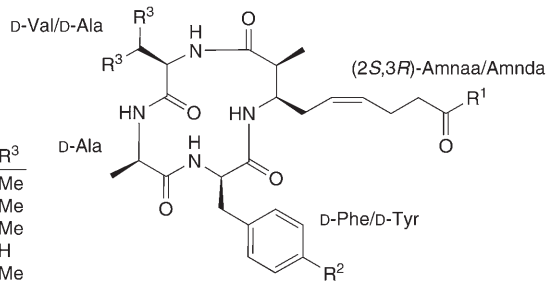
D-Pip-Containing apicidins

	R	R^1	R^2
Apicidin (**16**)	Et	MeO	$EtC(O)CH_2$
Apicidin A (**17**)	Et	H	$EtC(O)CH_2$
Apicidin C (**19**)	Me	MeO	$EtC(O)CH_2$
Apicidin D1 (**20**)	Et	MeO	$MeCH(OH)C(O)CH_2$
Apicidin D2 (**21**)	Et	MeO	$EtCH((S)\text{-}OH)CH_2$
Apicidin D3 (**22**)	Et	MeO	$MeCH(OH)CH_2CH_2$

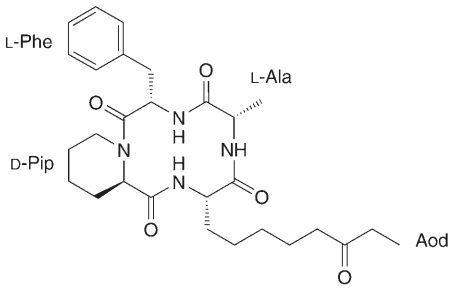


Apicidin B (**18**)

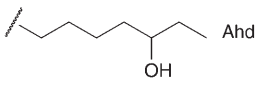
Fig. 4. *Structures of apicidins*



	R^1	R^2	R^3
Azumamide A (**23**)	NH_2	H	Me
Azumamide B (**24**)	NH_2	OH	Me
Azumamide C (**25**)	OH	OH	Me
Azumamide D (**26**)	NH_2	H	H
Azumamide E (**27**)	OH	H	Me



Microsporin A (**28**)



Microsporin B (**29**)

Fig. 5. *Structures of azumamides A–E* (**23**–**27**, resp.)*, and microsporins A and B* (**28** and **29**, resp.)

dermatophytes pathogenic to mammals, including humans [55]. Usually, they cause a single inflammatory skin or scalp lesion; invaded hairs show an ectothrix infection [56].

The sequences of microsporin A (**28**) was established as cyclo(-Aod-D-Pip-Phe-Ala-) and cyclo(-Ahd-D-Pip-Phe-Ala-), respectively. The structure of microsporin A (**28**) was further confirmed by solid-phase synthesis. Notably, the unusual lipoamino acid of microsporin B (**29**), (*2S*)-2-amino-8-hydroxydecanoic acid (Ahd), has not been described for cyclic tetrapeptides or cyclopeptaibiotics before. The configuration of the amino acids in the HCl hydrolysate was determined after derivatization with *Marfey*'s reagent and subsequent HPLC analysis of the diastereoisomers. Microsporins A and B (**28** and **29**, resp.) are potent inhibitors of histone deacetylase and showed cytotoxic activity against human colon adenocarcinoma (HCT-116) and other cancer cell lines [51].

Facts and Hypotheses on Structure–Activity Relationships. – Notably, all cyclic tetrapeptides reviewed here display the same building scheme. An uncommon, branched or unbranched lipoamino acid is linked to a Pro or Pip residue, respectively. Two additional amino acids are required to form a four-membered ring (*Figs. 1–5*). Most of the cyclic tetrapeptides contain one D-amino acid residue, which is likely to confer increased resistance to the ring system against proteolytic cleavage. This resistance is further increased by the presence of one Aib residue in the cyclopeptaibiotics. The occurrence of lipoamino and D-amino acids strongly suggests a non-ribosomal peptide biosynthesis as it is known from linear peptaibiotics [57][58] and larger cyclic peptides of fungal origin such as the L-α-aminobutyric acid (Abu)-containing cyclosporins from *Tolypocladium inflatum* [59][60].

The potent antiprotozoal activity of apicidins **16**–**22**, was demonstrated to be caused by inhibition of parasite histone deacylase [46–48]. The antitumor effects of WF-3161 (**13**) [42] and trapoxins [44] may be explained by irreversible inhibition of histone deacetylase 1 [61]. Contrary to what is known from linear peptaibiotics [1][23], ionophoric activities of cyclic tetrapeptides and cyclopeptaibiotics have not yet been reported in literature. The lumen formed by such a four-membered ring system is too small to act as a channel. Currently, the potent bioactivities of these peptides are best explained by the inhibition of histone deacetylases [61].

Synthetic, Non-Natural Aib-Containing Cyclic Peptides. – Although this review is focussed on naturally occurring cyclic peptides, it is worth mentioning that several non-natural cyclic hexa-, hepta-, and octapeptides containing a number of sterically constrained Aib-residues have been prepared by solution-phase synthesis. Crystal structures of some of them have been determined by X-ray-analysis [62–66].

Neither synthetic Aib-containing analogues of natural astins representing anti-tumor cyclic pentapeptides [67] and macrocyclic analogues of linear natural peptides such as neuropeptide Y (NPY) [68], nor the vast number of Aib-containing cyclic peptides filed in the patent literature, are treated in this review. We propose that use of the term 'cyclopeptaibiotics' should be restricted to biologically active, native peptides.

Future Prospects. – Structural diversity of cyclic tetrapeptides and cyclopeptaibiotics is expected to increase in the future. Recently, microheterogeneity has been

impressively demonstrated for apicidins [46] and azumamides [50]. Therefore, a similar structural diversity, as it is already known from linear peptaibiotics [69–71], can be postulated for cyclic tetrapeptides and cyclopeptaibiotics. Considering structural homologies known from linear peptaibiotics, it is not excluded that, for instance, HC-toxins containing Aib or Vxx (Val, and D- or L-Iva) instead of Ala will be detected. Such result would confirm previous observations for trichobrachins TB III A and TB III B: These are hexapeptides, the N-terminus of which has not been assigned yet. Remarkably, Aib could not be determined in those peptides [71][72].

The method of peptaibiomics [73] which has been successfully applied to screen peptaibiotics of *Trichoderma* sp. and its *Hypocrea* teleomorphs [3][74][75] is also recommended as a pivotal tool for screening cyclic tetrapeptides and cyclopeptaibiotics. Formation of such cyclic tetrapeptides and cyclopeptaibiotics might be interpreted as an adaptation to the highly specialized life style of the producers as plant pathogens, egg pathogens of nematodes or insects, or as plant endophytes. Therefore, screening of fungi from such ecological niches should considerably contribute to the diversification of cyclic tetrapeptide and cyclopeptaibiotic structures.

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REVIEW

Peptaibiomics: Towards a Myriad of Bioactive Peptides Containing C^{α}-Dialkylamino Acids?

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Dedicated to Prof. Dr. *Günther Jung*, University of Tübingen, a pioneer in the field of peptaibiotic research, on the occasion of his birthday.

Fungi are generally regarded as a literally infinite resource of bioactive secondary metabolites displaying remarkable structural diversity. Research on a particular group of linear and cyclic peptide antibiotics comprehensively named peptaibiotics – as result of the abundance of the non-proteinogenic C^{α}-dialkylated α-amino acids α-aminoisobutyric acid (Aib) and isovaline (Iva) – has been started 50 years ago. These peptides have gained constantly increasing interest because of their unique bioactivities and conformations. This review, reflecting the history of peptaibiotic research from 1958 to 2008, is focussed on introducing both the structural diversity and natural microheterogeneity of the peptaibiotics, as well as the biodiversity of their fungal producers. Recently introduced state-of-the-art methods for rapid screening and sequencing of peptaibiotics, such as peptaibiomics and intact-cell MALDI-TOF mass spectrometry, are discussed. Finally, future prospects in peptaibiotic research are presented. Owing to the ubiquity and biodiversity of the fungal producers in the biosphere, the discovery of a myriad of peptaibiotics within the next decade is predicted.

Contents

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1. Introduction. – Fungi are generally regarded as a literally infinite resource of bioactive secondary metabolites displaying remarkable structural diversity. Peptide antibiotics constitute a considerable part of these metabolites. During the past two decades, a constantly growing group of peptide antibiotics, the **peptaibiotics**, has started to regain particular interest because of their unique bioactivities and conformations [1]. Peptaibiotics are defined as linear or cyclic polypeptide antibiotics 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 α-aminoisobutyric acid (Aib); *iii*) are characterized by the presence of other non-proteinogenic amino acids and/or lipoamino acids; *iv*) possess an acylated N-terminus, and *v*) in the case of linear peptides, have a C-terminal residue that, in most of them, consists of a free or acetylated amide-bonded 2-amino alcohol.

The C-terminus might also be an amine, amide, free amino acid, 2,5-dioxopiperazine, or sugar alcohol. The majority of Aib-containing peptides carries a C-terminal residue representing a 2-amino alcohol, and this subgroup is, therefore, referred to as **peptaibols** [2][3]. In strongly lipophilic peptaibols, the N-terminus is acylated by octanoic, decanoic, or *cis*-dec-4-enoic acid, and these are named **lipopeptaibols** [4]. In the third subfamily of **lipoaminopeptides** (also named aminolipopeptides), the N-terminus is substituted by unbranched, α- or γ-methyl-branched, saturated or unsaturated C_4–C_{15} fatty acids. An L-proline-, *trans*-4-hydroxy-L-proline, or *cis*-4-methyl-L-proline residue is found in position 1 of the peptide chain, and, in most cases, it is followed by a lipoamino acid residue in position 2. To our present knowledge, this compound, 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD), has only been detected in this subfamily. A fourth subfamily comprises all other linear peptaibiotics that cannot be classified in any of the other three preceding subfamilies [2]. Recently, a fifth subfamily has been introduced. Not considering linearity as a necessary prerequisite anymore, extension of the above definition of 'peptaibiotics' to encompass also cyclic, Aib- or Iva-containing peptides of natural origin was proposed [3].

A review introducing nine subfamilies according to structural homologies of peptaibiotics has been published in 2001 [5], but these need to be revised and updated [6], because more than half of the currently known sequences of peptaibiotics have been discovered since then, including more than 200 found in our group [7–12]. Peptaibiotics are usually classified according to their main chain length as long-chain (17–21 residues), medium-chain (11–16 residues), and short-chain (5–10 residues) sequences. Although not yet reported in literature, *Degenkolb et al.* [2] claimed the detection of very short chain (< 5 residues) peptaibiotics that may represent drastically truncated sequences.

2. 1958–2008: Fifty Years of Peptaibiotic Research. – 2.1. *Discovery of the First Aib-Containing Peptide Antibiotic, ICI 13959.* The first report on the isolation of peptide-bound Aib from a fungal source was already published in 1958 [13], when an HCl hydrolysate of the non-crystalline peptide antibiotic ICI 13959 was investigated by ascending two-dimensional paper chromatography and elementary analysis of the resulting ninhydrin-positive spots. This antibiotic, produced by a taxonomically unidentified strain of the genus *Paecilomyces*, has shown remarkably high activity against *Trypanosoma congolense* infections in mice. Five ninhydrin-positive spots, A–E, were analyzed as Leu, Aib, γ-methylproline (γ-MePro), β-hydroxyleucine (β-Hyleu), and β-alanine (β-Ala), respectively. Furthermore, 'basic substances' were produced during hydrolysis the structures of which have not been elucidated [13]. Subsequently, spot D was further characterized as L-threo-β-hydroxyleucine [14], whereas spot C was determined as *cis*-4-methyl-L-proline [15]. Taken together, these results strongly suggest that antibiotic ICI 13959 might have consisted of a mixture of homologous leucinostatins. These lipoaminopeptides were subsequently isolated from *Paecilomyces lilacinus* A-267 [16–20], No. 1907 [21], P168 [22–25], and other strains of this species, as well as from *P. marquandii* 699/ISS [26–31]. To the best of our knowledge, neither reports to complete the structural elucidation of ICI 13959, nor bioactivity data were subsequently published.

2.2. *The First Two Decades of Peptaibol Research*. The history of research on peptaibiotics dates back to 1966, when suzukacillin (SZ), a mixture of two TLC-separable components, SZ-A and SZ-B, from *Trichoderma viride* 63 C-I, was obtained in crystalline form [32]. The unknown amino acid detected in the HCl hydrolysate was subsequently isolated and identified as Aib [33]. In 1967, the first report on the Aib-containing antibiotic U-22324 from *Trichoderma viride* NRRL 3199 was published [34]. This polypeptide antibiotic, later renamed alamethicin (ALM), is generally regarded as the most thoroughly investigated peptaibiotic, thus often serving as the classical paradigm of a peptaibol [8][10][12][35][36]. The antiprotozoal activity of antiamoebin (AAM) was published later the same year [37]. In 1968, its action on rumen microorganisms [38], polypeptidic nature, including the presence of non-proteinogenic amino acids such as Aib and Hyp [39][40], were recognized as well as the presence of an amino alcohol, determined as L-2-amino-3-phenylpropan-1-ol (L-phenylalaninol, Pheol) [41]. After the breaking publication by *Mueller* and *Rudin*, who reported on the induction of action potentials in experimental bilayer lipid membranes (BLMs) and cationic conductance induced by ALM [42], the interest in similar membrane-active peptide antibiotics increased remarkably – both SZ [43][44] and TXT A-40 [45][46] were subsequently reported to display similar channel-forming activity. Furthermore, the haemolytic activity of SZ, ALM, and TXT-A40 were published [47]. Non-targeted antimicrobial screening approaches led to the isolation of the following peptide antibiotics, which subsequently could be demonstrated to belong to the family of peptaibiotics:

- Stilbellin from a coprophilous (deer dung) isolate of *Stilbella* sp. [48], which later deceased;
- Trichotoxin A (TXT A) from '*Trichoderma viride*' NRRL 5242 and Trichotoxin B (TXT B) from '*Trichoderma viride*' NRRL 5243, two secondary invaders of maize cultures infested with *Helminthosporium maydis* (= *Bipolaris maydis*, teleomorph *Cochliobolus heterostrophus*) race T, the causal agent of Southern Leaf Spot of Maize [49];
- Emerimicins I–IV from *Emericellopsis microspora* 333 [50];
- Zervamicins I and II from *Emericellopsis salmosynnemata* 336 [51];
- Samarosporin from a mycelial culture of the ascomycete '*Samarospora* sp.' FERM-P 224 (F-7762) [52] that ceased the production of the antibiotic and is no longer deposited.

2.3. *Mass Spectrometry Becomes a Decisive Tool in Peptaibol Research*. During the first decade of research in this field, structure elucidation of peptaibols was remarkably hampered by the presence of an acylated N-terminus and a C-terminus that represents a 2-amino alcohol. Owing to the negative ninhydrin reaction, a cyclic structure was originally proposed for ALM [34], AAM [40], and TXT-A [49]. Linearity of ALM, including the presence of an acetylated N-terminus, Ac-Aib, was first recognized by NMR [53]. However, the correct, C-terminal position of the phenylalaninol (Pheol) residue in AAM, ALM, and emerimicins III and IV was assigned by a combination of a cation-exchange technique and field-desorption high-resolution mass spectrometry (FD-HR-MS), gas chromatography/high-resolution electron impact mass spectrometry (GC/HR-EI-MS), and gas chromatography/field ionization mass spectrometry

(GC/FI-MS), which also confirmed the presence of an N-terminal Ac-Aib residue [54–57]. Notably, *Pandey et al.* were the first to define a new family of peptide antibioitics, the so-called **peptaibophols**, as **pept**ide antibiotics, which contain the marker amino acid **Aib** and a C-terminal **ph**enylalanin**ol** (Pheol) residue [55]. Subsequently, combinations of FD-MS, GC/EI-MS, and CI-MS analyses of trifluoroacetylated and esterified partial hydrolyzates, as well as reduced and trimethylsilylated peptide fragments were introduced. These approaches proved to be a pivotal tool accelerating the sequence determination, as exemplified for TXT-A40 [58] – one of the two TLC-separable fractions of trichotoxin, TXT-A40 and TXT-A50. The importance of a chiral GC/MS approach could subsequently been demonstrated when hydrolysates of AAM, emerimicin IV, SZ, and TXT-A40 were shown to also contain D-Iva [59]. GC/MS Analysis of its methyl ester was also used to unambiguously establish the presence of AcAib in ALM F30, ALM F50, TXT-A40, TXT-A50, and SZ-A after selective trifluoroacetolytic scission of the above peptide antibiotics [60].

Since additional 2-alcohols such as valinol (Valol) in TXT-A40 [58] and leucinol (Leuol) in hypelcin from *Hypocrea peltata* [61] were detected as C-terminal constituents, the above definition of peptaibophols needed to be revised and updated. To appropriately characterize this family of peptide antibiotics, the technical term **peptaibol** was – independently from each other – proposed by *Benedetti et al.* [62], and by *Brückner* and *Graf* [63] for those **pept**ide antibiotics, which contain the marker amino acid **Aib** and a C-terminal amino alcoh**ol** (Xol) residue.

The introduction of fast atom bombardment mass spectrometry (FAB-MS) is to be considered as a further milestone for peptide sequencing [64][65]. FAB-MS impressively demonstrated the sequence identity of leucinostatin A [20], which is present as a constituent of the following antibiotics: A-267 [16], No. 1907 [21], P168 [22–25], and CC-1014 [66]. A multiple analytical approach combining HPLC, gas–liquid chromatography (GLC), FD-MS, and FAB-MS was used to confirm the identity of stilbellin and samarosporin with emerimicin IV [67]. Similar approaches, including selective *in situ* hydrolysis, were subsequently used to characterize the microheterogeneous mixtures of zervamicin [64], paracelsin (PC), TXT-A40, and TXT-A50 as well as gliodeliquescin [58][68–71].

By the middle of the 1980s, it was generally recognized that most of the hitherto known peptaibol antibiotics and related, Aib-containing structures such as trichopolyns [72][73], efrapeptins [74], elvapeptins [75], and hypelcins [76][77] represent microheterogeneous mixtures.

Although of microcrystalline structure, these Aib-containing peptide antibiotics still consist of a more or less complex mixture of homologous and positionally isomeric components. Microheterogeneity was postulated to originate from non-ribosomal peptide biosynthesis as indicated by the presence of non-proteinogenic amino acids [78] such as Aib, D-Iva, β-Ala, Hyp, Hyleu, MePro, as well as lipoamino acids such as AHMOD [17], C-terminal amino alcohols or amines. Non-ribosomal biosynthesis of ALM was already established in 1967, as the addition of cycloheximide to a growing culture of *Trichoderma viride* NRRL 3199 was found to block ribosomal peptide synthesis, but it did not interfere with the biosynthesis of ALM [78]. Moreover, the *in vitro* synthesis of ALM was not influenced by puromycin or RNase and found to be thiotemplate-associated [79].

In 1985, high-pressure liquid chromatography/fast-atom-bombardment mass spectrometry (HPLC/FAB-MS) with jet sprayer and moving belt interface was introduced. The idea behind this was to first separate the amphiphilic and thermally labile peptide antibiotics, including peptaibols, on C_{18} columns, which are subsequently sequenced by FAB-MS. [80]. The LC/MS interface was subsequently improved replacing the jet sprayer by a frit. HPLC/Frit-FAB-MS confirmed the identity of leucinostatin A [16][19][20] with antibiotic No. 1907 [21], P168 [22–25], and CC-1014 [66]. Furthermore, it revealed the structures of four new minor components of the leucinostatin family [81]. Finally, HPLC/Frit-FAB-MS was used to separate and sequence the microheterogeneous P168 mixture, which consisted of at least 20 homologous and positionally isomeric leucinostatins [25].

2.3.1. *New MS-Based Screening Approaches in Peptaibol Research*. The Aib-containing peptide antibiotics treated above were randomly discovered in bioactivity-guided screenings. By 1987, *ca*. 60 individual sequences of peptaibols and related Aib-containing linear peptides have been described in literature. Notably, all of them were isolated from hypocrealean fungi, belonging to different genera of the family Hypocreaceae (*Trichoderma*/*Hypocrea*, *Gliocladium*, *Emericellopsis*, *Stilbella*, and *Paecilomyces*) or Clavicipitaceae (*Tolypocladium*). As half of the sequences known at that time were produced by members of *Trichoderma* (and its *Hypocrea* anamorphs), this genus was the first one to be systematically screened for new peptaibol producers.

Crude organic extracts of fungal cultures were hydrolyzed; the free amino acids were converted into the corresponding pentafluoropropionyl-1-propyl esters; and the sample was subsequently analyzed for the presence of the PFP-Aib-1-propyl ester by GC-FID. Because a huge number of new Aib-positive species and strains of *Trichoderma* has been detected [82][83], this method was subsequently broadened, and both GC-FID and GC/MS were used for screening of *Stilbella*, *Emericellopsis*, *Gliocladium*, *Trichoderma*, and *Hypocrea* [84–86]. For resolution of DL-Iva on *ChirasilVal*™, conversion into *N*-acetyl-isovaline-1-propyl esters was found to be superior [87]. HPLC Analysis after precolumn derivatization of the total hydrolysate with *o*-phthaldialdehyde-3-mercaptopropionic acid (OPA/MPA) was introduced at the same time [85]; and subsequently different chiral variations of *Marfey*'s reagent were used for HPLC analysis of amino acids [88–90]. Regardless of a number of subsequent attempts undertaken to validate the previously reported detection of Aib in hydrolyzed extracts of *Metarhizium anisopliae* [91], *Penicillium roquefortii* [84], and *P. nalgiovense* [85], those three species could not be confirmed as producers of peptaibiotics. Furthermore, our isolate of *M. anisopliae* CBS 597.80 deceased; and this strain is no longer deposited in the culture collection of CBS. Thus, the isolation of metanincin [91] could not be reproduced. Notably, none of the subsequently examined strains and species of *Metarhizium* produced Aib-containing peptides. Therefore, taxonomic identity of *M. anisopliae* CBS 597.80 either had to be doubted, or that culture was contaminated by *Trichoderma* sp. The latter assumption was supported by the fact that *ca.* 1.4 g (!) of a peptaibol mixture, structurally related to the paracelsins, was isolated from a submerged fermentation of the fungus. Despite these three counter-examples, it should be emphasized here that most of the fungal strains and species we have reported positive for Aib and/or DL-Iva [92][93] were unambiguously confirmed as producers of peptaibiotics. Subsequent attempts undertaken by us or

other groups led to the isolation and complete chemical characterization of these compounds.

From the middle of the 1980s on, a huge number of fungal strains and species were systematically screened for the production of Aib-containing polypeptide antibiotics. Research was mainly focused on previously uninvestigated species of *Trichoderma* and its *Hypocrea* teleomorphs, which proved to be the richest sources of peptaibols and related Aib-peptides: In 1985, trichorzianine AIIIc was isolated from *Trichoderma harzianum* ATCC 20672 [94] – a strain from which another eight neutral 19-residue trichorzianins A [95] and seven acidic 19-residue trichorzianins B [96] could subsequently be obtained. *Fujita et al.* were the first to demonstrate that *T. polysporum* TMI 60146 produced a mixture of different subfamilies of Aib-containing polypeptide antibiotics [97]. In addition to the previously described ten-residue lipoaminopeptides trichopolyns I and II, eleven 20-residue peptaibols, named trichosporins B, were isolated [98][99]. The same phenomenon was reported for *T. longibrachiatum* MNHN 3431: this strain produced a mixture of two 20-residue peptaibols tricholongins B [100], and the eleven-residue trichogin A IV. The latter was shown to carry an N-terminal octanoyl (Oc) instead of an acetyl (Ac) residue. Thus, trichogin A IV was the first representative of a new subfamily of the peptaibiotics, named **lipopeptaibols**, as they carry an N-terminal acyl instead of an Ac residue [101]. Further eleven-residue lipopeptaibols, trikoningin KB I and KB II, and the 19-residue peptaibol trikoningin KA V were isolated from *T. koningii* MNHN 903589 [102].

In view of the constantly growing structural diversity of Aib-containing polypeptide antibiotics, *Brückner et al.* introduced the technical term **peptaibiotic** [103][104] to encompass all Aib-containing polypeptide antibiotics that did not match the classical definition of a peptaibol anymore [62][63].

Four neutral 20-residue peptaibols were isolated from *T. saturnisporum* MNHN 903578 [105]. *Ca*. 31 trichobrachins of different building scheme and chain length were reported from *T. longibrachiatum* CBS 936.69, and another seven 14-residue trichovirins from *T. viride* NRRL 5243 [106]. Seven of the eleven trichokindins, Iva-containing 18-residue peptaibols from *T. harzianum*, were shown to carry the rare C-terminal isoleucinol (Ileol) residue [107]. The same strain was subsequently shown to produce four eleven-residue trichorozins [108]. Trichodecenins I and II, two seven-residue lipoeptaibols with an N-terminal (*Z*)-dec-4-enoyl group [109], 24 eleven-residue trichorovins [110], eight 20-residue trichocellins A, and two 20-residue trichocellins B [111] were reported from a strain of *T. viride*. Harzianins HC, a mixture of eleven 14-residue peptaibols, was reported from strains of *T. harzianum*, *viz*. M-903614 and M-903603 [112]. The latter also produced the six 18-residue trichorzins HA [113][114] and the eleven-residue harzianin HB I [115]. Further peptaibols isolated from *T. harzianum* include three 18-residue trichorzins MA from strain M-922835 [113][114]. Furthermore, paracelsin E was obtained from *T. saturnisporum* CBS 330.70 [116]. Besides the eleven-residue peptaibol harzianin HK VI [117], two additional peptaibiotics with modified C-termini were isolated from *T. pseudokoningii* MVHC 662: the ten-residue pseudokonin KL III terminating in prolinamide (Pro-NH_2), and the nine-residue pseudokonin KL VI carrying an unusual 2,5-diketopiperazine, namely *cyclo*-Aib-L-prolinal. Notably, harzianin HK VI, and pseudokonins KL III and KL VI display the same sequence of the N-terminal heptapeptide [118]. Nine 20-residue

hypelcins A [119] and five 20-residue hypelcins B [120] some of which carry the rare C-terminal Ileol residue were isolated from the ascomycete *Hypocrea peltata*.

The search for novel sources of peptaibiotics also comprised previously uninvestigated genera and species of fungi such as:

- *Tolypocladium inflatum* (syn. *T. niveum*) and *T. geodes*, producing six insecticidal 16-residue efrapeptins C–H that carry an unusual C-terminal amine, *N*-peptido-1-isobutyl-2-(2,3,4,6,7,8-hexahydro-1-pyrrolo[1,2-*a*]pyrimidinio)ethylamine (PIHPPE) [121–124], and
- the ascomycetous *Verticimonosporium ellipticum* D 1528, the producer of aibellin, a 20-residue peptaibiotic carrying a diamino alcohol residue, 2-[(2-amino-3-phenylpropyl)amino]ethanol [125].

2.3.2. *Electrospray Ionization MS* (ESI-MS) *Revolutionizes the Research on Peptaibiotics.* Spray ionization (SI) techniques, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), introduced at the beginning of the 1990s, revolutionized the search for peptaibiotics. Contrary to the classical ionization techniques mentioned above, these 'soft' ionization techniques usually do not generate intense fragment ions but mostly pseudomolecular ions such as $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[2M+H]^+$, and $[2M+Na]^+$, or $[M-H]^-$ in the negative-ion mode, thus facilitating the recognition of the molecular weight and significantly reducing sample requirement. In most cases, sequence information is not obtained until collision-induced dissociation (CID) is performed in the skimmer region of the MS, or, more specifically, single precursor ions are selected and subsequently fragmented by collision with Ar, He, or ultrapure N_2, thus generating product ions in the collision cell – a technique, which is referred to as MS/MS or MS^2 [126][127].

Within three years, triple-quadrupole MS/MS impressively revealed its superiority for sequencing of peptaibiotics: for instance, a fungicolous strain of *Trichoderma koningii*, isolated from the bracket fungus *Ganoderma lucidum*, which has been used in Traditional Chinese Medicine for more than 4,000 years, was shown to produce nine 20-residue trichokonins and one 19-residue trichkonin [128–130]. The polysporins A–D, four alamethicin-like 20-residue peptaibols, were isolated from a strain reported as *T. polysporum* [131].

Notably, further mycoparasitic fungi outside *Trichoderma*/*Hypocrea* were successfully screened for the presence of peptaibiotics: *Mycogone rosea* DSM 8822, the producer of two nine-residue lipoaminopeptides, helioferin A and B [132]; *Sepedonium chrysospermum* DSM 7444 (teleomorph *Hypomyces chrysospermus*, syn. *Apiocrea chrysosperma*), biosynthesizing four 19-residue chrysospermins A–D [133]; *Sepedonium ampullosporum* DSM 10602, source of the neuroleptic 15-residue ampullosporin A [134], and further seven minor components, ampullosporins B–E_4 [135]. This strain also produced the five-residue peptaibolin, the shortest peptaibol reported in literature to date [136].

2.4. *Towards Structural Diversification: from 1996 to 2008.* 2.4.1. *New Peptaibols and Lipopeptaibols from Soil Fungi.* In 1996, three novel eleven-residue lipopeptaibols, LP237-F5, -F7, and -F8, were obtained from *Tolypocladium geodes* LP237, a soil fungus collected in the Pennine Mountains (UK). The N-terminus of LP237-F5 and -F8 is protected by an octanoyl residue, whereas LP237-F7 carries an decanoyl residue, thus

being the first lipopeptaibol to display this particular structural element. Another unique feature of LP237-F5 and -F8 is the presence of one α-ethylnorvaline (EtNor) residue, the methylene homologue of isovaline [137][138]. Four 15-residue bergofungins A [139] and B–D [140] were isolated from *Emericellopsis donezkii* HKI 0059. The 17-residue peptaibiotic XR586 obtained from the tropical soil fungus *Acremonium persicinum* X21488 was shown to terminate in Gly [141].

2.4.2. *New Peptaibiotics with Unusual N- and C-Termini*. In 1997, a peptaibiotic displaying a mannitol residue as a unique C-terminal substituent was isolated from the soil fungus *Clonostachys* sp. F5898: the 15-residue clonostachin is the only peptaibiotic described in literature so far the C-terminus of which is esterified to the carboxy group of the penultimate Ile residue [142]. Six eleven-residue hypomurocins A and another six 18-residue hypomurocins B were isolated from strain IFO 31288 [143]. This strain has originally been misidentified as *Hypocrea muroiana* but was recently proven to be *Trichoderma atroviride* [10].

The same year, two 21-residue peptaibiotics, texenomycins A and B, were claimed to be isolated from the basidiomycete *Scleroderma texense* DSM 10601 (syn. *S. bovista*). The N-terminal Pro residue of the texenomycins is protected by a β-keto-2-methyltetradecanoyl (MOTDA) residue, whereas arginol (Argol) is found as a so far unique C-terminal constituent [144]. However, ecophysiological and chemotaxonomic considerations rather suggest an unrecognized infection of the investigated strain by a mycoparasite of basidiomycete affiliation that could be attributed as the producer of these peptaibiotics [2][122]. Lipohexin, an inhibitor of prolyl endopeptidase, representing a six-residue N-terminal deletion sequence of texenomycin, was isolated from three fungal strains, originally identified as *Moeszia lindtneri* HKI-0054, and two *Paecilomyces* sp., HKI-0055 and HKI-0096 [145][146]. The former strain was subsequently reclassified as *Acremonium lindtnerei* DSM 11119 (teleomorph *Hypomyces chrysostomus*), and the latter two were recently reidentified as *Mariannaea elegans* CBS 120677 and CBS 120687 [2].

Four nine-residue peptaibiotics, MS-681a, b, c, and d, which act as inhibitors of myrosin light chain kinase, were isolated from *Myrothecium* sp. KY6568 [147]. Notably, the configuration of the Iva^8 and the Iva^2 residues was shown to be L, whereas Iva^5 was assigned the D-configuration. The C-terminus of all four homologues is protected by the same polyamine, (2′*RS*)-N^1-(2-amino-3-phenylpropyl)spermidine, another novel constituent of peptaibiotics [148].

The same year, a commercial sample of paracelsin from *T. reesei* QM 9414 was reinvestigated by high-energy CID tandem mass spectrometry, which led to the detection of four minor homologues, *viz*. PC-F, -G, -H-, and -I [149].

2.4.3. *Discovery of New, Iva-Rich Peptaibols.* In 1998, the sequences of the new minor components of paracelsin could independently be confirmed by HPLC coupled to an ESI-ion-trap MS [150]. Further diversification of the trichorzianine A mixture was also achieved by high-energy CID tandem mass spectrometry, leading to the detection of five new sequences in addition to the nine previously reported [151].

Moreover, seven Iva-rich 18-residue peptaibols, trichorzins PA, were isolated from *T. harzianum* M-902608 [152]. When the latter strain was grown on Aib-supplemented medium, two new compounds, trichorzin PA_U4 and harzianin PC_U4, were obtained in addition to the seven trichorzins PA mentioned above. Fermentation of *T. longi-*

brachiatum M-853431 on a synthetic medium led to the production of four 20-residue neutral longibrachins A I–IV and two acidic longibrachins B II and III. Growth of that strain on Aib-supplemented medium resulted in the simplification of the peptaibol mixture produced [153][154].

2.4.4. *From Friend or Foe, Host or Mycoparasite? – The Origin of New Peptaibiotics Found in Fruiting Bodies of Basidiomycetes.* In 1999, the isolation of the 19-residue boletusin [155] and the 15-residue tylopeptin [156] from fruiting bodies of basidiomyces, *viz*. *Boletus* sp. and *Tylopilus neofelleus*, was reported. However, the isolation of a peptaibiotic from a fruiting body of a member of the Boletales should be regarded as highly ambiguous.

Boletaceous fungi are known to be preferentially parasitized by members of the genus *Sepedonium* (with teleomorphs in *Hypomyces*). It should be emphasized again that the peptaibiotics produced by this genus exhibit a remarkably high structural homology with boletusin or tylopeptin. This phenomenon was extensively discussed by *Degenkolb et al.* [2][127]. Finally, *Neuhof et al.* [157] demonstrated that the fungicolous *Sepedonium chalcipori*, which exclusively parasitizes the Peppery Bolete *Chalciporus piperatus*, produced tylopeptins A and B in solid-state fermentation. These authors suggested that tylopeptins, as reported by *Lee et al.* [156], were in fact produced by an ascomycetous mycoparasite. Thus, the conclusions by *Kiet et al.* could be corroborated who attributed the detection of chrysospermins A–D in fruiting bodies of the new Vietnamese species *Xerocomus langbiannensis* to an unrecognized infection of this bolete by *Sepedonium* sp. [158].

A reinvestigation of the trichopolyn mixture produced by the fungicolous isolate *T. polysporum* TMI 60146 revealed the presence of three minor components, trichopolyns III–V in addition to the previously reported trichopolyns I and II. Notably, the N-terminal Pro residue of trichopolyn V carries a novel N-terminal substituent, 3-hydroxy-2-methyldecanoic acid (HMDA) [159].

Trichoderma viride NRRL 5243, currently deposited as *T.* cf. *harzianum* [10], has been reported in 1972 as the producer of trichotoxin B [49]. This strain was reinvestigated, ESI-ion trap MS^n being the method of choice for sequencing of homologous and positionally isomeric peptaibiotics. Even chromatographically inseparable peptides can be sequenced by the generation of different generations of product ions [126][127]. Trichotoxin B had been renamed to trichovirin in the meantime and found to consist of the groups trichovirins I and II [106]. The latter were shown to be a microheterogeneous mixture of twelve 18-residue peptaibols [160].

Trichotoxin A-40 produced by *Trichoderma viride* NRRL 5242, now reidentified as *T. asperellum* [161][162], was shown to consist of six homologous and positionally isomeric 18-residue peptaibols [163].

2.4.5. *New Producers of Antiamoebins and of Lipoaminopeptides.* In 2000, antiamoebin mixtures obtained from different fungi and from two commercial preparations of AAM were reinvestigated by LC/ESI-ion-trap MS^n and dipeptide analysis by GC/MS. Finally, this approach disclosed the impressive microheterogeneity of AAM, leading to the introduction of 14 new sequences. The following strains were confirmed as producers of AAM: *Stilbella erythrocephala* (syn. *S. fimetaria*) ATCC 28144, *S. fimetaria* CBS 548.84, *Gliocladium catenulatum* (syn. *Clonostachys rosea* f. *catenulata*) CBS 511.66, *Emericellopsis synnematicola* CBS 176.60, and *E. salmosyn-*

nemata CBS 382.62 [164]. Notably, *Lehr et al.* were – six years later – first to unequivocally prove that antiamoebins were responsible for antibiosis in colonized herbivore dung [165]. Four strains of *Stilbella fimetaria* (syn. *S. erythrocephala*) were isolated from dung of wild rabbits (D99026, D01024, and D03001) or of the tortoise *Testudo hermanni* (D03012). Furthermore, dung pellets naturally colonized by *S. fimetaria* were collected in the field, lyophilized, and extracted. The same was carried out with presterilized dung that has been artificially inoculated with the four strains mentioned above. As expected, AAM could be detected in all liquid cultures. To continue, the total AAM concentration – both in wild and artificially inoculated dung – was 126–624 μg/g fresh weight, with minimum inhibitory concentrations against most other coprophilous fungi being at or below 100 μg/ml. It should be pointed out that this is the first report describing the detection and isolation of peptaibiotics from natural substrates. The diterpene antibiotic myrocin B, not previously described from *S. fimetaria*, was also produced, but only at low, nonfungicidal levels (5.3 μg/g). As no other antifungal substances could be detected, a decisive role of the broad-spectrum antimycotic AAM during colonization of dung was proposed [165]. The 15-residue heptaibin was also isolated from *Emericellopsis* sp. BAUA8289, a strain colonizing the roots of a rice plant [166]. Heptaibin, displaying seven Aib-residues, is considered one of the Aib-richest peptaibols reported to date.

An ESI-ion-trap MS^n approach was shown to be decisive for sequencing of the roseoferin complex, a mixture of at least 16 homologous and positionally isomeric nine-residue lipoaminopeptides produced by *Mycogone rosea* DSM 12973. [167]. The liopaminopeptides of the helioferin/roseoferin complex are characterized by the presence of an N-terminal Pro residue that is protected by α-Me-branched fatty acids – either 2-methyloctanoic (MOA), or 2-methyldecanoic acid (MDA). A secondary or tertiary amine, either 2-(2′-aminopropyl)-aminoethanol (AAE), or 2-(2′-aminopropyl)-*N*-(methylamino)-ethanol (AMAE) is found at the C-terminus.

Further peptaibols reported in 2000 comprise two antiviral 19-residue peptaivirins A and B from an unidentified fungal strain KGT142 [168] and three atroviridins A–C, 20-residue peptaibols, from *Trichoderma atroviride* [169].

2.4.6. *Microheterogeneous Stilboflavins, Antihelmintic Cephaibols, Antimycoplasmic Longibrachins, and a Peptaibiotic Terminating in Free Aib*. In 2001, three microheterogeneous groups of peptaibols, stilboflavins A–C, were isolated from the soil fungus *Stilbella flavipes* CBS 146.81. Stilboflavin A consists of seven acidic (Glu^{18}–Gln^{19}) 20-residue peptaibols terminating in Valol or Leuol. Stilboflavin B, however, is a complex of ten neutral (Gln^{18}–Gln^{19}) 20-residue peptaibols, eight of them displaying a C-terminal Valol or Leuol residue. Notably, two of them carry the rare Ileol terminus [170].

Cephaibols, two groups of antihelminthic 16- or 17-residue peptaibiotics, were isolated from *Acremonium tubakii* DSM 12774 and shown to be highly active against ectoparasites. The 16-residue cephaibols A, A2, B, C, D, and E represent peptaibols with a C-terminal Pheol residue, whereas cephaibols P and Q are peptaibiotics carrying a Phe^{16} and terminating in a free Ser^{17} residue. Notably, cephabols E and D were shown to be identical with antiamoebins III and VI, respectively [171].

Two antimycoplasmic 20-residue peptaibols, longribrachin LGB II and LGB III, were isolated from *Trichoderma longibrachiatum* LCP-853431 [172].

Two antifungal peptaibiotics, SCH 466457 and SCH 466456, carrying an N-terminal 2-methyl-3-oxotetradecanoyl (MOTDA) residue were isolated from a taxonomically unidentified fungus. The first compound, SCH 466457, represents a 17-residue peptaibiotic, which terminates in free Ala. The second compound, SCH 466456, exhibits the same amino acid sequence in positions 1–16, and another Ala residue follows in position 17. Notably, a free Aib residue is found in the C-terminal position 18. Both compounds were active against *Candida* ssp., *Aspergillus* ssp., and *Trichophyton* dermatophytes [173]. To the best of our knowledge, this is the first report describing the occurrence of a free Aib residue as the C-terminal constituent of a peptaibiotic.

2.4.7. *Integramides,* D- *and* L-*Iva-Containing Inhibitors of HIV Integrase I. Diversification of the Lipoaminopeptide Pattern by Cofermentation of Two Producers.* In 2002, two novel inhibitors of HIV-1 integrase, the 16-residue integramides A and B, were isolated from extracts of the fungus *Dendrodochium* sp. MF6888. Remarkably, integramides contain an acetylated N-terminal D-Iva and a total of five (A), or six (B) D- or L-Iva residues. Recently, the complete chiral sequence of integramide A has definitely been assigned [174]. Among the natural peptaibiotics, integramides are those that contain the largest proportion of Iva described to date. Free Gly is found at the C-terminus of both peptaibiotics [174].

Four 18-residue neotroviridins A, B, C, and D, and three atroviridins A, B, and C were isolated from *T. atroviride* F80317 [175][176].

Trichoaureocin, a microheterogeneous mixture of nine 20-residue peptaibols, was isolated from *T. aureoviride* IMI 91968. This strain has been isolated from bark of *Fagus sylvatica* and was recently reclassified as *T. citrinoviride* [6]. Notably, the very rare Ac-Ala is found as the N-terminal constituent of trichoaureocin 1a [177].

Further structural diversification of peptaibiotic pattern was achieved by co-cultivation of two lipoaminopeptide-producing strains. The roseoferin producer *Mycogone rosea* DSM 12973 [161] and the leucinostatin-producing endophyte *Acremonium* sp. Tbp-5 [178] were grown in dual cultures. Notably, neither glucosylation of leucinostatin [178], nor glucosylation of roseoferin was observed. However, the (4*S*,2*E*)-4-methylhex-2-enoyl (MeHA) residue protecting the N-terminus of leucinostatins A, B, and K was substituted by the 2-methyldecanoyl (MDA) residue that is found as an N-terminal substituent of the roseoferins, thus leading to three novel ten-residue lipoaminopeptides, acremostatins A, B, and C [179].

2.4.8. *Sequences of Alamethicins Reconsidered and New Peptaibiotics from* Paecilomyces. In 2003, the sequences of ALM F30 and ALM F50 were thoroughly reconsidered and reconciled [8]: HPLC separation of ALM F30 using an acidic gradient revealed ten individual sequences of ALM F30/1–10. Basically, the same gradient was used to separate ALM F50 but omitting the addition of TFA to the eluents. As a result, 13 individual peptides, ALM F50/2, 3a–3c, 4a, 4b, 5, 6a, 6b, 7, and 8a–8c could be sequenced.

Recently, the same material used by *Kirschbaum et al.* [8] was further analyzed by nonaqueous capillary electrophoresis/mass spectrometry (NACE/MS): eleven amino acid sequences were identified, and characterized by the exchange of Ala to Aib in position 6, Gln to Glu in position 7 or 19. To continue, two novel ALM were detected, which were characterized by the loss of the C-terminal Pheol residue, thus terminating in Gln. Notably, two truncated 14-residue sequences carrying an N-terminal pyroglu-

tamyl group (commonly abbreviated as Glp, pGlu, or Pyr) were found. Overall, seven new sequences are reported compared to [8]. To the best of our knowledge, this is the first report on the occurrence of Glp as a constituent of fungal peptides in nature. Literature search did not reveal any previous publications regarding the isolation of peptide-bound Glp from a fungal source. To exclude the artificial formation of the Glp-peptaibols during workup or long-term storage, degradation studies were performed. Although neither treatment increased the amount of the truncated Glp peptides compared to untreated samples, further studies have to be performed in order to unequivocally prove the origin of the truncated Glp peptaibols [36].

Furthermore, two eleven-residue trichofumins A and B, as well as two 13-residue trichofumins C and D were obtained from *Trichoderma* sp. HKI 276, displaying neuroleptic activity in rodents [180].

Two antifungal 21-residue peptaibiotics, the N-terminus of which are also blocked by a MOTDA residue, were isolated from *Paecilomyces variotii* SCF 1559 [181]. They exhibit some structural homology with the 21-residue texenomycins A and B [144], as well as the six-residue lipohexin, which represents a deletion sequence of the texenomycins [145]. However, the configuration of the amino acids has not been determined.

2.4.9. *Cervinins, New Sequences from* Mycogone cervina, *a Mycoparasite on Helvellaeae.* In 2004, *M*ycogone *cervina* A09–02 was isolated as a parasite of the Vinegar Cup *Helvella* (*Paxina*) *acetabulum* and shown to produce two twelve-residue peptaibol antibiotics both exhibiting Ac-Leu as a new N-terminal motif. Leuol is found as the C-terminal residue of cervinin I. Cervinin II, however, has the same sequence of amino acids, but its C-terminal Leuol residue is acetylated [182]. As no comments were made with respect to the origin of the latter C-terminus, it cannot be completely excluded that cervinin I could have been partly acetylated during the process of workup, thus generating cervinin II. Surprisingly, no attempts were made to determine the configuration of the amino acids. We also have to comment on the authors' statement that peptaibols exclusively contain L-amino acids. The latter assertion is not acceptable, because a number of well-known counter-examples concerning the occurrence of D-Iva in peptaibiotics has been published and discussed in [2] and [127]. To the best of our knowledge, this is the first report on the isolation and structure elucidation of secondary metabolites from *M. cervina*. Surprisingly, no lipoaminopeptides have yet been recorded for that species [2][186].

2.4.10. *Cicadapeptins, New Peptaibiotics from an Entomopathogenic Fungus.* In 2005, two eight-residue peptaibiotics, cicadapeptins I and II, were isolated from *Cordyceps heteropoda* ARSEF#1880, a parasite of the Australian cicada *Cicadetta puer*. The N-terminus of these peptaibiotics is blocked by an decanoic acid residue, and the C-terminus is amidated by 1,2-diamino-4-methylpentane (DMAP) [183].

2.4.11. *Sequences of Suzukacillins A Reconsidered. Neoefrapeptins and Acretocins – the First Two Groups of Peptaibiotics Containing the Rare 1-Aminocyclopropane-1-carboxylic Acid* (Acc). In 2006, the microheterogeneous mixture of suzukacillin A could be further separated by HPLC, yielding 13 individual peaks. Nevertheless, two of them, SZ-A10 and -A11, still represented a microheterogeneous mixture. Thus, 15 individual 20-residue peptaibols, SZ-A1–A9, -A10a/b, -A11a/b, and SZ-A12 and -A13, were shown to be present in total. Despite the microheterogeneity, complete sequences

of all SZ-A peptaibols and configuration of the individual amino acids have been determined by HPLC/MS^n and GC/EI-MS approaches [9]. In this context, the previously established presence of D-Iva [59] was confirmed. However, both L-Val/D-Iva and L-Leu/L-Ile were detected in HCl hydrolysates of SZ-A by chiral GC/MS, thus increasing the previously established sequence combinations for the main C-terminal heptapeptides of SZ-A [184]. However, it appears as if the suzukacillin-producing strain is no longer deposited, – neither in industrial, nor in public culture collections.

Ten 16-residue and two 13-residue neoefrapeptins A–N with insecticidal activity [185] were isolated from *Geotrichum candidum* SID 22780. It should be pointed out that this is the first report regarding the isolation and structure elucidation of a peptaibiotic from a yeast-like fungus. Neoefrapeptins revealed two uncommon structural elements that are newly described for peptaibiotics: 1-aminocyclopropane-1-carboxylic acid (commonly abbreviated as Acc) and (2*S*,3*S*)-3-methylproline (*cis*-3-L-MePro) were identified. Configuration analysis of the 16-residue neoefrapeptins revealed L-Iva^{15}. Except for the Aib-containing neoefrapeptins D and N, Iva is present in position 4. Some of the neoefrapeptins also contain Iva^5 and Iva^{10}. If present in position 4 and/or 5, the Iva residue was assigned the D-configuration, whereas the Iva residues in positions 10 and 15 were shown to possess the L-configuration. The L-configuration was assigned to all pipecolic acid (Pip) residues found in neoefrapeptins. Notably, papainic cleavage and ESI-MS of the enzymatically generated fragments were applied as a novel approach for sequence determination.

Another group of peptaibiotics, acretocin (ACR) [186], that is very closely related to the efrapeptins (including tolypin) [186] has been detected in *Acremonium crotocinigenum* CBS 217.70 – a species that is known as a facultative parasite of a number of polypores (= bracket fungi) and agarics. Unlike efrapeptins which carry L-Iva (*S*-Iva), the presence of D-Iva (*R*-Iva) has been established for acretocins. Remarkably, acretocins I–VI differ from the efrapeptins C–G by a mass difference of 2 Da, indicating the presence of an amino acid residue of *m*/*z* 83 [186]. Such an observation led to the assumption that acretocins could also contain an Acc^9 residue. This hypothesis was subsequently confirmed by GC/MS analysis of the hydrolyzed acretocin mixture, comparing retention time and fragmentation behavior with those of a standard sample of TFA-Acc-isopropyl ester [2].

Four ten-residue lipoaminopeptides, culicinins A–D, were characterized from an Australian isolate of *Culicinomyces clavisporus* (Hypocreales, Clavicipitaceae). Strain LL-12I252 was isolated from larvae of the biting midge *Forcipomyia marksae* (Diptera, Ceratopogonidae). Culicinins exhibit a number of common structural features: their N-terminal Pro residue is protected by butanoic acid (BTA). In 2-position, 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD) is found. Another lipoamino acid, 2-amino-4-methyldecanoic acid (AMD), is situated in position 5, while β-alanine (3-aminopropionic acid; APA) is located in position 9 of the peptide chain. The C-terminus is substituted by 2-(2′-aminopropyl)-aminoethanol (AAE), here referred to as APAE. The lipoamino acid AHMOD is present in position 2 of almost all lipoaminopeptides, being the characteristic structural element of this subgroup of the peptaibiotics. C-Terminal APAE has previously been described for trichopolyns, helioferins, and roseoferins. Notably, BTA and AMD have been detected in peptaibiotics for the first time, thus representing novel structural elements. Selective

inhibitory activity against PTEN-negative MDA468 tumor cells has been observed [187]. Furthermore, eight new eleven-residue peptaibols, trichobrachins A I–IV and B I–IV, as well as one trichorovin [110] were reported from *T. longibrachiatum* MMS 151, a marine strain that has been isolated from the cockle *Mytilus edulis* [188].

2.4.12. *New Sequences and Bioactivities: Inhibition of Amyloid β-Peptide Formation by Peptaibols.* In 2007, *T. longibrachiatum* MMS 151 was reinvestigated by LC/MS, and a total of 30 eleven-residue trichobrachins A and C were sequenced, 21 of which were regarded as new [189].

Notably, *Hosotani et al.* [190] demonstrated that sophisticated approaches may lead to the detection of novel or previously unrecognized biological activities of peptaibiotics. These authors reported on the inhibition of amyloid β-peptide formation in primary guinea pig cerebral cortex neuron cell cultures by a 14-residue peptaibol, SPF-5506-A. It is known that increased accumulation of amyloid β-peptides plays a decisive role in the pathogenesis of neurodegenerative dementia such as *Alzheimer*'s disease. Produced by *Trichoderma* sp. SPF-5506, peptaibol SPF-5506-A_4 can be considered as a positional isomer of the 14-residue harzianin HC [112]. Thus, it can be hypothesized that harzianins and structurally related peptaibols might display similar bioactivities.

Two 20-residue peptaibiotics, septocylindrins A and B, have been isolated from the fungus LL-Z1518 [191]. Amino acid residues 1–19 and configurations of the amino acids are identical with those reported for alamethicin F30/3 of alamethicin F50/5, respectively. Septocylindrins differ from alamethicins only in the structure of the C-terminal alcohol, which is 2-[(2-amino-3-phenylpropyl)amino]ethanol (Phaol). Such C-terminus has only been described for aibellin before [125]. However, the identification of the producer as *Septocylindrium* sp. is questionable as the authors did not provide taxonomic data to verify its identity [2].

2.4.13. *Cyclopeptaibiotics: a New Subfamily of Peptaibiotics.* In 2008, a new subfamily of peptaibiotics, the so-called cyclopeptaibiotics, was introduced comprising all cyclic Aib- and DL-Iva-containing cyclic peptides of natural origin that have been described from 1974 until present [3]. Currently, seven cyclotetrapeptides and two cycloheptapeptides are found in this small subgroup. Chlamydocin [192] can be regarded as the classical paradigm of a cyclopeptaibiotic. For detailed information on sequences and bioactivities, and ecophysiological and taxonomic importance of cyclopeptaibiotics and related cyclic peptides, the reader is referred to the exhaustive review [3].

3. 'Peptaibiomics' and 'Intact Cell MALDI-TOF-MS' – Two State-of-the-Art Techniques for Rapid Screening and Sequencing of Peptaibiotics. – During the past two years, peptaibiomics and 'Intact cell MALDI-TOF-MS' (IC-MS) have increased considerably the screening efficiency for peptaibiotics but also contributed to progress in chemotaxonomy of *Trichoderma*/*Hypocrea* as outlined below [193]:

3.1. *Peptaibiomics.* The method of **peptaibiomics** is aimed on the characterization of the entirety of peptaibiotics produced by a fungal strain under defined culture conditions, the so-called **peptaibiome** [194]. It also may include the time-dependent dynamics of biosynthesis and degradation of peptaibiotics as exemplified for the extremely microheterogeneous trichobrachins from *T. ghanense* CBS 936.69 [7].

The workflow comprises solid-phase extraction (SPE) of peptaibiotics on C_{18}-cartridges, followed by on-line reversed-phase (RP) HPLC coupled to an ion-trap electrospray tandem mass spectrometer (ES-MS). Such LC/MS-based peptaibiomics approach is based on the generation of so-called 'in-source fragments' by 'collision-induced decomposition' mass spectrometry (CID-MS) to the skimmer region of an ESI mass spectrometer. Varying voltages are applied to the skimmer, thus providing the possibility of clearly differentiating various types of ions. The CID-MS technique provides a possibility to partially sequence peptaibiotics. The most diagnostic difference to screen for is Δ (m/z) 85, which indicates the presence of the α-aminoisobutyric (Aib) residue that can be observed in the *b*- or *y*-series of acylium fragment ions. Another diagnostic difference is Δ (m/z) 99, indicative for a Vxx residue (*i.e.*, isomeric valine or D- or L-isovaline). Complete sequencing is achieved by a combination of data from CID-MS, MS/MS, QTOF-MS, and MS^n (in the case of ion-trap instruments). These data are generally needed to be combined with HPLC-elution profiles, the so-called fingerprints of the samples. Peptaibiomics was first used to analyze the peptaibiome of recently described species of *Hypocrea* and *Trichoderma*. Peptaibiotics produced by the following strains were partially sequenced and compared to published sequences: *Hypocrea muroiana* MUCL 28442, *H. nigricans* MUCL 28439, *H. gelatinosa* CBS 724.87, *H. dichromospora* CBS 337.69, H. *vinosa* CBS 247.63, *H. semiorbis* CBS 244.63, and *H. citrina* (syn. H. *lactea*) CBS 853.70; *Trichoderma asperellum* CBS 433.97, *T. aggressivum* f *europaeum* CBS 100526, *T. inhamatum* CBS 345.96, and *T. stromaticum* CBS 101875 [194].

Trichobrachin (TB) has been isolated from strain CBS 936.69. This particular *Trichoderma* strain has originally been identified as *T. longibrachiatum* but is now reclassified as *Trichoderma ghanense* (syn. *T. parceramosum*). Three major groups designated TB I, TB II, and TB III could be separated and isolated by preparative TLC on silica gel. The trichobrachin mixture comprises ten 19-residue peptides with free C-terminal Gln residues (TB I peptides), two 18-residue peptides with free C-terminal Gln residues (TB II 1 und TB II 2), seven 20-residue peptides with C-terminal amide-bonded Pheol (TB II 3–10), and 34 eleven-residue peptides with either C-terminal Leuol, Ileol, or Valol (TB III 1–34). TLC Analysis of the dynamics of TB formation and degradation unequivocally demonstrated that those two 18-residue TB I and TB II peptides with a free carboxy terminus resulted from enzymatic C-terminal degradation of 20-residue TB II peptides [7].

Three strains of *Trichoderma brevicompactum* (CBS 109720, CBS 119569, and CBS 119570) and another four, which are closely related to that species (*Trichoderma* cf. *brevicompactum* CBS 112445, CBS 119576, CBS 119577, and NRRL 3199) were analyzed for the formation of peptaibiotics. These isolates were selected because of an antagonistic potential against *Eutypa* dieback and *Esca*, fungal diseases of grapevine (*Vitis vinifera*) trunks that have not yet been investigated for the production of peptide antibiotics. All strains were found to produce membrane-active alamethicins F30 (see above). In addition to that, novel peptaibols were detected, namely fourteen twelve-residue trichocryptins B, twelve eleven-residue trichocryptins A, nineteen eleven-residue trichobrevins A and B, six ten-residue lipoaminopeptides – trichoferins, and seventeen seven-residue trichocompactins. Taken together, the differential patterns of alamethicin production as well as the production of different trichothecene-type

mycotoxins clearly support DNA-sequencing results. Both molecular and chemotaxonomic approaches indicated the existence of – at least - two species within what has been called *Trichoderma brevicompactum*, so far [10].

3.2. *Intact-Cell MALDI-TOF-MS* (IC-MS). This method permits detection of biomolecules in unfractionated biomass down to the attomolar level. The standard IC-MS sample preparation process involves suspending of a few μg of fungal mycelium or spores in a mixture of organic solvents that is immediately mixed with the matrix solution and subsequently spotted onto a well of the MALDI target plate. Finally, the air-dried samples are analyzed by MALDI-TOF-MS. On the basis of the facile sample preparation avoiding extensive workup procedures that may potentially generate artefacts, this method is recommended for high-throughput screening approaches.

In the case of fungal samples, the method is routinely used for analysis of hydrophobin patterns [195], but it also permits detection of peptaibiotics in the range of $m/z > 800$ [6][12].

Using IC-MS, *Neuhof et al.* demonstrated that the production of chrysospermins is rather widespread within the genus *Sepedonium*, although not all species screened have been found to produce peptaibiotics. *S. chrysospermum*, *S. ampullosporum*, *S. laevigatum*, and *S. brunneum* were positively screened, whereas *S. chlorinum* and *S. tulasneanum* did not produce peptaibiotics under the conditions investigated. Notably, *S. chalcipori* produced two novel 15-residue chalciporins A and B in addition to chrysospermins [157].

3.3. *A Multiphasic Approach: Linking Peptaibiomics and Intact-Cell MALDI-TOF-MS with State-of-the-Art Approaches in Chemotaxonomy and Molecular Biology.* Recently, IC-MALDI-TOF-MS, peptaibiomics, and LC/HR-ESI-MS mycotoxin screening of *Trichoderma*/*Hypocrea* were linked to morphological, molecular taxonomic, and phylogenetic methods. This multiphasic approach was aimed at resolving the inconsistencies within the different lineages of the '*Trichoderma brevicompactum* complex' mentioned above [10]. It culminated in the introduction of a new lineage in *Trichoderma*/*Hypocrea*, the *Brevicompactum* clade. This clade included, in addition to *T. brevicompactum*, three new species in the *T. brevicompactum* complex, and an additional new species, *Hypocrea rodmanii*.

Profiling additional isolates of *T. brevicompactum* (CBS 112444, CBS 112446, CBS 112447, and CBS 121154), and the new species *T. arundinaceum* (CBS 119575 and CBS 121153), *T. protrudens* (CBS 121320), and *T. turrialbense* (CBS 122554), 27 new eleven- and twelve-residue trichocryptins and trichobrevins were sequenced. In this context, the presence of alaninol (Alaol) as a new C-terminal constituent of trichocryptins C and D was established. Notably, the two cultures of *H. rodmanii*, CBS 109719 and CBS 120895, displayed a completely different inventory of peptaibiotics, namely two 20-residue trichokonins, five new 14-residue peptaibols, hyporodicins A–E, and six new hypocompactins I–VI. Four of the latter could be assigned as seven-residue lipopeptaibols, carrying a C_8 mono-unsaturated N-terminal fatty acid residue, probably octenoyl [12].

By a similar profiling approach, *Stoppacher et al.* [196] sequenced 15 novel trichoatrokontins, the C-terminus of which has not been elucidated yet. The trichoatrokontin-producing strain, *T. atroviride* ATCC 74058, was previously shown to produce 16 trichorzianins [197].

4. Outlook and Future Prospective – Towards a Myriad of Peptaibiotics! – Currently, more than 850 individual sequences of peptaibiotics are reported in literature. Approximately half of them have been discovered within the past five years; and the number of peptaibiotics to be discovered within the next decade is expected to rise exponentially.

4.1. *Modern Methods of Mass Spectrometry to Detect Femtogram Amounts of Peptaibiotics.* A first, major reason to explain this phenomenon is that the analytical equipment used for screening and structural elucidation of peptide antibiotics is becoming more and more sophisticated, as illustrated by the recently introduced methods of peptaibiomics and IC-MS. For IC-MS, one single loop of conidia or mycelium from a fully grown *Petri* dish is sufficient; for a standard peptaibiomics approach, the material grown on a single slope or *Petri* dish is required.

For trace analysis, in general, HPLC-MS/MS on triple quadrupole instruments is state-of-the-art, because both the sensitivity (down to low fg levels) and specificity are excellent. Ion-trap mass spectrometers enable the detection and sequencing of homologues and positional isomers, even if present in trace amounts. Thus, triple quadrupole MS, ion-trap MS, and MALDI-TOF-MS should be regarded as complementary methods and combined – whenever possible.

4.2. *Exploring the World of Fungal Biodiversity.* Second, screening of fungi from highly specialized habitats such as fungicolous [128–130][132–136][159][182], coprophilous [164][165], and entomopathogenic [183][187] species remarkably enhances the probability to find new producers and sequences of peptaibiotics. During the last few years, peptaibiotic-producing fungi from marine habits have gained increasing interest [188][189][198–201]. Considering that the vast majority of peptaibiotics known to date was isolated from terrestrial fungi, one has to keep in mind that more than 70% of the earth's surface is covered by oceans. Consequently, we have to admit that we are just beginning to understand and exploit aquatic habits as a proliferating source of novel bioactive secondary metabolites, including peptaibiotics.

Increased sampling also revealed endophytic fungi as promising sources of new peptaibiotics, such as *Trichoderma stromaticum* [11] and *T. protrudens* [12]. The discovery and characterization of new fungal species in previously unexplored regions [202–204], and in ecological niches such as woody tissues of trees [205–208], will undoubtedly lead to the discovery of new producers, novel constituents, and eventually uncommon building schemes of peptaibiotics.

For instance, the genus *Stilbella* containing many coprophilic species can still be regarded as a promising source of peptaibiotics. Besides the antiamoebin- and stilboflavin-producing cultures mentioned above, the following strains were positively screened for Aib and/or Iva: *S. aciculosa* CBS 546.84 and CBS 476.85, *S. albocitrina* CBS 649.83, *S. annulata* CBS 450.85, CBS 185.70, and CBS 545.85, *S. fimetaria* CBS 558.84 and CBS 117.84, IMI 080670 and IMI 090138 as well as *S. fusca* CBS 826.85, and *Remersonia thermophila* (syn. *S. thermophila*) CBS 409.63 [86].

Less common species of ascomycetous fungi have previously been screened positively for the presence of Aib and/or Iva in the pioneering work of *Reinecke* [92] and *Becker* [93] but not yet investigated for peptaibiotics, which were recently compiled and commented by *Degenkolb et al.* [2]. As most of those strains belong to families

outside the Hypocreaceae and Clavicipitaceae, the detection of novel building schemes and structural elements of peptaibiotics can be expected.

In this context, it has to be emphasized that peptaibiotics are not only biosynthesized by highly specialized fungi occupying ecological niches. It always should be considered that they are also produced by ubiquitous, often cosmopolitan fungi, occurring both in temperate and tropical soils that are highly competitive habits [12].

It appears worth mentioning here that the occurrence of Aib and racemic Iva in sediments close to the the cretaceous–tertiar (K/T) boundary has been reported [209]. The authors attributed their occurrence to the impact of a carbonaceous meteorite. Some meteorites, such as the *Murchison*, contain Aib as well as non-racemic mixtures of chiral C^{α}-dialkylamino acids, indeed [210]. However, based on the abundance of data presented here, a microbial origin of Aib and Iva in sediments cannot be excluded [12][104][165]. Notably, recently peptaibiotics were partially sequenced in fresh and frozen marine sediments collected in France [200].

4.3. *Fungal Taxonomy Revolutionized by DNA Sequencing*. Third, substantial progress in the taxonomy of *Trichoderma*/*Hypocrea*, the most important source of peptaibiotics, was achieved after the introduction of DNA sequencing in the middle of the 1990s, providing a precision in species identification and species boundaries that was lacking in purely morphological taxonomy. Prior to 1969, only one single species of *Trichoderma*, *T. viride*, was known. Taxonomy of the genus was first revised by *Rifai* who distinguished nine species [211]; and *ca.* 40 species were described in 1991 [212–215]. In 2005, a total of 88 species was recognized [216], and, one year later, already 100 *Trichoderma* species were characterized by molecular methods [217]. Currently, almost 140 species are distinguished, and *Trichoderma* stands as one of the few species-rich genera of microfungi for which all known species have been cultured, and for which sequences of two or more genes have been deposited in GenBank [193]. Recently, the species concept of *Trichoderma brevicompactum*, for instance, was thoroughly revised, thus leading to the introduction of the *Brevicompactum* clade, which comprises, in addition to *T. brevicompactum*, three closely related species, *viz*. *T. protrudens*, *T. turrialbense*, and *T. arundinaceum*. It should be emphasized here that *T. viride* NRRL 3199, the classical source of alamethicins, was reidentified as *T. arundinaceum* [12]. Considering these recent revisions, the identity of the suzukacillin-producing strain *Trichoderma viride* 63 C-I is also highly questionable, but the unavailability of this strain precludes its taxonomic reinvestigation. Notably, most of the peptaibiotics-producing species formerly included in *Gliocladium* [218] are now reclassified in *Trichoderma* [219]. Prior to 2000, peptaibiotics were in most cases attributed to four 'common' species, *T. viride*, *T. koningii*, *T. harzianum*, and *T. longibrachiatum*. However, the identity of those cultures that have sometimes even been deposited under a synonym such as *T. album*, '*T. todica*', *T. virgatum*, or *T. lignorum* [82][83] is generally in doubt, or has already shown to be incorrect. In fact, the identity of none of those *Trichoderma viride* cultures reported to produce peptaibiotics has been verified by molecular methods.

Furthermore, the identity of peptaibiotics-producing strains described and deposited as *Hypocrea* sp. need to be reinvestigated and, most probably, revised. While *ca.* 400 species of *Hypocrea* have been described over the past 175 years, few of them have

been seen since they were first described, and even fewer have been linked to *Trichoderma* stages.

Certainly, these authors cannot be criticized, as it has only been in the last few years that sufficient sequences of correctly identified *Trichoderma*/*Hypocrea* species have been generally available through the ISTH web site and GenBank [220]. Nevertheless, statements about *Trichoderma*/*Hypocrea* species must always be considered with great caution unless the authors of those reports describe how their cultures were identified.

In fact, most of the currently recognized species of *Trichoderma*/*Hypocrea* have not yet been systematically screened for the formation of peptaibiotics. Complete or partial sequences have been obtained from approximately 30 verified species; and another ten recently described species were positively screened for the presence of peptaibiotics by IC-MS [6].

Currently, other peptaibiotic-producing fungal genera such as *Paecilomyces* are in the ongoing process of fundamental taxonomic revision based on DNA sequencing. Some entomopathogenic species formerly included in *Paecilomyces* have already been shown to group in the monophyletic Isaria clade [221] and were thus reclassified in the genus *Isaria*. For instance, *P. lilacinus* [16–25][222] and *P. marquandii* [26–31] will soon be redescribed as two separate species. The leucinostatin-producing '*P. abruptus*' [223] seems to be closely related to *P. lilacinus*; however, it appears that this species has never been validly described [224].

5. Conclusions. – To date, peptaibiotics have been reported from *ca.* 25 genera of fungi [2][3][127], but we currently know several thousand fungal genera, which are represented by an exponentially growing number of species. Considering the intra-specific genetic variability, an infinite number of strains from different geographic regions and habitats can be predicted – just waiting to be exploited. Owing to the ubiquity and biodiversity of their producers, we predict that a myriad of new sequences of peptaibiotics will be discovered within the next decade!

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Note Added in Proof: Applying a novel screening approach, which combines HPLC/bioactivity profiling-microtiter plate fractionation with subsequent capillary NMR analysis [225], a new 15-residue peptaibol, chrysaibol, from the fungicolous strain CANU E609 was characterized. The producer has been isolated from a decaying fruiting body found in the Kamai Ranges near Kaitaki, Bay of Plenty, New Zealand, and reported as *Sepedonium chrysospermum* [226]. Chrysaibol exhibits structural homology with ampullosporin A [134], both displaying the common motif Gln-Aib-Aib-Aib-Gln-Leu-Aib and the N-terminal AcTrp residue. Because of the latter, we predict a neuroleptic activity for chrysaibol, in addition to the cytotoxic (P388 murine leukemia cells) and antimicrobial effects (*Bacillus subtilis*) reported. Chrysaibol [225] is one of the very few peptaibols [10][12] known to date, which carry a C-terminal Alaol residue. The marine fungus *Acremonium* sp., strain 021172cKZ from Papua New Guinea, associated with the sponge *Teichaxinella* sp. (Halichondria, Axinellidae) [199], was shown to produce a mixture of five efrapeptins – E, F, G, E_a, and H, the latter two being new homologues [227]. The sequence reported here as efrapeptin H was supposed to be identical with the peak displaying a molecular ion m/z 1662 that has previously been assigned as efrapeptin H [123][228]. Notably, this strain coproduced two cycloheptapeptaibiotics, scytalidamide A and B [3][229]. This observation led to a reinvestigation of the

two scytalidamide-producing strains 021172cKZ and CNC310 by methods of molecular taxonomy, confirming that both isolates belong to the genus *Acremonium* [227]. A new down-regulator of the molecular chaperone GRP78, efrapeptin J, displaying a molecular ion *m*/*z* 1620, was recently isolated from a marine fungus, *Tolypocladium* sp. AMB18 [230]. Further, two efrapeptin peptaibiotics exerting interesting bioactivities have been described: adenopeptin from *Chrysosporum* sp. induces apoptotic cell death in cells transformed with the adenovirus oncogens [231]; this peptaibiotic contains an unusual Ac-Pro N-terminus as well as four Iva-residues and five Aib-residues; and efrapeptin J from a marine *Tolypocladium* sp. represents as a new down-regulator of the molecular chaperon GRP78 [232].

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Aib and Iva in the Biosphere: Neither Rare nor Necessarily Extraterrestrial

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Fourty-nine species and strains of filamentous fungi of the genera *Acremonium*, *Bionectria*, *Clonostachys*, *Emericellopsis*, *Hypocrea/Trichoderma*, *Lecythophora*, *Monocillium*, *Nectriopsis*, *Niesslia*, *Tolypocladium*, and *Wardomyces*, deposited with the culture collection of the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands, were grown on nutrient agar plates. Organic extracts of mycelia were analyzed after acidic total hydrolysis and derivatization by GC/SIM-MS on *Chirasil*-L-Val for the presence of Aib (= α-aminoisobutyric acid, 2-methylalanine) and DL-Iva (= isovaline, 2-ethylalanine). In 37 of the hydrolysates, Aib was detected, and in several of them D-Iva or mixtures of D- and L-Iva. Non-proteinogenic Aib, in particular, is a highly specific marker for a distinctive group of fungal polypeptides named **peptaibols** or, comprehensively, **peptaibiotics**, *i.e.*, **pept**ides containing **Aib** and displaying (anti)b**iotic** activities. The biotic synthesis of these amino acids by filamentous fungi contradicts the still widespread belief that α,α-dialkyl-α-amino acids do not or rarely occur in the biosphere and, if detected, are of extraterrestrial origin. The abundant production of peptaibiotics by cosmopolitan species of microfungi has also to be considered in the discussion on the occurrence of Aib and Iva in ancient and recent sediments. The detection of trace amounts of Aib in ice samples of Antarctica that are devoid of meteorites might also be related to the presence of Aib-producing microorganisms, being either indigenous psychrophiles, or being transported and localized by mechanisms related to bioaerosols and cryoconites. The presence of microfungi being capable of producing α,α-dialkyl α-amino acids in terrestrial samples, and possible contamination of extraterrestrial materials are pointed out to be of relevance for the reliable interpretation of cosmogeochemical data.

1. Introduction. – Certainly, nobody could have predicted half a century ago that the detection of the non-proteinogenic α,α-dialkyl amino acid Aib (= α-aminoisobutyric acid or 2-methylalanine) in a peptide antibiotic produced by a taxonomically unidentified species of the microfungus *Paecilomyces* [1] would result in the characterization of *ca.* 850 microheterogeneous peptide sequences, produced by at least 23 genera of ascomycetous fungi or their anamorphs. Owing to its unique constituent, the acronym **peptaibiotics** became established for this group of **pept**ides containing **Aib**

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and showing (anti)b**iotic** activities. The ubiquitous anamorphic genus *Trichoderma* (with teleomorphs in *Hypocrea*) is generally regarded as the richest source of peptaibiotics. To date, the presence of Aib and/or Iva has been reported from *ca.* 32 genera of fungi (including those described here) that comprise hundreds of accepted species and a literally unlimited number of strains, being omnipresent in the biosphere [2].

Since the history and the current status of peptides containing Aib, its chiral homologue Iva, and some other α,α-dialkyl amino acids have extensively been treated in recent reviews, we refer to the literature, which also includes two special issues of journals and a book devoted to this unique group of peptides [3]. Structures and configuration of α,α-dialkyl amino acids detected in natural peptaibiotics are presented in *Fig. 1*.

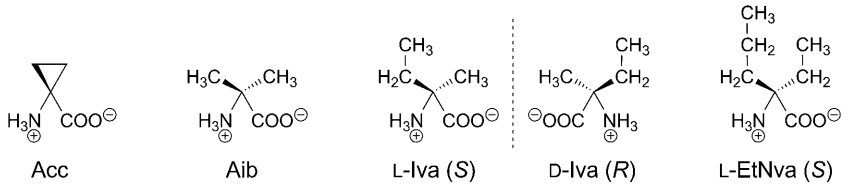

Fig. 1. *Structures and configurations of α,α-dialkyl amino acids*

Knowledge of the non-ribosomal synthesis of peptaibiotics and characterization of the multi-enzyme complexes involved, as well as the biosynthesis of their uncommon constituents [4] and the interaction of these helical peptides with other biomolecules is of interest not only for academia but also for the industry that is particularly interested in new biocides and plant-protecting agents [5].

Beyond such application-oriented considerations, one should keep in mind that fungi represent an ancient kingdom of eukaryotic, multicellular organisms, apart from plants and animals. Owing to their common feature of absorptive heterotrophy or chemoorganotrophy, *i.e.*, exploitation of nutrients from digestion of living or dead tissues, fungi play a decisive role in the biosphere that cannot, or only less effectively, be accomplished by other organisms.

Since *ca.* 1.5 million species of fungi are estimated to exist of which *ca.* 90,000 have been described [6], a multitude of bioactive secondary metabolites produced is waiting to be explored. Among those, the definite role of peptaibiotics and their constituting α,α-dialkyl amino acids in the life styles and survival strategies of their fungal producers in their natural habitats still has to be unravelled.

Microbiologists, chemists, and pharmacologists have become increasingly aware of the potential of the peptaibiotics in recent years. They are excited by the challenges of their sequence determination [7], problems encountered in the total syntheses of the natural peptides and synthetic analogues [8], biocidal activities [9], and their unique capability of forming transmembrane channels in lipid bilayer membranes [10].

Notably, the majority of astrobiologists as well as geo- and cosmochemists still consider Aib and Iva as 'exotic' amino acids, being absent or extremely rare in the biosphere and, if detected, of abiotic origin. Therefore, Aib and Iva are still regarded as

relatively safe markers for extraterrestrial materials, in particular carbonaceous meteorites [11].

In continuation of previous work and ongoing research, here, we present data resulting from the screening of filamentous fungi that have not or only sporadically been investigated for the production of peptaibiotics, and we discuss possible aspects regarding the occurrence and release of Aib and Iva in the biosphere.

2. Results. – Based on previously reported screening procedures for Aib and Iva [2], filamentous fungi were grown on solid agar media in *Petri* dishes. After vigorous growth had been observed, the cultures were treated with $MeOH/CH_2Cl_2$, the organic extracts were filtered, totally hydrolyzed, derivatized, and analyzed for the presence of Aib and Iva by using GC/SIM-MS. Although free Aib and Iva, serving as building blocks for the non-ribosomal enzymatic synthesis of peptaibiotics, are also detectable in unhydrolyzed extracts of the corresponding fungi, hydrolysis was performed in order to release peptide-bonded Aib and Iva.

Achiral Aib as well as Iva could be detected, and the enantiomers of the latter were partly (*N*-PFP-D,L-Iva-2-Pr esters) or completely (*N*-Ac-D,L-Iva-1-Pr esters) separated on *Chirasil*-L-Val capillary columns (*cf. Fig. 2*).

Table 1 comprises names of fungi studied; CBS accession numbers, geographic origin and substrates (if known), presence or absence of Aib and Iva, and the configuration of the latter, whenever possible. Sections of chromatograms displaying the presence of Aib and, in many cases, Iva are presented in *Fig. 2.* As can be seen, Aib is of higher abundance, and Iva is present either in (*R*) (=D)-configuration [12], or as a mixture of (*R*)- and (*S*)-(=L)-enantiomers. Using Ac-DL-Iva-1-Pr esters, resulting in baseline separation of enantiomers in *Clonostachys rosea* (syn. *Gliocladium roseum*, teleomorph *Bionectria ochroleuca*), 41.4% of D-Iva and 56.6% of L-Iva could be detected corresponding formally to an enantiomeric excess (ee) of 15.5% L.

As shown in *Table 1*, 37 strains of fungi were tested positively for the production of Aib and, in part, Iva. The geographic origins of the fungi given in the *Table* also demonstrate that they are cosmopolitan, and their natural habitats comprise in particular soil and decaying plant materials. These fungi had been selected mainly in view of morphological diversity and without emphasis on geographical diversity. Notably, three species of *Acremonium* were also involved in fungal infections or lesions of man.

Species of *Trichoderma* (with teleomorphs in *Hypocrea*) have already been recognized as abundant producers of peptaibiotics; many of these peptaibiotics have been sequenced, and their bioactivities have been characterized. In addition to *Trichoderma/Hypocrea*, species and strains of the genera *Acremonium*, *Bionectria*, *Clonostachys* (incl. *Sesquicillium*), *Emericellopsis*, *Lecythophora*, *Monocillium*, *Nectriopsis*, *Niesslia*, *Tolypocladium*, and *Wardomyces*, which have not or less intensively been screened for the production of Aib and Iva, are shown here to be potential producers of peptaibiotics and are listed in *Table 1.*

The fungi were grown in surface culture on standard media selected to maintain morphological characteristics. Consequently, growth represents just a small section of the life style of the corresponding organism. To exploit the complete potential of peptaibiomics, *i.e.*, the sequential characterization and dynamics of the entirety of

Table 1. *CBS Strains Investigated in This Study*

Species	CBS[a])	Habitat; geographic origin	Aib[b])	Iva[b])	CM[c])
Acremonium berkeleyanum	234.70	*Fomes fomentarius*; Poland, Białowieża National Park	+	–	1
A. charticola	219.70	Old leaf of *Tussilago farfara*; Germany, Kiel-Kitzeberg	+	D	1
A. crotocinigenum	217.70	Decaying wood; Germany, Kr. Plön, Schüttbrehm	+	D	1
A. chrysogenum	144.62	Soil; India, Maharashtra, Pimpri-Poona	+	–	1
A. curvulum	523.72	Leaf litter of *Acacia karroo*; South Africa	+	–	1
A. curvulum	430.66	Wheat-field soil; Germany, Kiel-Kitzeberg	–	–	1
A. curvulum	384.70A	Greenhouse soil; Netherlands	+	–	1
A. inflatum	305.74	Cereal stem; UK, Rothamsted	–	–	1
A. ochraceum	797.69	Decaying leaf of *Canna indica*; Netherlands, Baarn	–	–	1
A. radiatum	142.62	Soil; India, Karad	–	–	1
A. recifei	362.76	Soil under *Saccharum officinarum*; Taiwan	–	–	2
A. recifei	402.89	Forest soil; Brazil, Pará, Capitão Poço	+	D	2
A. recifei	188.82	*Ulcus corneae* of man; Netherlands, Akad. Ziekenhuis, University of Amsterdam	+	D	2
A. recifei	485.77	Granulomatous lesion, man; India	+	–	2
A. recifei	647.93	Bark and wood of declining *Macadamia integrifolia*; USA, Hawaii	+	–	2
A. recifei	442.66	Nut of *Bertholletia excelsa*	+	–	1
A. sclerotigenum	384.65	Deformed toe nail of man	+	D	1
A. strictum	376.70 H	Peat bog; Ireland	+	–	1
Bionectria pityrodes	322.78	*Vitis*; Venezuela, Edo Miranda, near El Bachiller	+	D,L	2
Clonostachys rosea (anamorph)/	906.72D	Decaying angiosperm wood; Netherlands	+	D,L	3
Bionectra ochroleuca (teleomorph)	194.57	Decaying bulb of *Lilium auratum*; USA	+	D,L	2
Clonostachys candelabrum (syn. *Sesquicillium candelabrum*)	205.69	*Equisetum hyemale*; dead stem	+	D,L	2
Emericellopsis glabra	376.64	Soil, under *Solanum tuberosum*; Netherlands	+	D	2
E. minima	107.64	Soil; Turkey	+	–	1
E. minima	716.73	*Upjohn Comp.*; USA, Kalamazoo	+	D	1
Hypocrea atrogelatinosa	237.63	*Bondarzewia berkeleyi*; New Zealand, Auckland, Mamaku	+	D	1
H. citrina var. *citrina*	977.69	*Fomitopsis pinicola*; Canada, British Columbia, North Vancouver	+	–	1
H. tawa	246.63	*Coprosma robusta*; New Zealand, Little Barrier Island	+	–	2

Table 1 (cont.)

Species	CBS[a])	Habitat; geographic origin	Aib[b])	Iva[b])	CM[c])
H. vinosa	247.63	New Zealand, Auckland, Te Aroha	+	D	1
Lecythophora mutabilis	303.62	Soil; Netherlands	+	–	3
Monocillium mucidum	306.70	Soil	+	–	1
M. nordinii	101.63	Wood of *Pinus contorta* v. *latifolia*; Canada, Alberta, Stracham	+	–	1
M. nordinii	116.70	Wood of *Pinus contorta* var. *latifolia*; Canada, Alberta, Kananaskis Forest Exp. Station	–	–	1
Nectriopsis candicans	627.72	*Leocarpus fragilis*, on *Prunus laurocerasus*; Netherlands, Doorwerth	+	D,L	1
Niesslia aemula	556.75	Burnt soil; Netherlands, Noordoostpolder, Schokland	+	D	1
N. exigua	152.68	*Chamaerops humilis* in greenhouse; Netherlands, Baarn, Cantonspark	+	–	1
N. exilis	560.74	Decayed needle of *Pinus sylvestris*; UK, England, Warwicksh., Hay Wood	+	D	1
N. exilis	389.70A	Wooden clog; Netherlands, Andijk	–	–	1
N. ilicifolia	459.74	Decaying leaf of *Ilex aquifolium*; UK, Warwicksh., Banzey, Mockley Wood	–	–	1
N. subiculosa	326.77	Rachis of *Cyathea dealbata*; New Zealand, Auckland Prov., Thames Co., Coromandel Forest Park, Kawaeranga Valley	–	–	1
N. exosporioides	359.70	Dead leaf of *Canna indica*; Netherlands, Baarn, Cantonspark	–	–	3
Tolypocladium cylindrosporum	717.70	Litter of *Pteridium aquilinum*; UK, England, Cumbria, Roudsea Wood	+	D	2
T. nubicola	944.72	Alpine tundra soil; Canada, Alberta, Rocky Mountains	+	D	3
Trichoderma pseudokoningii	817.68	Soil; Sri Lanka, Agalawatta, near Dartonfield, Rubber Research Institute	+	–	1
Wardomyces anomalus	299.61	Air cell of an egg, in cold storage in salt solution; Canada, Ontario, Ottawa, National Research Council Laboratory	–	–	1
W. columbinus	233.66	Sandy soil; Germany, Giessen	+	–	1
W. humicola	368.62	Wheat-field soil; Germany, Kiel-Kitzeberg	+	–	2
W. inflatus	367.62	Greenhouse soil under *Lycopersicon esculentum*; Belgium, Heverlee	+	–	2
W. inflatus	216.61	Decaying wood of *Acer* sp.; Canada, Quebec, Gatineau County, Ste Cécile de Masham	–	–	1

[a]) CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. Numbers refer to accession numbers of CBS; names listed in *Table 1* are used according to the CBS filamentous fungi database currently found at http://www.cbs.knaw.nl/databases/. Note that the identity of CBS 523.72, 305.74, and 647.93 has not yet been verified by DNA sequencing, and CBS 485.77 has to be placed outside *Acremonium* but still within the Bionectriaceae. [b]) Production/absence of Aib and Iva is denoted with + and –, resp.; D,L refers to a mixture of enantiomers detected, and D to single enantiomer (*cf. Table*). [c]) CM, culture medium: 1, oatmeal agar; 2, cornmeal agar; 3, malt extract agar.

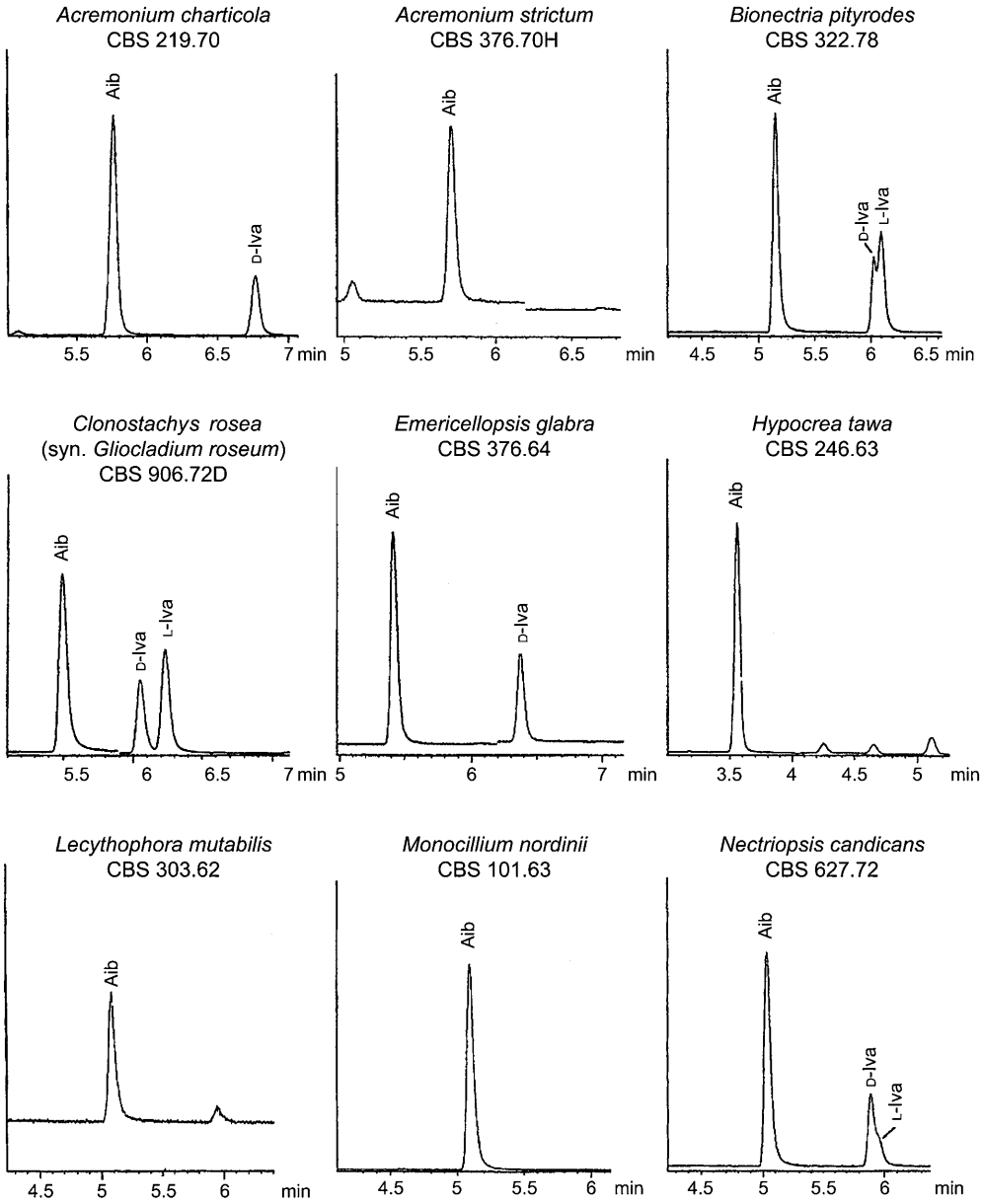

Fig. 2. *Detection of Aib and Iva enantiomers in derivatized total hydrolysates of organic extracts of fungal mycelia.* Sections of GC/SIM-MS of representative fungi from *Table 1* are shown. Numbers under the names of fungi refer to CBS strain numbers. Amino acids were analyzed as *N*-PFP-2-propyl esters on *Chirasil*-L-Val with the exceptions of CBS 906.72D that was analyzed as *N*-acetyl-1-propyl ester, and CBS 246.63 that was analyzed as *N*-TFA-1-propyl ester on a *Lipodex E* column.

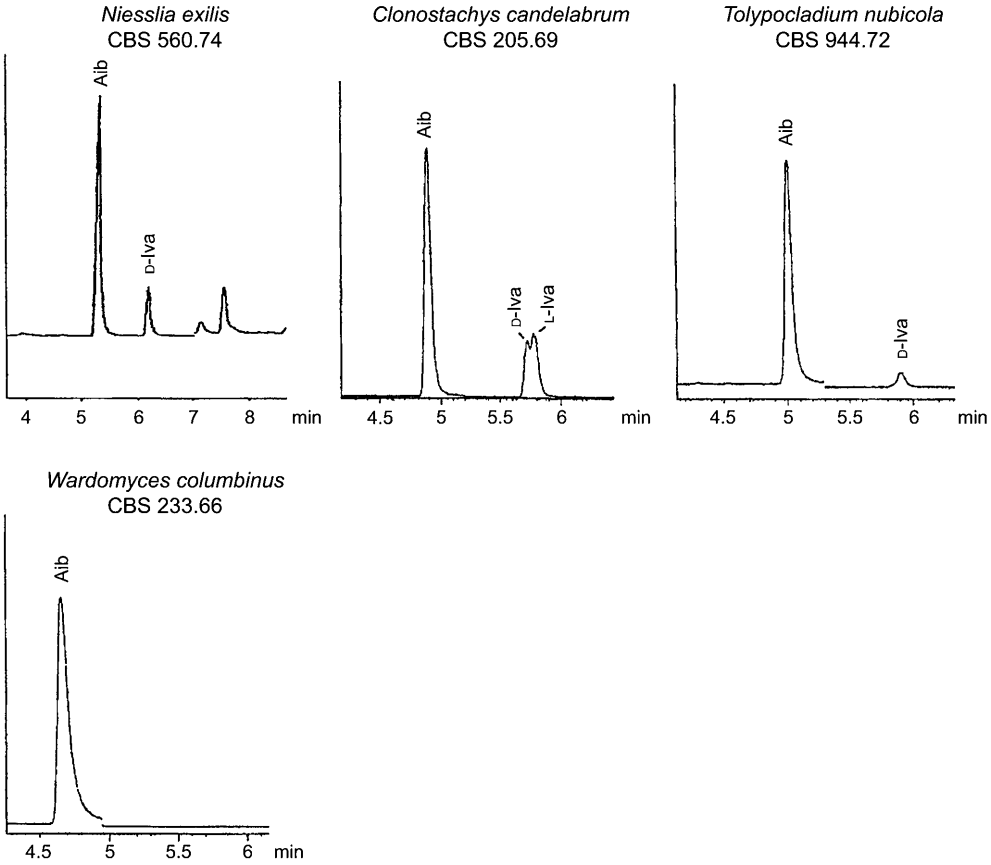

Fig. 2 (cont.)

peptides synthesized under defined conditions, fungi should be grown under varying culture conditions, including submerged fermentation.

3. Discussion. – 3.1. *Aib and Iva in Filamentous Fungi*. Total hydrolysates of fungal extracts were used for screening for Aib and Iva by GC/SIM-MS because of specificity and sensitivity for these marker amino acids. Instead of chiral stationary phases, use of capillary columns coated with thermally stable silicon oils, or complementary methods based on HPLC are possible [13]. The methods described enable the rapid screening of fungi for the presence of peptaibiotics prior to techniques of higher experimental and financial expenditure such as LC/ESI-MS^n or MALDI-TOF-MS. The latter methods, however, are the basis of the technical approach named peptaibiomics, *i.e.*, the complete sequential characterization of the entirety of individual peptaibiotics synthesized by a fungus under well-defined conditions in the laboratory or in its natural habitat [2].

Considering the microheterogeneity of these peptides, *ca.* 850 sequences of linear and cyclic peptaibiotics are currently reported in literature, many of which have not yet been included in the peptaibol database [2]. Consequently, Aib and Iva cannot be considered *a priori* as abiotic or rare in the biosphere. Remarkably, the CH_2 homologue of Iva, *i.e.*, 2-ethylnorvaline (EtNva; sometimes abbreviated EtNor) [14] as well as the cyclic analogue of Aib, *i.e.*, 1-aminocyclopropane-1-carboxylic acid (Acc) have also been detected as constituents of peptaibiotics [15]. This indicates that further α,α-dialkyl amino acids are fairly common in fungi. Thus, we predict that many more peptaibiotics are still waiting to be discovered by established screening methods. Despite the fact that fungi are preferably grown or fermented aseptically as pure cultures, production of antiamoebins (*cf. Fig. 1*) [16] by the coprophilous *Stilbella fimetaria* (syn. *S. erythrocephala)* has also been proven when growing on rabbit dung in the field. Production of up to 624 μg peptide/g fresh weight was observed [17]. Species of *Stilbella* and *Emericellopsis* produce particularly large amounts of peptaibiotics [18].

Names and sequences of representative peptaibiotics containing Iva in either the (*R*)- or (*S*)-configuration, corresponding to D- and L-Iva, respectively, or even both enantiomers, are compiled in *Table 2*. This *Table* also comprises all hitherto known peptaibiotics displaying Acc or EtNva residues. Fungal producers of the sequences are given in the *Table*'s legend.

The production of peptaibiotics, without exceptions, was also demonstrated by a recent thorough investigation of *Trichoderma/Hypocrea,* comprising *ca.* 30 contemporary, accepted as well as newly described species. They were characterized by modern methods of DNA sequencing and analyzed by 'Intact-Cell' (IC) MALDI-TOF mass spectrometry [19]. This work supports our considerations outlined below.

3.2. *Occurrence and Global Distribution of Filamentous Fungi as Potential Producers of Aib and Iva*. Biosynthesis of the α,α-dialkyl amino acids and peptaibiotics is correlated with growth and life styles of the fungal producers in their natural terrestrial or aquatic habitats. Like bacteria, fungi are found almost everywhere in the biosphere and constitute a considerable part of the terrestrial and aquatic biomass, particularly taking into account mycorrhizal and soil fungi. In forest soils, fungi exceed by far the volume of bacteria and may account for up to 445 kg dry mycelial matter per hectare.

For instance, *Trichoderma* spp., known as potential producers of peptaibiotics, were isolated from litter, soils, and the rhizosphere of *Populus* sp. and *Salix* sp. in the Danube National Park in Austria, representing a primeval floodplain-forest, and identified at the species levels by morphological and molecular techniques [20]. Consequently, global distribution of fungi and localization even in extreme environments as outlined below is to be expected, and long-range distribution of fungal materials has been established. Active discharge of spores of Asco- and Basidiomycota, resulting in the formation of biogenic aerosols in the atmosphere, is considered to account for a large proportion of the air particulate matter, reaching up to 100 μm in size, *e.g.*, in tropical rainforest regions. Such biogenic aerosols and airborne fungi are ubiquitous in the Earth's atmosphere, and thus play an important role in the spread of living microorganisms [21][22]. Air-borne propagules can serve as nuclei to initiate the formation of clouds, fog, and rain and, depending on temperature, also for snow, hail, and ice crystals [23]. Sand and dust loaded with fungi are globally distributed, and even

Table 2. *Selected Sequences of Peptaibiotics Containing Acc,* D-*Iva* (=(*R*)), L-*Iva* (=(*S*)), *and/or EtNva*[a])

		Residue[b])							
		1	2	3	4	5	6	7	8
Acretocin									
1a	Ac	Pip	Aib	Pip	D-**Iva**	Aib	Leu	*β*-Ala	Gly
1b	Ac	Pip	Aib	Pip	Aib	Aib	Leu	*β*-Ala	Gly
2	Ac	Pip	Aib	Pip	D-**Iva**	D-**Iva**	Leu	*β*-Ala	Gly
3	Ac	Pip	Aib	Pip	D-**Iva**	Aib	Leu	*β*-Ala	Gly
4	Ac	Pip	Aib	Pip	D-**Iva**	D-**Iva**	Leu	*β*-Ala	Gly
5	Ac	Pip	Aib	Pip	D-**Iva**	Aib	Leu	*β*-Ala	Gly
6	Ac	Pip	Aib	Pip	D-**Iva**	D-**Iva**	Leu	*β*-Ala	Gly
Neoefrapeptin									
A	Ac	Pip	Aib	Pip	D-**Iva**	Aib	Leu	*β*-Ala	Gly
C	Ac	Pip	Aib	Pip	D-**Iva**	Aib	Leu	*β*-Ala	Gly
D	Ac	Pip	Aib	Pip	Aib	Aib	Leu	*β*-Ala	Gly
E	Ac	Pip	Aib	Pip	D-**Iva**	D-**Iva**	Leu	*β*-Ala	Gly
Efrapeptin G	Ac	Pip	Aib	Pip	L-**Iva**	Aib	Leu	*β*-Ala	Gly
Adenopeptin	Ac	Pro	Iva	Iva	*β*-Ala	Gly	Aib	Aib	Iva
Cephaibol A	Ac	Phe	Aib	Aib	Aib	Aib	Gly	Leu	D-**Iva**
Cephaibol P	Ac	Phe	D-**Iva**	Gln	Aib	Ile	Thr	Aib	Leu
Integramide A	Ac	D-**Iva**	Hyp	Ile	L-**Iva**	Leu	Aib	Aib	Aib
Clonostachin	Ac	Aib	Hyp	Leu	D-**Iva**	Hyp	Leu	L-**Iva**	Hyp
Chrysospermin D	Ac	Phe	Aib	Ser	Aib	D-**Iva**	Leu	Gln	Gly
Boletusin	Ac	Phe	Aib	Ala	Aib	D-**Iva**	Leu	Gln	Gly
Tylopeptin A	Ac	Trp	Val	Aib	D-**Iva**	Ala	Gln	Ala	Aib
XR 586	Ac	Trp	D-**Iva**	Gln	Aib	Ile	Thr	Aib	Leu
Antiamoebin I	Ac	Phe	Aib	Aib	Aib	D-**Iva**	Gly	Leu	Aib
Emerimicin II B	Ac	Trp	Ile	Gln	D-**Iva**	Ile	Thr	Aib	Leu
Emerimicin III	Ac	Phe	Aib	Aib	Aib	Val	Gly	Leu	Aib
Zervamicin I A	Ac	Trp	Ile	Glu	D-**Iva**	Va	Thr	Aib	Leu
Bergofungin A	Ac	Val	Aib	Aib	Aib	Val	Gly	Leu	Aib
Trichotoxin									
A-40/5	Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib
A-50/E	Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib
Peptavirin A	Ac	Phe	Aib	Ala	Aib	L-**Iva**	Leu	Gln	Gly
Harzianin HB I	Ac	Aib	Asn	Leu	Ile	Aib	Pro	D-**Iva**	Leu
Harzianin HCIII	Ac	Aib	Asn	Leu	Aib	Pro	Ser	Val	Aib
Trichokindin-Ia	Ac	Aib	Ser	Ala	Aib	Aib	Gln	D-**Iva**	Leu
Trichokonin VII	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib
Saturnisporin SA II	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib
Trichosporin B-IVd	Ac	Aib	Ala	Ala	Ala	Aib	Aib	Gln	Aib
Trichovirin II 6b	Ac	Aib	Gly	Ala	Leu	D-**Iva**	Gln	D-**Iva**	Val
Trichorzin HA VII	Ac	Aib	Gly	Ala	Aib	D-**Iva**	Gln	Val	Val
Trichorzin MA III	Ac	Aib	Ser	Ala	Aib	D-**Iva**	Gln	D-**Iva**	Leu
Trichorzin PA VI	Ac	Aib	Ser	Ala	Aib	D-**Iva**	Gln	Aib	Val
Trichorzin PA VII	Ac	Aib	Ser	Ala	D-**Iva**	D-**Iva**	Gln	Aib	Val
Trichorzianine A VII	Ac	Aib	Ala	Ala	Aib	D-**Iva**	Gln	Aib	Aib
Trichorzianine B VII	Ac	Aib	Ala	Ala	Aib	D-**Iva**	Gln	Aib	Aib
Longibrachin A IV	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib
Longibrachin B III	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib
Trichobrachin I B	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib
Atroviridin C	Ac	Aib	Pro	Aib	Ala	Aib	Aib	Gln	Aib
Neoatroviridin D	Ac	Aib	Gly	Ala	Leu	D-**Iva**	Gln	D-**Iva**	Leu
Hypomurocin A-2	Ac	D-**Iva**	Gln	Val	Val	Aib	Pro	Leu	Leu
Hypomurocin B-5	Ac	Aib	Ser	Ala	Leu	Aib	Gln	Aib	Val
Trichocellin A-II	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib
Trichocellin B-II	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib
Hypelcin A-VII	Ac	Aib	Pro	Aib	Ala	Aib	Ala	Gln	Aib
Hypelcin BIII	Ac	Aib	Pro	Aib	Ala	Aib	Aib	Gln	Aib
MS-681d	Ac	Phe	L-**Iva**	Aib	Ala	D-**Iva**	Gly	Phe	L-**Iva**
Trikoningin KB II	Oc	D-**Iva**	Gly	Val	Aib	Gly	Gly	Val	Aib
LP237-F 8	Oc	Aib	Pro	Phe	Aib	Gln	Gln	Aib	**EtNva**
LP237-F 5	Oc	Aib	Pro	Tyr	Aib	Gln	Gln	Aib	**EtNva**
Chlamydocin	cyclo	(Aoe	D-Pro	Phe	Aib)				
FR235222	cyclo	(Aoh	*t*-4-D-MePro	Phe	L-**Iva**)				
Scytalidamide A	cyclo	(Phe	Leu	Pro	*N*-MeLeu	Aib	Phe	*N*-MePhe)	

9	10	11	12	13	14	15	16	17	18	19	20
Acc	Aib	Pip	Aib	Gly	Leu	Aib	PIHPPE				
Acc	Aib	Pip	Aib	Gly	Leu	**Iva**	PIHPPE				
Acc	Aib	Pip	Aib	Gly	Leu	Aib	PIHPPE				
Acc	Aib	Pip	Aib	Gly	Leu	**Iva**	PIHPPE				
Acc	Aib	Pip	Aib	Gly	Leu	**Iva**	PIHPPE				
Acc	Aib	Pip	Aib	Ala	Leu	**Iva**	PIHPPE				
Acc	Aib	Pip	Aib	Ala	Leu	**Iva**	PIHPPE				
Acc	Aib	Pip	Aib	Gly	Leu	L-**Iva**	PIHPPE				
Acc	L-**Iva**	Pip	Aib	Gly	Leu	L-**Iva**	PIHPPE				
Acc	Aib	Pip	Aib	Gly	Leu	L-**Iva**	PIHPPE				
Acc	Aib	Pip	Aib	Gly	Leu	L-**Iva**	PIHPPE				
Aib	Aib	Pip	Aib	Ala	Leu	L-**Iva**	PIHPPE				
Aib	Aib	Aib	Pip	Iva	PIHPPE						
Aib	Hyp	Gln	D-**Iva**	Hyp	Aib	Pro	Pheol				
Aib	Hyp	Gln	Aib	Hyp	Aib	Pro	Phe	Ser			
Hyp	Leu	L-**Iva**	Aib	Hyp	L-**Iva**	D-**Iva**	Gly				
Aib	L-**Iva**	Aib	Hyp	D-**Iva**	Ile	Mannitol					
Aib	Aib	Ala	Ala	Aib	Pro	D-**Iva**	Aib	Aib	Gln	Trpol	
Aib	Aib	Ala	Ala	Aib	Pro	Aib	Aib	Aib	Gln	Trpol	
Ser	Aib	Ala	Leu	Aib	Gln	Leuol					
Aib	Pro	Gln	Aib	Hyp	D-**Iva**	Pro	Phe	Gly			
Aib	Hyp	Gln	D-**Iva**	Hyp	Aib	Pro	Pheol				
Aib	Hyp	Gln	Aib	Hyp	Aib	Pro	Pheol				
Aib	Hyp	Gln	D-**Iva**	Hyp	Aib	Pheol					
Aib	Hyp	Gln	Aib	Hyp	Aib	Pro	Pheol				
Aib	Hyp	Gln	L-**Iva**	Hyp	Aib	Pheol					
Aib	Ala	Aib	Aib	Pro	Leu	Aib	D-**Iva**	Glu	Valol		
Aib	Ala	Aib	Aib	Pro	Leu	Aib	D-**Iva**	Gln	Valol		
Aib	Aib	Ala	Ala	Aib	Pro	L-**Iva**	Aib	Aib	Gln	Trpol	
Aib	Pro	Leuol									
Pro	D-**Iva**	Leu	Aib	Pro	Leuol						
Aib	Ala	Aib	Aib	Pro	Leu	Aib	Aib	Gln	Ileol		
Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	D-**Iva**	Gln	Gln	Pheol
Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	D-**Iva**	Gln	Gln	Pheol
Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	D-**Iva**	Gln	Gln	Pheol
Aib	Gly	D-**Iva**	Aib	Pro	Leu	Aib	Aib	Gln	Leuol		
Aib	Gly	Leu	Aib	Pro	Leu	Aib	D-**Iva**	Gln	Leuol		
Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	Valol		
Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	Pheol		
Aib	Gly	Leu	Aib	Pro	Leu	Aib	Aib	Gln	Trpol		
Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	Gln	Gln	Pheol	
Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	Gln	Glu	Pheol	
Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	D-**Iva**	Gln	Gln	Pheol
Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	D-**Iva**	Glu	Gln	Pheol
Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	D-**Iva**	Gln	Gln	
Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	D-**Iva**	Gln	Gln	Pheol
Aib	Gly	D-**Iva**	Aib	Pro	Leu	Aib	Aib	Gln	Leuol		
Aib	Pro	Leuol									
Aib	Gly	D-**Iva**	Aib	Pro	Leu	Aib	Aib	Gln	Leuol		
Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	D-**Iva**	Gln	Gln	Pheol
Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	D-**Iva**	Glu	Gln	Pheol
Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	D-**Iva**	Gln	Gln	Leuol
Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	D-**Iva**	Glu	Gln	Leuol
APPS											
Gly	Ile	Leuol									
Gln	Ala	Leuol									
Gln	Ala	Leuol									

Table 2 (cont.)

[a]) List of producers: **acretocin**, *Acremonium crotocinigenum* CBS 217.70 (note that the configuration of Iva[15] has not been assigned definitely!); **neoefrapeptin**, *Geotrichum candidum* SID 22780; **efrapeptin**, *Tolypocladium inflatum* (syn. *T. niveum*, teleomorph: *Elaphocordyceps subsessilis*) IMI 202309, *T. inflatum* 1897, *T. inflatum* ARSEF 616, 3277, 3278, 3279, 3280, 3281, 3321, 3394, 3395, 3396, 3398, 3432; *T. geodes* ARSEF 2684, 3271, 3272, 3274, 3275, 3276; *T. cylindrosporum* 705, 961, 962, 963, 976, 1028, 1580, 2005, 2912, 3392, 3393, 4561, 4562, 4563, 4996; *T. tundrense* ARSEF 3401; *T. parasiticum* (syn. *Pochonia parasitica*) ARSEF 3436; *Tolypocladium* sp. AMB18; *Acremonium* sp. 021172cKZ, *Acremonium* sp. CNC-310; **adenopeptin**, *Chrysosporium* sp. PF1201; **cephaibol**, *Acremonium tubakii* DSM 12774; **integramide A**, *Dendrodochium* sp. MF 6888; **clonostachin**, *Clonostachys* sp. F5898; **chrysospermin**, *Sepedonium chrysospermum* DSM 7444, *S. chrysospermum* S39, *S. microspermum* S6, *S. ampullosporum* S158, *S. laevigatum* S13, *S. brunneum* S171; **boletusin/tylopeptin**, *Sepedonium* sp. (the production of both peptaibols was originally misattributed to fruiting bodies of *Boletus* sp. and *Tylopilus neofelleus* being preferred hosts of *Sepedonium* sp.); **XR 586**, *Acremonium persicinum* X21488; **antiamoebin**, *Stilbella fimetaria* (syn. *S. erythrocephala*) ATCC 28144, CBS 548.84, *Clonostachys rosea* f. *catenulata* (syn. *Gliocladium catenulatum*) CBS 511.66; *Emericellopsis synnematicola* CBS 176.60; *E. salmosynnemata* CBS 382.62, *Verticillium epiphytum* (syn. *Cephalosporium pimprima*), *Emericellopsis poonensis*; **emerimicin**, *Emericellopsis microspora* 333; **zervamicin**, *E. salmosynnemata* 336; **bergofungin**, *E. donezkii* HKI 0059; **trichotoxin A40/A50**, *T. asperellum* NRRL 5242 (originally misidentified as *T. viride*); **peptavirin**, *Sepedonium* sp. KCTC 8921P; **harzianin HB I**, *T. harzianum* MNHN-903603; **harzianin HC**, *T. harzianum* MNHN-903603, MNHN-903614; **trichokindin**, *T. harzianum*; **trichokonin**, *T. koningii*; **saturnisporin**, *T. saturnisporum*; **trichosporin**, *T. polysporum* TMI 60146; **trichovirin I/II**, *T.* cf. *harzianum* (originally misidentified as *T. viride*); **trichorzin HA**, *T. harzianum* MNHN-903602; **trichorzin MA**, *T. harzianum* MNHN-922835; **trichorzin PA**, *T. harzianum* MNHN-902608; **trichorzianine A/B**, *T. harzianum* ATCC 20672; **longibrachin**, *T. longibrachiatum* LCP-853431; **trichobrachin**, *T. ghanense* (syn. *T. parceramosum*) CBS 936.69; **atroviridin/neotatroviridin**, *T. atroviride* F80317; **hypomurocin A/B**, *T. atroviride* IFO 31288 (originally misidentified as *Hypocrea muroiana*); **trichocellin A/B**, *T. viride*; **hypelcin A/B**, *Hypocrea peltata*; **MS-681**, *Myrothecium* sp. KY6568; **trikoningin KB II**, *T. koningii* MNHN-903589; **LP237**, *Tolypocladium geodes* LP 237; **chlamydocin**, *Pochonia chlamydosporia* S 3440 (originally described as *Diheterospora chlamydosporia*); **FR235222**, *Acremonium* cf. *murorum* 27082 (= strain FERM BP-6539); **Scytalidamide A**, *Acremonium* sp. CNC-310 (originally misidentified as *Scytalidium* sp.). Note that the identity of most producers originally reported as *T. viride*, *T. harzianum*, *T. longibrachiatum*, or *T. koningii* has not been verified by molecular methods. [b]) List of Abbreviations not explained in the text: Pip, L-pipecolic acid; PIHPPE, *N*-**p**eptido-1-**i**sobutyl-2-(2,3,4,6,7,8-**h**exahydro-1-**p**yrrolo[1,2-*a*]**p**yrimidinio)**e**thylamine; APPS, (2′*R*,*S*)-N^1-(2-**a**mino-3-**p**henyl**p**ropyl)**s**permidine; Aoe, L-2-**a**mino-9,10-**e**poxy-8-**o**xodecanoic acid; Aoh, L-(2*S*,9*R*)-2-**a**mino-9-**h**ydroxy-8-**o**xodecanoic acid; *t*-D-4-MePro, *trans*-4-D-MePro = (2*R*,4*S*)-4-MePro.

marine aerosols have been found to contain viable fungi [24]. Aircraft, balloon, and rocket measurements have shown that such particles are ubiquitous over land and oceans. They also are transported to high altitudes (up to 80 km) and over long distances.

3.3. *Extraterrestrial Aib and Racemic Iva in Ancient Marine Sediments Close to the K-T Boundary.* The detection of Aib together with racemic Iva in outcrop sediments at Stevns Klint, Denmark, above and below a very thin layer of clay, named the cretaceous-tertiary (K-T) boundary [25] attracted some attention. This boundary marks the extinction of estimated 75% of organisms, including dinosaurs, *ca.* 65 million

years ago. The presence of Aib as well as Iva in these sediments was attributed to the impact of a large bolide, as proposed by *Alvarez et al.* [26]. Alternatively, influx of abiotic Aib and Iva by comet dust has also been suggested [27].

Curiously enough, Aib and racemic Iva are absent in the boundary clay layer itself, but present in the layers above and below it, for which diffusion or geochromatography were considered as feasible explanations [28].

3.4. *Aib in Non-Marine Sediments Close to the K-T Boundary.* In subsequent analyses, sediments from the originally coastal K-T-boundary sites of Stevns Klint as well as non-marine sediments from Starkville South/Colorado and Raton Pass/New Mexico were investigated for the presence of Aib as well as common proteinogenic amino acids (Iva and enantiomers could not be assigned definitely). Presence of Aib was reported for Stevns Klint and Starkville South but not for Raton Pass [29]. However, no $\delta(^{13}C)$ values of Aib were determined, and Iva could not be detected by HPLC after derivatization with *o*-phthaldialdehyde. By GC on a *Chirasil*-Val column, Aib and Iva were only tentatively assigned in the elution profiles of the TFA-amino acid-2-propyl esters, and the configuration of Iva was not determined. From the presence of proteinogenic L-amino acids and the D-enantiomers of Ala, Asp, and Glu, as well as their ^{13}C-compositions, terrestrial, biogenic origin of these amino acids was concluded.

3.5. *Possible Formation of Abiotic Aib and Iva in Sediments as Result of Volcanic Action.* Intrusion of volcanic magma into natural coal beds could have led to a geothermal gasification and formation of hydantoins in the presence of water in a *Bucherer* hydantoin reaction, which provides an alternative explanation for the formation of Aib and racemic Iva. The resulting 5,5-dimethyl- and 5-ethyl-5-methylhydantoins may serve as precursors for Aib and racemic Iva [30], which may subsequently be formed by aqueous or enzymatic hydrolysis [31]. This scenario, in principle, might also be applied to contemporary volcanic activities, thus leading to the formation and local or global distribution of abiotically formed amino acids following the mechanisms outlined here.

3.6. *Aib and* D,L-*Iva in Young Marine Sediments of Tokyo Bay, Japan.* Aib and D,L-Iva were also detected by HPLC in young marine sediments (<100 y) of Tokyo Bay, Japan [32]. Based on the lack of evidence for volcanic action and meteoritic origin, the occurrence of Aib and Iva enantiomers in surface samples as well as in 30–35-cm depth of marine sediments was attributed to the inlet of industrial waste waters containing 5,5-dialkyl-substituted hydantoins that are used as pharmaceutical drugs or as raw materials for polymers. The authors used GC/MS of *N*-(trifluoroacetyl)-butyl esters on *Chirasil*-L-Val capillary columns for the detection of Aib, and separation of D- and L-Iva. Because of the unavailability of optically pure standards of Iva, the elution order of the enantiomers could not be assigned (but D-Iva is eluted prior to L-Iva on *Chirasil*-L-Val, according to our experience; see *Exper. Part*). The ratio of the former peak area to the latter one was estimated to be *ca.* 0.9 in a surface sample, indicating that L-Iva actually was present in small excess.

3.7. *Isolation of Peptaibiotics from* Trichoderma *in Recent Marine Sediments.* Peptaibiotic-producing species of *Trichoderma* were isolated from recent marine sediments of Fier d'Ars at the French Atlantic coast, and bioaccumulation of these peptides in Blue Mussels (*Mytilus edulis*) was reported [33]. Therefore, biotic synthesis

of Aib and Iva by marine fungi is an alternative explanation for the occurrence of these amino acids in hydrolysates of Tokyo Bay sediments.

3.8. *Possible Fungal Origin of Aib and Iva in Ancient Sediments.* In the light of the data and arguments presented here, fungal origin of Aib and Iva in ancient sediments has to be considered. Regarding the mass extinction marked by the K-T boundary, it is of interest that fungal proliferation ('fungal spike'), indicated by fungal spores and hyphae, was detected in the Moody Creek Mine, Greymouth Coalfield, New Zealand, coincident with the iridium anomaly that marks the extinction event [34]. This is interpreted as dramatic and global dieback of photosynthetic organisms, and replacement by saprotrophic fungi that dominated the biosphere for a certain period of time. These findings indicate at least the capability of fungi to occupy and even dominate favorable ecosystems. The palaeomicrobial records indicate that fungi were abundant at the geological time periods discussed [35–38]. However, since outcrop sediments at the K-T boundary at Stevns Klint and related sites are still exposed, and have been exposed for long periods of time to the environment, and since samples have neither been collected nor stored under aseptic conditions, biotic origin of Aib and Iva from recent fungi in such samples cannot be excluded. Indeed, lipid biomarkers indicating microbial activity have been detected in K-T boundary sediments of Stevns Klint and related sites [39]. Notably, peptaibiotics are extremely stable towards proteolytic enzymes [2].

3.9. *Abiotic Aib and Iva in Meteorites.* There is consensus, and we completely agree about the conclusion that the abundance of organic materials in carbonaceous meteorites, such as the *Murchison* or the *Murray*, is the result of cosmochemical, abiotic synthesis. In particular the α,α-dialkyl amino acids are the results of basically known synthetic pathways such as the *Strecker* cyanohydrin synthesis or its variations. The building blocks (synthons) of amino acids abundantly occur in space [40].

Whereas Iva in K-T-boundary sediments was reported to be completely racemic, Iva as well as other α,α-dialkyl amino acids in carbonaceous *Murchison* and *Murray* meteorites were found to be non-racemic, and even result in a moderate excess of the L-enantiomers [11][41].

Some proteinogenic L-amino acids that are usually detected together with non-proteinogenic α,α-dialkyl α-amino acids in the 'classical' carbonaceous meteorites are considered to be the result of terrestrial, in particular, microbial contamination, since these meteorites have been exposed to the terrestrial environment for extended periods of time and have not been stored under aseptic conditions [42]. It is not unlikely that peptaibiotic-producing fungi had or have the opportunity to grow in such meteorites. Then, certain amounts of biotic α,α-dialkyl amino acids might be added to those formed abiotically, and even result in changes of the ratio of Iva enantiomers. The ee excess of the series of chiral non-proteinogenic α,α-dialkyl amino acids of these meteorites has been ascribed to discrimination by circularly polarized light [43] or, recently, to enantiomeric amplification mechanisms related to the *Soai* reaction [44]

3.10. *Aib in Meteorites and Ice Samples from Antarctica.* Aib and Iva have also been detected in carbonaceous meteorites collected in Antarctica, serving as a giant collector and freezer thus considered to minimize terrestrial, in particular, microbial, contamination [45].

Besides solid pieces of carbonaceous meteorites, the so-called Antarctic micrometeorites (AMMs), typically ranging between 100–200 μm in size, were collected from molten ice, together with non-meteoritic materials [46].

Remarkably, Aib and Iva have been detected in solid pieces of carbonaceous meteorites collected from ice fields of Antarctica [47][48][49] but also from an ice block extracted from the La Paz region, Antarctica, that was devoid of solid pieces of meteorites [49]. Therefore, AMMs were considered as potential sources for Aib, provided that at least parts of those amino acids survived the heating process on entering the atmosphere [49][50]. Indeed, steady influx of interplanetary dust and micrometeorites in the terrestrial atmosphere takes place, and materials accreted in Antarctica and the Arctic have been analyzed [51]. Recently, cosmic dust has been detected together with the much more common terrestrial dust in an ice core drilled in Dronning Maud Land, Antarctica, covering the time period from 6,800 to 29,000 ybp, using $^4He/^3He$ isotope ratios [52]. Therefore, and in extension of our considerations, it is reasonable to assume that cosmic dust particles, together with terrestrial materials, form part of the 'aerosols' in the atmosphere.

3.11. *Possible Biotic Origin of Aib in Ice Samples Devoid of Meteorites.* Localized deposits of materials originating from Aib-containing bio- or mycoaerosols or related dust particles serving as ice nuclei as described above might be an explanation, with Aib being released and concentrated as result of lyophilization or/and freeze-concentration of solutes. Active fungal growth and biosynthesis of Aib under conditions favoring freeze-thaw cycles is also to be expected.

Indeed, viable microorganisms, including fungi, have been detected in all ice samples and sites analyzed for their presence.

Two expeditions to Antarctica were aimed at answering the question whether biotic materials in ice could contaminate carbonaceous meteorites. Microorganisms, including fungi, were present in all ice samples from Patriot Hills, Thiel Mountains, and South Pole Station, and cryoconite ice also contained viable microorganisms [53]. In meteorites and ice samples collected in the blue ice fields near Pecora Escarpment, it was shown that microbial life was present in every ice sample [54]. In this context, it is noteworthy that psychrophilic (cryophilic) fungi have been isolated from Arctic as well as Antarctic soils, cryopegs (*i.e.*, lenses of supercooled water in permafrost regions), and Antarctic lakes [55].

Presence of fungi entombed in ancient glacial ice has also been recognized. Among those, species of *Acremonium*, *Geomyces pannorum*, and *Cladosporium* have been identified, and, among many other fungi, viable *Lecytophora hoffmannii* AY 781227 was isolated from an ice core from Greenland estimated to originate from <500 ybp [56].

In cold regions, the dark, powdery dust deposited on the surface of snow or ice (cryoconite) might accumulate in cryoconite holes. Consequently, a localized deposit and release of Aib and Iva from airborne dust loaded with relevant fungi can be expected.

3.12. *Fungal Taxa from Antarctica as Potential Producers of Aib, Iva, and Peptaibiotics.* Production of Aib and Iva, and peptaibiotics from recent psychrophilic microorganisms, reported to occur in Antarctica, is likely. Indeed, some of the Aib-producing fungal genera presented in *Table 1* have been detected in Antarctica as well as extremophiles in other cold regions.

Acremonium strictum, *Isaria farinosa* (syn. *Paecilomyces farinosus*), and *Verticillium* sp. have been isolated, among other fungi, from soil and biomaterials from the Victoria Land in Antarctica [57]. The isolation of *Acremonium antarcticum*, *Acremonium psychrophilum*, *Paecilomyces variotii*, and *Trichoderma* sp. was reported from soils of Schirmacher Oasis, East Antarctica [58], and Antarctic microfungi serve as models for exobiology [59].

The fungi from North-east Bailey, Windmill Islands, continental Antarctica, have been investigated in detail [60]. Among others, *Acremonium crotocinigenum* (*cf. Table 1* and *Fig. 1*) was isolated from the lichens *Xanthoria candelaria*, *Umbilicaria decussata*, and *Usnea sphacelata*, as well as an *Acremonium* sp. from the moss *Grimmia antarctica*. Further, *Hydropisphaera peziza* (syn. *Nectria peziza*) was isolated from the mosses *Bryum pseudotriquetrum* and *Grimmia antarctica*, and two *Trichoderma* species, then identified as *T. harzianum* and *T. 'pseudokoningii'*, were isolated from soil. It is expected that carbonaceous meteorites, owing to the low temperatures, are not less prone to vigorous microbial growth. However, the formation of water as a result of thaw-freeze cycles on the surface and/or supercooled water in micropores might enable growth of psychrophilic microorganisms, both fungi and bacteria, feeding on these potential sources of organic C, N, P, and S. Notably, these considerations might be extended even to meteorites sunken at depths up to several meters in the 'Blue Ice' of Antarctica owing to the relative transparency of that ice and the absorption of solar radiation by solids that can promote formation of liquid water [45].

4. Conclusions and Perspectives. – Screening of filamentous fungi for the production of Aib, and D- and L-Iva, grown under standard conditions on agar plates, revealed the biosynthesis of peptaibiotics in taxa not recognized as producers before. Owing to the ubiquity of these fungi in the biosphere and the abundance of sequence data compiled in the literature and rapidly expanding databases, these amino acids are no longer considered rare in the biosphere. [2][3].

We dare predict that Aib and related α,α-dialkyl amino acids are to be expected in all environments associated with peptaibiotic-producing fungi, and the detection of Aib and Iva is just dependent on knowledge and the application of advanced analytical techniques as described here and elsewhere.

Detection of trace amounts of Aib and Iva in sediments, soil, or ice samples from Antarctica devoid of carbonaceous meteorites as potential sources of abiotically formed Aib and Iva should be considered in the light of the results and arguments presented here. In particular, the concomitant presence of carbonaceous meteorites or cosmic dust and of fungi, being potential producers of peptaibiotics, will make the distinction difficult and requires methods to distinguish among abiotic and biotic Aib and Iva. It is highly recommended that analyses of meteoritic and palaeogeological samples for Aib and other α,α-dialkyl α-amino acids should be accompanied by a thorough evaluation of the microbial flora of these materials. Besides standard methods such as light-microscopic inspection or scanning electron microscopy, and growth studies of microorganisms in suitable culture media, advanced state-of-the-art molecular-diagnostic techniques that are specific for the detection of contaminating microorganisms should be applied. Examples comprise rRNA-based PCR amplifica-

tion or DNA-specific fluorescence staining possibly combined with confocal laser scanning electron microscopy [61].

Finally, it should be emphasized that the considerations outlined here on the fungal occupation of materials and production of peptaibiotics can be extended to other microorganisms, including bacteria that might also be associated in symbiosis or biocoenosis. Examples are lichenized fungi, fungicolous algae, microbial mats, biofilms, and (crypto)endolithic communities, all of which represent biosystems that should be screened for the production of α,α-dialkyl amino acids. Yeasts, in particular, are potential candidates as has already been shown with the yeast-like fungus *Geotrichum candidum* (Saccharomycetales) – notably also occurring in Antarctica – that incorporates Aib as well as equal amounts of D- and L-Iva in certain neoefrapeptin sequences [15].

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Experimental Part

General. Chemicals. MeOH, PrOH, i-PrOH, CH_2Cl_2, AcCl, Ac_2O, pentafluoropropionic acid anhydride (PFPAA) (*Merck*, D-Darmstadt), *Dowex 50W-X8* (*Serva*, Heidelberg, Germany), NH_3 (24%); HCl in PrOH was prepared by slowly adding AcCl (3 ml) to PrOH (7 ml) with chilling. Aib was from *Senn Chemical Comp.*, CH-Liesberg. Enantiomers L- and D-Iva for comparison as reference standards were prepared from DL-Iva synthesized in our laboratory according to the *Strecker* procedure and converted into *N*-monochloroacetyl(MCA)-DL-Iva. Treatment with porcine renal acylase released L-Iva that was separated from the remaining MCA-D-Iva by ion-exchange treatment according to [12][62].

Strains and Culture Conditions. Strains with CBS numbers are accessible from the *Centraalbureau voor Schimmelcultures* (CBS), NL-Utrecht. Cultures were transferred aseptically onto agar plates (9.5-cm diameter *Petri* dishes) and grown at r.t. for 9 d under ambient daylight. Sterilized culture media used were [g/l]: OA (oatmeal 30, agar 15), CMA (cornmeal 2 (*Difco*, Detroit, USA), agar 15; pH 6.0), MA2 (malt extract 20 (Servabacter Light, *Serva*, D-Heidelberg), agar 15).

Extraction of Peptides from Mycelia. MeOH/CH_2Cl_2 1:1 (*v*/*v*; 5 ml) was added to vigorously growing fungi on agar plates, the org. layers were filtered through fluted filter papers (125-mm diameter, *Schleicher and Schüll*, D-Dassel), and the filtrate was evaporated to dryness with a rotary evaporator. MeOH (2 ml) was added to the remaining residue, and the soln. was filtered through an *Anatop® 10* disposable filter disk (0.2 μm, *Merck*, D-Darmstadt) into 10-ml conical flasks and evaporated to dryness. Accordingly, blanks were prepared from culture media in *Petri* dishes not inoculated with fungi and analyzed as described below.

Total Hydrolyses of Extracts. 6M HCl (0.5 ml) was added to the residues, and the resulting solns. transferred into 1-ml-thick-walled reaction vials (*Reacti-Vial®*, Wheaton, Milville, USA). The vials were closed with *Teflon*-lined screw caps and heated for 24 h at 110°. The acid was removed in a stream of N_2, 0.1N HCl (1 ml) was added to the dry residue, and the resulting solns. were passed through a glass column (5-cm length, 0.6-cm diameter) filled with cation exchanger. The resin was washed with dist. H_2O (4 ml), and amino acids adsorbed were eluted with 4N aq. NH_3 (4 ml). The eluate was evaporated to dryness *in vacuo*, 0.1N HCl (0.5 ml) was added, and the soln. was transferred into reaction vials as described above and evaporated to dryness in a stream of N_2.

Derivatization Procedures for GC. HCl in i-PrOH (or PrOH) was added to the dry residue for esterification, and the closed vial was heated for 1 h at 100°. The solvent was removed in a stream of N_2, then PFPAA (10 μl) and CH_2Cl_2 (250 μl) were added to the residue, and the closed vial was heated for 20 min at 100°. Accordingly, Ac derivatives were prepared by derivatization of hydrolysates with HCl in PrOH and subsequent treatment with Ac_2O. Derivatizing reagents were carefully removed in a cold

stream of N_2 at r.t., CH_2Cl_2 (30 µl) was added, and 2–5 aliquots were subjected to GC/MS analysis. The split ratio was set 1:30. Between analyses, CH_2Cl_2 was injected into the GC, and the complete temp. program was run, followed by inspection of the baseline in order to detect possible contamination or carryover of Aib and Iva from previous analyses.

Instruments. For GC/MS, a *GC-17A* instrument (*Shimadzu*, Tokyo, Japan) was used equipped with *a*) *Chirasil*-L-Val cap. column (25 m × 0.25 mm ID, *Chrompack*, NL-Middelburg) or *b*) in one case (CBS 246.63) a *Lipodex E* cap. column (25 m × 0.25 mm ID; *Macherey-Nagel*, D-Düren). Temp. and pressure programs used for *a*) and *b*) were: initial temp. 70°, then 2.5°/min to 90°, then 7°/min to 190°, held for 10 min; pressure 5 kPa for 5 min, then 0.2 kPa/min to 6.7 kPa, then 0.6 kPa/min to 15 kPa, held for 10 min. The MS detector was a *QP 5000* instrument (*Shimadzu*) used in the selected ion monitoring (SIM) mode; detector gain was 1.5 kV, sampling rate 0.2 s.

Characteristic mass fragments used for SIM-MS of PFP-Aib-2-Pr esters were at *m/z* 204 ($[M-COOCH(CH_3)_2]^+$) and for PFP-Iva-2-Pr esters at *m/z* 218 ($[M-COOCH(CH_3)_2]^+$). For analysis of CBS 906.72D (*cf. Fig. 2*), Ac-Aib-1-Pr ester at *m/z* 100 ($[M-COOC_3H_7]^+$) and for Ac-Iva-1-Pr ester at *m/z* 114 ($[M-COOCH_3H_7]^+$) were used.

The elution order of enantiomers of DL-Iva from *Chirasil*-L-Val (D-Iva eluting prior to L-Iva) was determined by comparison with pure enantiomers (see above). Blanks were made from hydrolyzed org. solvent extracts of batches of nutrient agar used for *Petri* dishes not inoculated with fungi, and analyzed as described above.

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Studies on the Selective Trifluoroacetolytic Scission of Native Peptaibols and Model Peptides Using HPLC and ESI-CID-MS

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Representative members of a group of linear, *N*-acylated polypeptide antibiotics (peptaibols) containing α-aminoisobutyric acid (Aib) and, in part, isovaline (Iva), as well as proteinogenic amino acids and a C-terminal-bonded 2-amino alcohol, were treated with anhydrous trifluoroacetic acid (TFA) at 37° for 0.5–26 h. The resulting fragments were separated by HPLC and characterized by electrospray ionization collision-induced dissociation mass spectrometry (ESI-CID-MS). The following 16–20-residue peptaibols were investigated: natural, microheterogeneous mixtures of antiamoebins and alamethicin F50, uniform paracelsin A, and synthetic trichotoxin A50/E. In the natural peptides, bonds formed between Aib (Iva) and Pro (Hyp) were rapidly and selectively cleaved within 0.5 h. Furthermore, TFA esters of the C-terminal amino alcohols were formed. Depending on time, release of C-terminal tri- and tetrapeptides as well as amino acids from the major fragments was observed. Synthetic homooligopeptides, namely Z- and Ac-$(Aib)_{10}$-O^{t}Bu and Z-$(Aib)_7$-O^{t}Bu, were analyzed for comparison. On treatment with TFA, a regular series of Z-$(Aib)_{10-5}$-OH from Z-$(Aib)_{10}$-O^{t}Bu were detected within 0.5 h, and, after 3 h, release of a regular series of Z-$(Aib)_{7-3}$-OH from Z-$(Aib)_7$-O^{t}Bu were observed. Moreover, concomitant release of the series of H-$(Aib)_{10-3}$-OH from the decapeptide occurred. From these data, a repetitive cleavage mechanism *via* intermediate formation of C-terminal oxazolones on trifluoroacetolysis is proposed. Furthermore, their formation and stability in native peptaibols are correlated with subtle structural differences in protein amino acids linked to Aib. From the conspicuous concordance of the formation and abundance of regular series of trifluoroacetolytic fragments and of positive ions of the *b*-series in CID-MS, the generation of intermediate oxazolonium ions in both gas and liquid phase is concluded.

1. Introduction. – The carboxamide groups in peptides and proteins are remarkably stable against treatment with organic acids, and use of protecting groups and linkers, which are selectively cleaved by acids of varying strengths under controlled conditions, is of major importance in both solution and solid-phase peptide synthesis. However, several unexpected and selective cleavage reactions of peptide bonds formed in particular by non-proteinogenic amino acids have been reported. Consequently, such reactions have to be taken into account not only when planning classical or advanced,

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fully automated syntheses, but also in the course of workup and purification of synthetic and natural peptides, as well as their sequence determination. Regarding the aim of the work presented here, we focus on more recent results of the selective acidolytic amide-bond cleavage of special amino acids. However, readers interested in the selective nonenzymatic cleavage of proteins and peptides are referred to the classical review [1].

Recently, *Rubini et al.* [2] reported that synthetic peptides containing consecutive Pip[2]) residues, *e.g.*, H-Pip[1]-Pip[2]-Pip[3]-Leu[4]-Pip[5]-Pip[6]-Lys(Boc)[7]-Pip[8]-Lys(Boc)[9]-Phe[10]-O-*Wang* resin, have been cleaved between Pip[5]-Pip[6] on treatment with 95–50% CF_3COOH (TFA) and scavenger.

Contrary to that, no cleavage of the Pip bonds was observed by treatment with 1% TFA in CH_2Cl_2 when using the extremely acid-sensitive 2-chlorotrityl chloride resin. Analogously, the peptide Ac-Pip[1]-Pip[2]-Pip[3]-Leu[4]-Pip[5]-Pip[6]-Lys(Ac)[7]-Pip[8]-Lys(Ac)[9]-Phe[10]-O-*Wang* showed cleavage of the Pip[1]-Pip[2] as well as the Pip[5]-Pip[6] bond on treatment with 95–50% TFA.

Jiang et al. [3] observed that the C-terminal amide of a series of *N*-acyl-*N*,α,α-trialkylglycine amides (acyl, benzoyl; amide, Pmb or cyclohexyl), such as *N*-acyl-(dibenzylglycine)cyclohexylamide, was cleaved on treatment with 5% TFA in MeCN. *Mannekens et al.* [4] reported that the amide bond of (*S*)-2-pivaloyl-Tic and structurally related derivatives, such as (1*R*,3*S*)-1-[3-(benzyloxy)benzyl]-2-pivaloyl-Tic-Phe-OBn, were easily cleaved by treatment with MeOH, saturated with HCl_g at 0°.

Creighton et al. [5] found that the cyclic hexapeptides c[(NMe)Aib-Phe-D-Trp-Lys-Thr-Phe] and c[(NMe)α Ac[5]c-Phe-D-Trp-Lys-Thr-Phe] as well as c[Phe-Ser(Bn)-Ser(Bn)-Phe-NMeAib] showed unexpected amide bond cleavage on the terminal side of (NMe)Aib or its analogue (NMe)Ac[5]c on treatment with a mixture of anhydrous TFA/CH_2Cl_2 1:1 at 0° for 1 h. This mixture was used for the simultaneous removal of the Boc and *t*-Bu-ether protecting groups in the last steps of the syntheses. To get insight into the cleavage mechanisms, a series of benzoyl-dipeptide derivatives of the general structure *p*-X-C_6H_4C(O)-(NMe)Aib-Phe-OMe were synthesized. The X-group was varied from NO_2 to Cl. Fast cleavage and release of Phe-OMe was observed in 1:1 mixtures of TFA/CH_2Cl_2 or TFA/MeCN. Extremely fast amide-bond cleavage was observed for *p*-Me-C_6H_4C(O)-(NMe)Aib-Phe-OMe using 2% TFA in MeCN.

Anteunis and *van der Auwera* [6] reported on the acidolytic cleavage of a large number of protected di- and tripeptides. They found that, in peptides containing C-terminal N-methylamino acid carboxamides, such as Z-Pro-(NMe)Ala-NMe_2, the Me_2NH is already cleaved by TFA within 30 min.

In the course of their intensive studies on the use of the so-called azirine/oxazolone method [7] for the synthesis of Aib-containing peptides and peptaibols [8], the group of *Heimgartner* recognized that the amide bond in the dipeptide Z-Ala-Aib-NMe_2 was selectively cleaved by treatment with 3M HCl in H_2O/THF 1:1 within 12 h at 35°, and Z-Ala-Aib-OH was released in 87% yield [9].

Selective, repetitive amide cleavage of N-acyl-$(Aib)_n$-NMe_2 using HCl_g/toluene and reaction of the resulting *N*-protected peptide acids with 3-(dimethylamino)-2,2-dimethyl-2*H*-azirine was used for the synthesis of oligo-Aib-peptides [10].

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[2]) For abbreviations, see *Exper. Part.*

Except for the above azirine/oxazolone approach, cleavages of amide bonds were considered as rather undesired side reactions. In contrast to that, the selective scission of peptide bonds formed by Aib and Pro attracted considerable attention, because the former is the characteristic and predominant constituent of a group of polypeptides comprehensively named **peptaibiotics**, *i.e.*, **pept**ides containing **Aib** and displaying antib**iotic** as well as a plentitude of other b**iotic** activities [11].

Notably, it was already recognized by *Closse* and *Huguenin* in 1974 [12] that the cyclotetrapeptaibiotic chlamydocin could be selectively cleaved on the carboxy side of Aib by treatment with TFA at 50° for 2 h, resulting in the formation of the linear peptide H-Phe-D-Pro-Aoe-Aib-OH, which has subsequently been sequenced by *Edman* degradation.

Selective trifluoroacidolysis of the Aib–Pro bond was also used by *Pandey et al.* for the mass spectrometric sequence determination of alamethicins and antiamoebins [13][14].

Subsequently, analytical and preparative trifluoroacetolysis of the peptaibols trichotoxin and suzukacillin was intensively used by *Jung* and co-workers [15][16]. It was shown that release of C-terminal peptides occurs, besides the fast scission of the Aib–Pro bond that is followed by cleavages of certain Aib–Aaa bonds (Aaa: Gly, Ala). This sequential work was accompanied by systematic investigations of the acid-sensitivity of synthetic peptides representing partial sequences of native **peptaibols** (*i.e.*, **pept**ides containing **Aib** and a C-terminal 2-amino alcoh**ol**). Recently, preferred acidolytic cleavage of the Aib(Iva)-Hyp bonds in the 16-residue peptaibiotics integramides A and B was observed. Treatment with 1N HCl for 1 h at 110° was used for chiral sequence determination of D- and L-Iva in fragments [17][18].

From the above reports, it was evident that peptide bonds formed by Aib and Pro are particularly sensitive to trifluoroacetolysis. Notably, bonds formed by other C(α),C(α)-dialkylated glycines, such as isovaline (Iva, 2-ethylalanine), and amino acids related to Pro, such as Hyp or Pip, show a similar cleavage behavior. However, the slow release of C-terminal Aib as well as tri- and tetrapeptides from *N*-acetylpeptide acids, resulting from preparative TFA scission of trichotoxin [15] and suzukacillin [16], had been recognized, indicating further cleavage preferences.

To evaluate the cleavage mechanisms proposed for small peptides and amino acid derivatives, a series of native and synthetic peptaibols were subjected to time-dependent trifluororacetolysis. Their cleavage behavior was compared to synthetic homooligo-Aib model peptides treated in parallel. Fast separation and reliable assignment of the acidolytic fragments was achieved by on-line-HPLC coupled to ESI-CID-MS.

2. Results. – 2.1. *Trifluoroacetolysis of Homooligo-Aib-Peptides.* Sequences of model peptides and peptaibiols and preferential cleavage sides are compiled in *Fig. 1.*

Z-(Aib)$_{10}$-O^{t}Bu (**1**). Peptide fragments resulting from the trifluoroacetolysis of **1**, separated by HPLC and analyzed by total ion current (TIC)-MS, are compiled in *Table 1*, together with their characteristic protonated molecular ions. The ESI-MS of **1**, establishing its identity and showing a regular series of acylium ions b_2–b_{10} with the characteristic difference of 85 Da for Aib residues, is presented in *Fig. 2*.

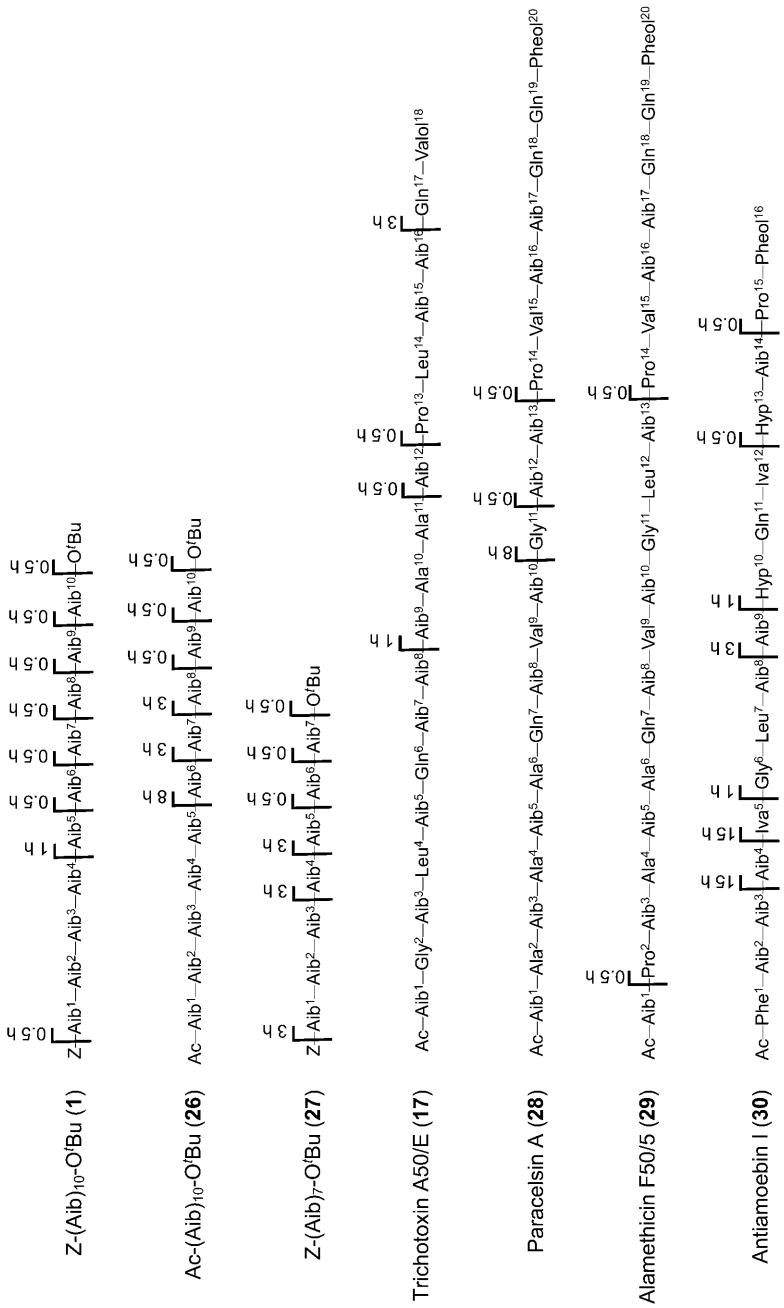


Fig. 1. *Structures of homooligo-Aib-peptides and peptaibols.* Preferred scission points at certain periods of time are indicated.

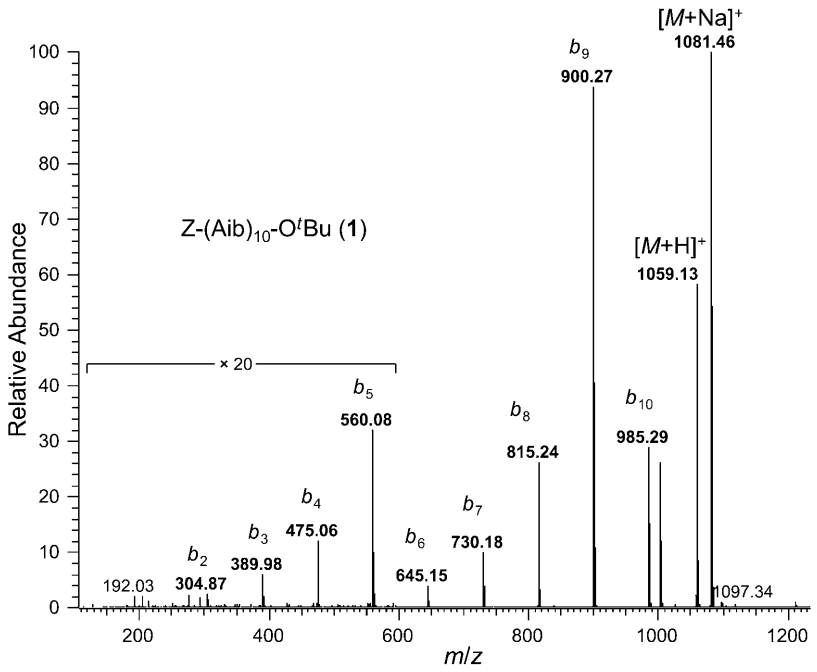


Fig. 2. *Mass spectrum of synthetic Z-(Aib)$_{10}$-O^{t}Bu* (**1**) s*howing the protonated molecular ion at* m/z *1059 and sodiated molecular ion at* m/z *1081.* Regular series of fragment ions b_2 to b_{10}, distinguished by the characteristic mass difference for Aib residues (85 Da), can be observed.

Table 1. *Peptides* **2**–**16** *Resulting from Trifluoroacetolysis of Z-(Aib)$_{10}$-O^{t}Bu* (**1**) *and Protonated Molecular Ions [*M + *H]$^+$ Used for the Characterization of Peptides*

	Detection after	Disappearance after	1	2	3	4	5	6	7	8	9	10		$[M+H]^+$ [m/z]
1		0.5 h	Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	O^{t}Bu	1059
2	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib		1003
3	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib			918
4	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib				833
5	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib					748
6	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib						663
7	0.5 h		Z	Aib	Aib	Aib	Aib	Aib							578
8	0.5 h	15 h		Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib		869
9	0.5 h	15 h		Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib			784
10	0.5 h			Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib				699
11	0.5 h			Aib	Aib	Aib	Aib	Aib	Aib	Aib					614
12	0.5 h			Aib	Aib	Aib	Aib	Aib	Aib						529
13	0.5 h			Aib	Aib	Aib	Aib	Aib							444
14	0.5 h			Aib	Aib	Aib	Aib								359
15	0.5 h			Aib	Aib	Aib									274
16	1 h		Z	Aib	Aib	Aib	Aib								493

After 0.5 h of trifluoroacetolysis, the starting material disappeared owing to the fast and complete cleavage of the ^{t}Bu group and the formation of the peptide acid Z-$(Aib)_{10}$-OH (**2**). Unexpectedly, concomitant generation of a mixture of individual peptides, *i.e.*, **3**–**16**, with decreasing ion intensities and retention times were observed. Peptides **2**–**10** were well resolved by HPLC, whereas peptides **11**–**15** co-elute. After 1 h, Z-$(Aib)_4$-OH (**16**), appeared, co-eluting with peptide **8**. Despite this, peptides **8** and **16**, as well as **11**–**15**, can be distinguished by their characteristic molecular ions (see *Table 1*). Z-Peptide acids **2**–**7** correspond to the series Z-$(Aib)_{10-5}$-OH, whereas peptides **8**–**10** correspond to the free peptides H-$(Aib)_{10-8}$-OH. The co-eluting free peptides **11**–**15** represent the mixture of H-$(Aib)_{7-3}$-OH. Inspection of *Fig. 3* shows that, with increasing reaction time, the intensity of the Z-decapeptide acid **2** decreases, and those of the series Z-$(Aib)_9$-OH (**3**) to Z-$(Aib)_5$-OH (**7**), increase. After 1 h, formation Z-$(Aib)_4$-OH (**16**; co-eluting with H-$(Aib)_{10}$-OH (**8**)) was also observed. This indicates successive cleavage of C-terminal Aib residues, one after the other. However, after 0.5 h reaction time, concomitant release of the series of free peptides **8**–**15** was observed, representing H-$(Aib)_{10-3}$-OH. This establishes that also the Z-group of the respective peptides is cleaved off. From the ion abundances, it appears, however,

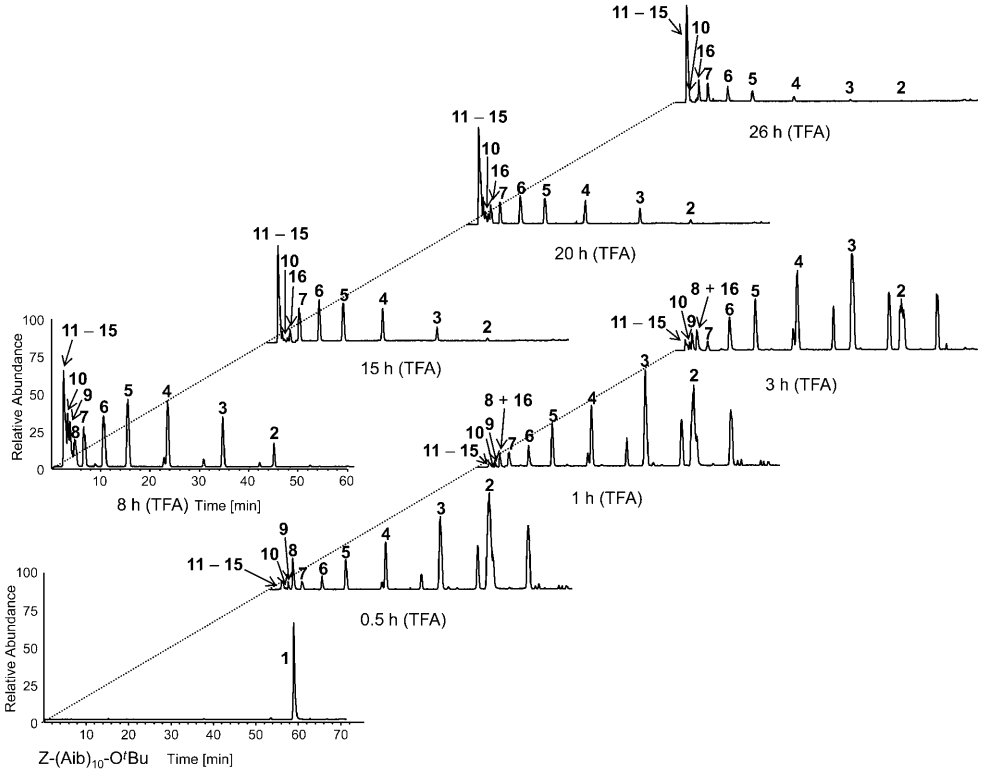


Fig. 3. *HPLC/TIC-MS of peptides* **2**–**16** *resulting from the trifluoroacetolysis of Z-(Aib)$_{10}$-O^{t}Bu* (**1**). Numbers refer to sequences presented in *Table 1*.

that cleavage of the Z-group proceeds slower, compared to the release of C-terminal Aib residues.

Ac-(Aib)$_{10}$-O^{t}Bu (**26**). Although this peptide contained some impurities resulting from the synthesis, the molecular ion at *m/z* 967 ($[M+H]^+$) could be used as the precursor, and the acidolytic cleavage kinetics could be analyzed. After 0.5 h, formation of the series Ac-(Aib)$_{10-8}$-OH was observed. After 3 h, Ac-(Aib)$_7$-OH and Ac-(Aib)$_6$-OH were recognized, and, after 8 h, formation of Ac-(Aib)$_5$-OH. After 15 h reaction time, Ac-(Aib)$_{10}$-OH and Ac-(Aib)$_9$-OH could not be detected anymore. In contrast to Z-(Aib)$_{10}$-OH, no formation of free H-(Aib)$_{10}$-OH or lower homologues from Ac-(Aib)$_{10}$-O^{t}Bu, even after longer reaction times (up to 26 h), could be observed. This indicates that the Ac-Aib terminus is more stable in comparison to the Z-Aib terminus. This is in agreement with the relative stability of acetylpeptides or Ac-Aib-OH released from peptaibols (see *Fig. 1*).

Z-(Aib)$_7$-OH (**27**). In analogy to the protected decapeptide, the *t*-Bu group was completely cleaved after 0.5 h reaction time, and formation of Z-(Aib)$_7$-OH was observed, as well as formation of the series Z-(Aib)$_6$-OH to Z-(Aib)$_5$-OH. After 3 h of acidolysis, formation of Z-(Aib)$_4$-OH and Z-(Aib)$_3$-OH were recognized as well as release of H-(Aib)$_7$-OH. This result clearly indicates that the Z-group was also cleaved in that case. Basically, the acidolytic cleavage behavior of the heptapeptide is in agreement with that of the decapeptide.

2.2. *Trifluoroacetolysis of Peptaibols. Trichotoxin* (**17**). The sequences of the peptaibols discussed in the following and fragments released are also presented in *Fig. 1*.

TICs of HPLC/MS resulting from time-dependent trifluoroacetolysis of the synthetic octadecapeptide **17** and the corresponding pseudomolecular ions of the resulting fragments recorded in CID off-mode are presented in *Fig. 4*.

Consecutive numbers of peptides resulting from trifluoroacetolysis and their corresponding sequences are presented in *Table 2*. Uniformity of trichotoxin (**17**) in HPLC and the peak at *m/z* 1690 ($[M+H]^+$) established the authenticity of the synthetic octadecapeptide. After 0.5 h of trifluoroacetolysis, the starting material **17** decreased, and the acetylpeptide acids **18** and **20**–**22**, as well as the TFA esters of **18** and the prolylhexapeptide **24**, appeared. After 1 h, peptide **23** and, after 3 h, peptide **19** was detected. The free prolylhexapeptide **25** was observed after 8 h.

Comparison with the sequence of trichotoxin (see *Fig. 1*) shows that, after 0.5 h, fast, but not complete, cleavage of the Aib–Pro bond takes place with release of the N-terminal dodecapeptide Ac-Aib^1-Aib^{12}-OH (**21**). Notably, concomitant formation of the trifluoroacetyl esters **18** of the C-terminal Valol from the intact trichotoxin (**17**), as well as the C-terminal prolylhexapeptide **24**, was recognized (see *m/z* in *Table 2* and *Fig. 4*). Furthermore, cleavage of the C-terminal Aib^{12} from the acetyldodecapeptide acid **21** and formation of Ac-Aib^1-Ala^{11}-OH (**22**) from that were observed. From **22**, the tripeptide Aib^9-Ala^{10}-Ala^{11} was subsequently released within 1 h, resulting in the formation of the acetyloctapeptide acid Ac-Aib^1-Aib^8-OH (**23**). After 3 h of acidolysis, cleavage of the C-terminal Gln^{17}-Valol^{18} from trichotoxin (**17**) was observed with release of the acetylhexadecapeptide acid Ac-Aib^1-Aib^{16}-OH (**19**). Notably, some trichotoxin (**17**) still remained intact even after 26 h of trifluoroacetolysis. These data are in excellent agreement with those of the preparative trifluoroacetolysis of native

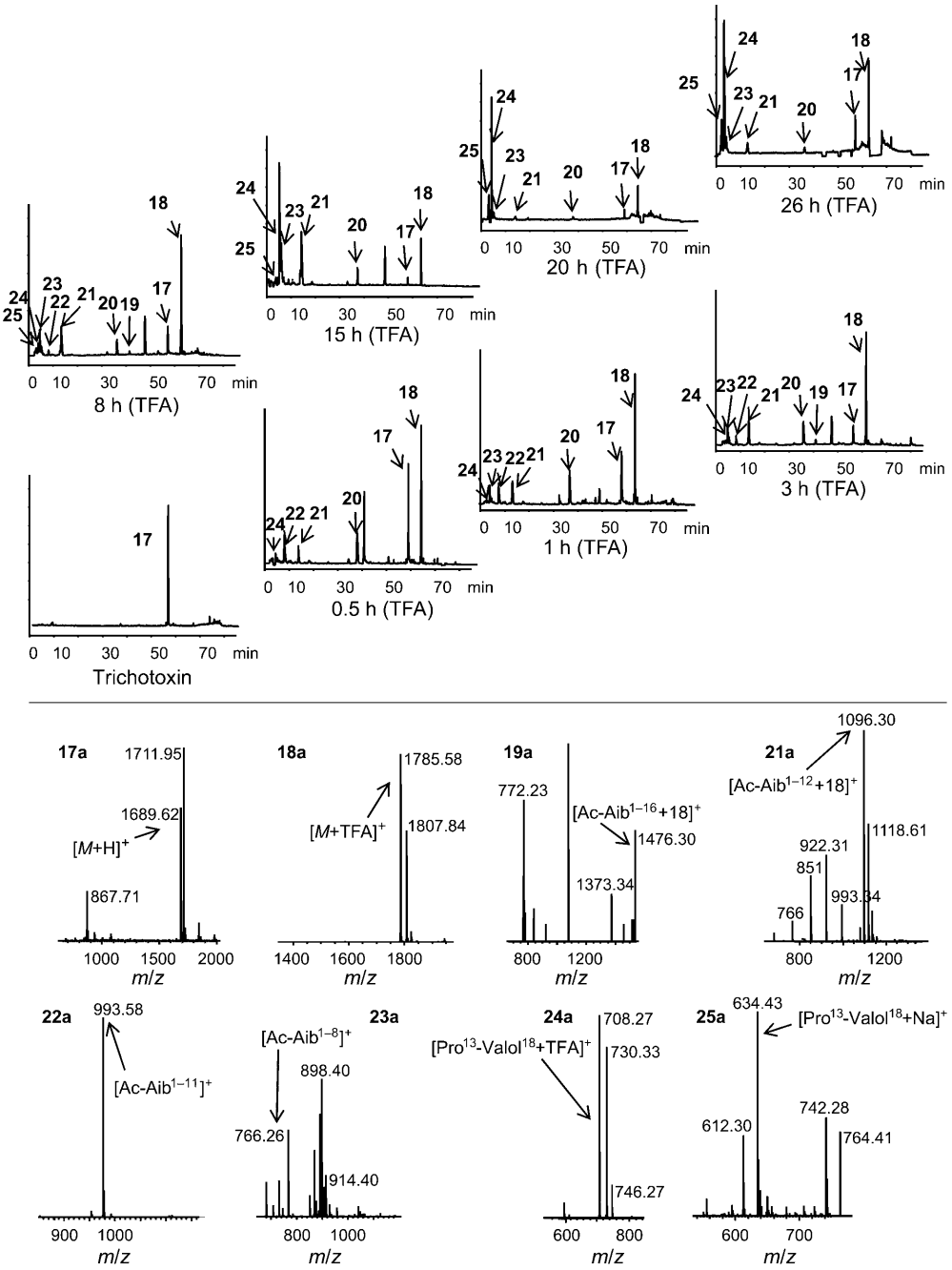


Fig. 4. *HPLC/TIC-MS of peptides* **17**–**25** *resulting from the trifluoroacetolysis of synthetic trichotoxin* (**17**; top), *and the corresponding diagnostic ions,* **17a**–**25a**, *used for their characterization* (bottom). Numbers refer to sequences presented in *Table 2*. Peptide **20** could not be assigned unambiguously.

Table 2. *Peptides* **20**, **21**, *and* **23**–**25**, *and TFA-esters* **18** *and* **22** *Resulting from the Trifluoroacetolysis of Trichotoxin* (**17**), *and Protonated Molecular Ions* $[M+H]^+$ *Used for the Characterization of Peptides*

	Detection after	Disappearance after	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		$[M+H]^+$ [m/z]
17			Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala	Aib	Pro	Leu	Aib	Aib	Gln	Valol		1690
18	0.5 h		Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala	Aib	Pro	Leu	Aib	Aib	Gln	Valol	TFA	1786
20	0.5 h																					
21	0.5 h		Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala	Aib								1096
22	0.5 h	15 h	Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala									1011
24	0.5 h														Pro	Leu	Aib	Aib	Gln	Valol	TFA	709
23	1 h		Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib											784
19	3 h	15 h	Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala	Aib	Pro	Leu	Aib	Aib				1476
25	8 h															Pro	Leu	Aib	Aib	Gln	Valol		612

trichotoxins [15], aimed on the isolation of fragments suitable for sequence determination. Compound **20**, however, appearing after 0.5 h and still detectable after 26 h, could not be characterized unambiguously. The acidolytic scission of the Gln^{17}-Valol^{18} C-terminus of **17** *via* simultaneous or subsequent formation of a pyroglutamic acid derivative has been treated in previous reports and, therefore, is not considered here [19].

Paracelsin (**28**). Fragments resulting from trifluoroacetolysis of peptaibols **28**–**30** are presented in *Fig. 1*, but TICs of HPLC/MS are not shown. Therefore, peptide fragments treated in the following paragraph are not assigned by numbers as done in the cases of Z-$(Aib)_{10}$-O^{t}Bu (**1**) and trichotoxin (**17**). After 0.5 h of trifluoroacetolysis, cleavage between Aib^{13}-Pro^{14} was observed with release of the acetyltridecapeptide acid Ac-Aib^1-Aib^{13}-OH and the prolylheptapeptide H-Pro^{14}-Pheol^{20}. In addition, scission of the C-terminal dipeptide H-Aib^{12}-Aib^{13}-OH from Ac-Aib^1-Aib^{13}-OH, resulting in the formation of Ac-Aib^1-Gly^{11}-OH, was observed. After 8 h of acidolysis, cleavage of C-terminal Gly therefrom occurred, resulting in the formation of the acetyldecapeptide acid Ac-Aib^1-Aib^{10}-OH. After 15 h of acidolysis, no starting material remained, anymore.

Alamethicin (**29**). The major difference between paracelsin and alamethicin is that the latter contains an additional acid-sensitive Aib–Pro bond in the Ac-Aib^1-Pro^2 terminus (see *Fig. 1*).

After 0.5 h of acidolysis, partial formation of the TFA ester of **29** was observed as well as scission of the Aib–Pro bonds, resulting in the detection of H-Pro^2-Pheol^{20} and its TFA-ester, H-Pro^{14}-Pheol^{20} and its TFA-ester, as well as the free dodecapeptide H-Pro^2-Aib^{13}-OH. The N-terminal Ac-Aib-OH was not observed under the MS conditions applied, but its formation and use of this particular selective cleavage for sequencing was demonstrated previously [15][16]. After 3 h, the fragment H-Pro^2-Aib^{13}-OH increased in intensity, indicating the faster cleavage of the N-terminal Ac-Aib^1–Pro^2 bond in comparison to the Aib^{13}–Pro^{14} bond. After 8 h of acidolysis, starting material **29** was no longer detectable. The data are also in good agreement with those of isolated fragments resulting from the preparative trifluoroacetolysis of the alamethicin-related peptaibol suzukacillin [16].

Antiamoebin (**30**). Owing to its microheterogeneity and the presence of two Hyp as well as a C-terminal Pheol residue, separation of fragments and interpretation of mass spectra were more difficult in comparison to the above peptaibols. After 0.5 h of trifluoroacetolysis, formation of a mixture of mono-, di-, and tri-TFA esters, resulting from the esterification of the OH groups of the two Hyp residues and the C-terminal Pheol residue, were observed. Cleavage of Iva^{12}-Hyp^{13} and Aib^{14}-Pro^{15} was observed after 0.5 h. After 1 h, cleavage of Aib^9-Hyp^{10} with formation of Ac-Phe^1-Aib^9-OH was recognized. Also after 1 h, partial cleavage of Ac-Phe^1-Aib^9-OH between Iva^5(Aib^5)-Gly^6, resulting in the release of Ac-Phe^1-Iva^5(Aib^5)-OH, was observed (amino acids in parentheses indicate exchange positions in the natural peptaibol mixture used, resulting in the detection of the corresponding homologous fragments). After 3 h, further cleavage of C-terminal Aib^9 from remaining Ac-Phe^1-Aib^9-OH occurred, resulting in the release of Ac-Aib^1-Aib^8-OH. After 15 h, cleavage between Aib^4-Iva(Aib)^5 and Aib^3-Aib^4 was recognized, resulting in the formation of Ac-Phe^1-Aib^3-OH and Ac-Phe^1-Aib^4-OH.

3. Discussion. – The fast acidolytic cleavage of the Aib–Pro bond, the release of Aib and small C-terminal peptides from the C-termini of fragments, as well as the apparently successive scission of C-terminal Aib from the Z- or Ac-protected or free homooligo-Aib-peptides, require an explanation. The latter peptides, in particular, have the advantage of consisting of uniform amino acid constituents. Thus, subtle steric or electronic effects of the varying side chains as known from peptides that exclusively consist of proteinogenic amino acids must not be considered in this case.

Consequently, the multitude of information about the acidolytic scission of amino acid derivatives or simple model peptides reported in the literature or observed in our laboratory should be discussed. Thus, they can be taken into account for mechanistic considerations on homooligo-Aib-peptides and native peptaibols/peptaibiotics.

Rapid scission of the Aib–Pro bond by TFA or HCl/AcOH has also been reported for Ac-Aib-Pro-NH_2 [20], serving as a model for the N-termini of peptaibols such as alamethicin or hypelcin. Notably, the Aib–Pro bond is more stable if mixtures of TFA/CH_2Cl_2 are used at ambient or lower temperatures. This was established for Boc-Gly-Ala-Aib-Pro-Ala-Aib-Aib-Glu(OBzl)-Gln-OMe. Using a 9.3% solution in TFA/CH_2Cl_2 5 :2 at 22°, it has been observed that the Boc group was completely cleaved within 0.5 h. However, no prolylpeptides could be detected by TLC after that time [20].

Trifluoroacetolytic cleavage of the Aib–Aaa bond has previously been investigated using the synthetic model peptides Ac-Aib-Ala-OH, Ac-Aib-Gly-OH, Ac-Ala-Aib-OMe, and Ac-Ala-Aib-Ala-OMe in the laboratory of *Günther Jung*, Tübingen University, Germany [19].

Whereas *ca.* 50% of the peptide bonds in Ac-Aib-Ala-OH and Ac-Aib-Gly-OH were cleaved with TFA within 3 h at 37°, cleavage of the peptide Ac-Ala-Aib-OMe started slowly after 6 h of acidolysis, but little cleavage was observed even after 19 h. Notably, only cleavage of the peptide bond was observed but not release of the Ac group. The tripeptide Ac-Ala-Aib-Ala-OMe remained almost unaffected on treatment with TFA for 23 h.

Based on the model peptide Ac-(NMe)Aib-Phe-OMe, *Creighton et al.* [5] postulated the formation of the oxazolone *via* an intermediate, tetrahedral oxazolinolium ion, from which the amino acid is ejected. The resulting Ac-(NMe)AibOx cation was postulated to be rapidly hydrolyzed by traces of H_2O with release of Ac-(NMe)Aib-OH.

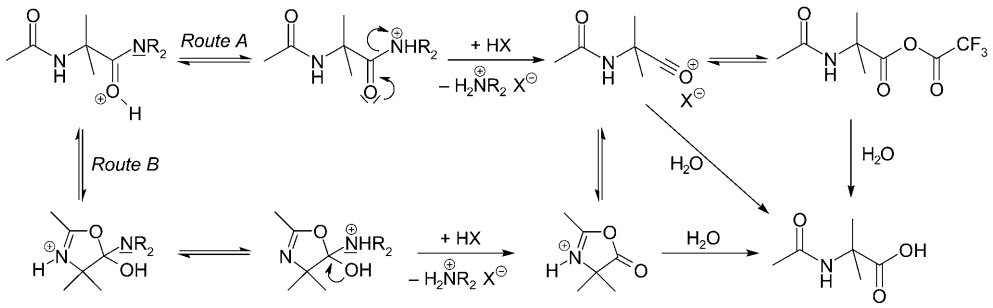
Notably, *Obrecht* and *Heimgartner* [10] had already demonstrated that $Me_2NH\cdot$HCl is released from peptides of the general structure Acyl-$(Aib)_n$-NMe_2 ($n = 1–3$), following treatment with HCl_g in toluene. Formation of intermediate oxazolones in this series was established by IR spectroscopy, as well as preparative isolation of the corresponding oxazolones. These authors also pointed out the experimental evidence for an equilibrium between the protonated oxazolone as well as the respective intermediate acylium ion or acid chloride.

This view is supported by the fact that the simple derivative Bz-Aib-NMe_2, considered as model for, *e.g.*, the Ac-Aib-Pro bond, is easily converted into the respective oxazolone by treatment with HCl_g in toluene at 80–100°. Such treatment resulted in the release of $Me_2NH\cdot HCl$ [9]. This oxazolone is easily hydrolyzed to Bz-Aib-OH by heating with mixtures of aqueous HCl/organic solvents. Analogously, the model peptides Z-Aaa-Aib-NMe_2 (Aaa: Ile, Ala) were directly converted into the

oxazolone by treatment with HCl_g/toluene (5–8 min, 100°). The corresponding dipeptide acids were obtained therefrom by treatment with 2N HCl for 0.5 h at room temperature. Intermediate formation of oxazolones in the latter cases was also deduced from the high degree of racemization of Ile and Ala as result of tautomerism. The problem of the racemization of the preceding chiral amino acids on prolonged activation of C-terminal Aib is well-known [21].

We found that treatment of Ac-Aib-NEt_2 with anhydrous TFA (10% solution, 15 min at 60°) resulted in the complete amide-bond cleavage with release of $Et_2NH\cdot$ TFA and Ac-Aib-OH within that time. This was evidenced by TLC and comparison with reference compounds. Notably, no formation of an oxazolone could be detected by TLC (*H. Brückner*, unpublished results). If one assumes protonation of the Ac O-atom in Ac-Aib-NEt_2 by TFA with formation of an oxonium ion, as has been proposed by *Klotz et al.* [22] for Ac-NHMe (*N*-methylacetamide), an intramolecular nucelophilic attack on the positive C-atom of the carboxamide group is not likely. In anhydrous TFA, representing a highly polar, ion-solvating solvent, however, concomitant release of the protonated amine and formation of an intimate ion-pair between the carbonium cation and trifluoroacetyl anion is favored that might result in an equilibrium with the respective covalently bonded mixed anhydride (*Scheme 1*, *Route A*). These intermediates are very reactive and will react quickly with traces of H_2O. This mechanism is related to that one formulated in [10], and, at least for Ac-Aib-NEt_2, does not require formation of an oxazolone, but excludes its formation neither directly (*Scheme 1*, *Route B*) nor *via* cyclization of an intermediate carbonium/acylium cation as shown in *A*. This mechanism might also be applied on the fast cleavage of N-terminal Ac-Aib-Pro bonds.

Scheme 1. *Possible Trifluoroacetolytic Cleavage of Ac-Aib-NEt_2* via *Intermediate Formation of an Ion Pair or Mixed Anhydride* (*Route A*) *or oxazolone* (*Route B*). R = Et, HX = TFA; $X^- = CF_3COO^-$.



However, most reports and studies presented in the introduction discuss or focus on the formation of an intermediate oxazolonium ion, indeed. This results from the intramolecular nucleophilic attack of the Aib C=O O-atom on the C-atom of the neighboring amide bond with release of the adjacent amino acid or substituted amine.

4. General Cleavage Mechanism. – Taking the aforementioned data into account, a general mechanism is proposed based on the facileness of the formation of the oxazolones and fragility of the protonated intermediates formed in Aib-peptides and their instability under the acidolytic cleavage conditions used.

The considerations on homooligo-Aib-peptides are extended to peptaibols and model peptides, and a mechanism is presented in *Scheme 2*. With regard to the following discussion, the encircled residue R represents a C-terminal Aib residue. The considerations can be extended on Pro (Hyp, Pip), or a disubstituted amide or, with limitations, to peptaibols containing Aib–Gly(Ala) bonds.

4.1. *Trifluoroacetolysis of Z- and Ac-(Aib)$_{10}$-O^{t}Bu and Z-(Aib)$_7$-O^{t}Bu*. Taking the large excess of TFA into account, the *t*-Bu groups of the peptides are cleaved immediately, and scission of the peptide bonds of the resulting peptide acids is discussed in the following.

Internal nucleophilic attack of the C=O O-atom of the carboxamide group of the second to last Aib on the carboxy group of the last Aib residue is probably favored by the *geminal* dimethyl or *Thorpe–Ingold* effect [7][23]. This leads to the fast formation of an unstable tetrahedral oxazolinolium intermediate [4], which is subsequently stabilized by proton transfer and release of the original C-terminal Aib. The resulting oxazolonium peptides are easily hydrolyzed by traces of H_2O which are always present, even in TFA labelled as anhydrous.

From the fast release of a complete series of Z-(Aib)$_{9–4}$ from Z-(Aib)$_{10}$-OH, a repetitive cleavage of the respective C-terminal Aib is proposed. The series ends with the formation of Z-(Aib)$_4$-OH. This might be explained by the acidolytic stability of tri- and tetrapeptides as a result of their particularly stable secondary structures.

This stability against trifluoroacetolysis of the smaller acylpeptides is also supported by the release of Ac-Phe^1-Aib-Aib^3-OH from antiamoebins after 15 h. This tripeptide, however, remained resistant against further cleavage (see *Fig. 1*).

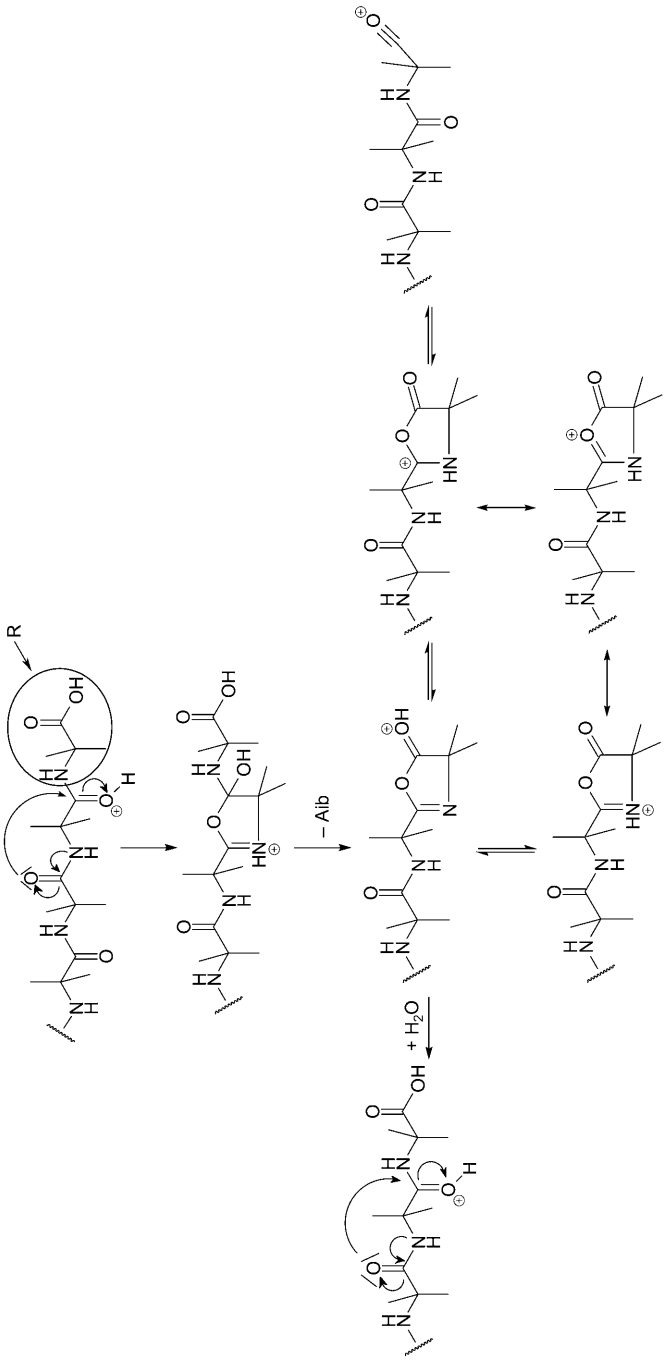
Remarkably, oxazolones are not formed from the C-terminal Aib but from the preceding ones. From a C-terminal oxazolone, the precursor peptide would be reconstituted on hydrolysis. Therefore, it is assumed that the C-terminal carboxy groups form H-bridges with TFA, and the resulting solvent clusters shield the C-atom of the carboxy group from nucleophilic attack of the O-atom by the adjacent carboxamide group. This effect might also explain the observed stability of Ac-Aib-OH in TFA.

However, concomitant cleavage of the Z-group leading to the series H-(Aib)$_{10–3}$-OH is also observed. This observation contradicts experiences from non-Aib-peptide chemistry: the Z-group, commonly used for orthogonal protection in peptide chemistry, is usually stable towards TFA under the conditions applied and requires, *e.g.*, boiling TFA for its removal. Unexpected cleavage of the Z-group from Z-Ala-Aib-NMe$_2$ on treatment with HCl_g in MeOH was also reported [9].

The release of Aib residues from the N-terminus of the free peptides is assumed not to take place because the protonated N-terminus decreases the nucleophilicity of the corresponding carboxamide group of Aib. This phenomenon is attributed to its electron-withdrawing properties.

From the data obtained from prolonged trifluoroacetolysis of peptaibols and release of C-terminal di- to tetrapeptides therefrom (see *Fig. 1*), formation of oxazolones at certain positions in the peptide backbone is also concluded according to the general mechanism proposed (in *Scheme 2*, encircled R in these cases represent C-terminal peptide sequences).

Scheme 2. *Proposed C-Terminal Cleavage Mechanism of Homooligo-(Aib)$_n$-Peptides* via *Repetitive Intermediate Formation of Oxazolinolium/Oxazolinium* (oxazolonium) *Ions after Treatment with TFA, Leading to Homooligo-(Aib)$_{n-1}$-Peptides* (left side) *and Related Formation of the* b-*Series of Positive Fragment Ions under Conditions of ES-MS* (right side). Encircled R represents an α-aminoisobutyryl or prolyl residue, or a disubstituted amine.



The considerations outlined for homooligo-Aib-peptides can be extended on peptide bonds formed between Aib and Pro or homologues. The scission of the amide bond proceeds fast and can take place at any position of the polypeptide chain.

4.2. *Trifluoroacetolysis of Peptaibols.* The above considerations can also be applied to *alamethicin* and *paracelsin*. In the case of *alamethicin*, fast cleavage of the Aib[1]–Pro[2] and Aib[13]–Pro[14] bonds with release of the respective fragments, according to the proposed mechanism, was observed. In paracelsin, fast cleavage of the Aib[13]–Pro[14] bond occurs. From the resulting Ac-Aib[1]-Aib[13]-OH, release of the dipeptide H-Aib[12]-Aib[13]-OH and of the tripeptide H-Gly[11]-Aib[12]-Aib[13]-OH on prolonged trifluoroacetolysis are also observed. Again, release of stable tripeptides and nucleophilic attack of constrained Aib on the sterically least constrained Gly were recognized. With the exception of this amino acid, sequence of -Aib-Aaa-(Aib-Aaa)$_n$-Aib- (Aaa, proteinogenic amino acid; exceptions C-terminal Aib-Gln-Xol bonds [15][19]) are stable against trifluoroacetolysis.

In *paracelsin*, release of C-terminal dipeptide Aib[12]-Aib[13] from Ac-Aib[1]-Aib[13]-OH (the latter resulting from scission of the C-terminal prolylheptapeptide) is assumed to proceed also *via* formation of an intermediate oxazolone. Cleavage of the Aib[10]–Gly[11] bond after 8 h indicates moderate acid-sensitivity of this bond.

In *trichotoxin*, the fragment Ac-Aib[1]-Ala[11]-OH formed on trifluoroacetolysis behaves conspicuously different because the C-terminal tripeptide Aib[9]-Ala[10]-Ala[11] was released therefrom and not the C-terminal Ala[11]. This is explained by the fact that the Ala residues, in contrast to Aib, contain C_α-hydrogens and oxazolone formation is not favored. Intramolecular attack of the carbonyl of Aib[8] on Aib[9], however, might proceed *via* formation of oxazolone and release of the tripeptide as outlined before. The stability of the tripeptide is in principal agreement with that of the model Ac-Ala-Aib-Ala-OMe mentioned above. The remaining Ac-Aib[1]-Aib[8]-OH remains stable in TFA for hours; possibly formed smaller fragments could not be detected.

Generally, cleavage of Aib-peptides is much slower if mixtures of TFA with organic solvents such as CH_2Cl_2 are used. This is explained by the decrease of the dissociation as well as concentration of TFA. Cleavage is also much lowered when acidic aqueous solvents are used or water is added to TFA. This is explained by a much lower capability to form oxazolones in aqueous solution. Heating at elevated temperature at prolonged time, however, enables acidolytic cleavage under these conditions (see examples in introduction). It is assumed that a change from the oxazolone mechanism to the common acid-catalyzed cleavage of amide bonds proceeds, with preference for disubstituted amides.

4.3. *Considerable Accordance of Trifluoroacetolytic Scission Pattern and Mass-Spectrometric Fragmentation*. Inspection of the trifluoroacetolytic fragments of Z-(Aib)$_{10}$-O^{t}Bu, resulting in the series Z-(Aib)$_{10-5}$-OH, and the Low-CID-MS of the decapeptide, shows that there is considerable agreement between the generation of homooligo-Aib-peptide fragments in the protic solvent and gas-phase dissociation, since corresponding regular series of *b* ions, corresponding to the Z-(Aib$_{10-2}$)$^+$ acylium (or oxonium/oxazolonium) cations (see *Fig. 2*), can be observed in the positive-ion mode (see *Scheme 2*).

Analogously, a regular series of b_2 to b_{10} cations is also generated from Ac-(Aib)$_{10}$-O^{t}Bu, and of b_2 to b_7 cations from Z-(Aib)$_7$-O^{t}Bu.

Notably, generation of acylium ions at low collision energy in peptides has been proposed to proceed *via* nucleophilic attack of the peptide bond C=O at the protonated carbonyl group of the preceding C-terminal amino acid, formation of an intermediate oxazolol, and proton transfer onto the N-atom of the amino acid that is released with formation of an oxazolonium/oxazonium cation [24–26]. Regarding peptaibols, prolyl residues are the best leaving groups and give rise to the respective intensive *y* series [27][28].

The abundances of the corresponding acylium ions are also in striking agreement: highest intensities are observed for ions resulting from Aib residues owing to the stabilization by the geminal dimethyl groups, lower intensities are observed for acylium ions from protein amino acids. For example, in the partial sequence -Aib^{9}-Ala^{10}-Aib^{11}-Aib^{12}-Pro^{13}- of trichotoxin A40/5, the resulting b_{12} acylium ion is most intensive, and the b_9 and b_{11} ions are comparatively more intensive than the b_{10} ion [27]. The notable moderate acid sensitivity of the -Aib–Gly- bond can also be observed in the positive-ion MS of suzukacillin A4. The MS/MS of the b_{13} fragment ion resulting from the partial sequence -Aib^{8}-Aaa^{9}-Aib^{10}-Gly^{11}-Aib^{12}- displays the highest ion abundance of b_{10}, and, lower abundance of b_9 and, in particular, of b_{11} in comparison to b_8 and b_{12} acylium ions [28].

Finally, it should be emphasized that the results presented here for oligo-Aib-peptides and peptaibols, and the mechanistic aspects deduced therefrom are in excellent agreement with the data and postulates resulting from the azirine/oxazolone approach promoted for many years by the group of *Heimgartner* [10].

5. Consequences and Outlook. – Beyond the mechanistic considerations outlined above, the potential acid-sensitivity of peptaibiotics has to be considered in the course of isolation and handling of natural products, as well as in planning of classical or solid-phase syntheses of peptaibiotics or peptides containing the special amino acids or analogues mentioned in the text.

From an applied point of view, the selective acidolytic scission of peptaibols and analysis of their fragments is of importance for their sequence determination in order to generate fragments suitable for the assignments of the position and configuration of amino acids, such as Iva enantiomers [15–18].

Use of proteases for the selective cleavage of peptaibiotics is of limited value, since bonds formed by repetitive Aib(Iva) residues or adjacent protein amino acids are completely resistant towards proteolytic degradation. For example, the 15-residue peptaibols of the native ampullosporin mixture displaying N-terminal Ac-Trp and C-terminal leucinol could neither be cleaved by the action of pepsin, chymotrypsin, or pronase, nor by treatment with liver homogenates [29].

This is in agreement with our and others' attempts to cleave trichotoxin or alamethicin with common proteases [30]. However, special peptaibiotics comprising bonds between consecutive proteinogenic amino acids, α-amino acids and β-amino acids, are cleaved at certain positions. This has been shown by the cleavage of the Gly^{13}–Leu^{14} and Leu^{6}–β-Ala^{7} bonds of neoefrapeptins with papain [31], and for synthetic ampullosporin analogues in which Aib residues had been replaced by a series of subsequent Ala residues [29].

However, since formation and degradation of peptaibiotics under fermentation conditions was observed for trichobrachins [32], fungal enzymes must exist being capable of cleaving Aib-peptide bonds. The search for such enzymes being capable of cleaving such sterically constrained bonds is thus encouraged. It might provide a novel class of enzymes being capable of degrading related polymers such as plastic fibres or detergents.

The extreme enzymatic resistance of the peptaibiotics, together with their microheterogeneity, might be the result of the defence strategy of their fungal producers [11] in order to overcome degradation of their arsenal of antibiotic and membrane-modifying peptides in their natural environments [33].

Experimental Part

Abbreviations. Aib, α-aminoisobutyric acid; Iva, isovaline (2-ethylalanine); Pheol, L-phenylalaninol; Valol, L-valinol; Xol, 2-amino alcohol; (NMe)Aib, 2-amino-*N*-methylisobutyric acid; (NMe)Ala, *N*-methylalanine; (NMe)αAc^{5}c, 1-amino-*N*-methylcyclopentanecarboxylic acid; Ser(Bn), serine-*O*-benzyl; Me_2N, dimethylamino; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Pmb, 4-methoxybenzyl; Bn, benzyl; Aaa, proteinogenic amino acids; oxazolone refers to oxazole-5(4*H*)-one and pseudoaromatic oxazolone intermediates; AibOx in the synthesis part refers to the oxazolone of the C-terminal Aib; Pip, pipecolic acid; SPip, γ-thiapipecolic acid; Aoe, L-2-amino-9,10-epoxy-8-oxodecanoic acid.

Chemicals. MeCN and MeOH were obtained from *Riedel-de Haën* (*Sigma Aldrich*, D-Seelze); CF_3COOH (TFA) was from *Fluka* (CH-Buchs) and used without further treatment.

Homooligo-Aib-Peptides. The homooligo-Aib-peptides were synthesized according to the oxazolone method [34–36] but instead of stepwise elongation of the peptide segments condensation was used [37]. Briefly, coupling of Z-Aib-Aib-AibOx and H-$(Aib)_2$-O^{t}Bu in butyronitrile at 100° for 24 h yielded Z-$(Aib)_5$-O^{t}Bu. Hydrogenolysis in MeOH in the presence of Pd/C provided H-$(Aib)_5$-O^{t}Bu, and treatment of Z-$(Aib)_5$-O^{t}Bu with TFA/CH_2Cl_2 for 1 h at r.t. yielded Z-$(Aib)_5$-OH. The pentapeptide was converted into the respective oxazolone by treatment with Ac_2O for 1.5 h at 100°.

Reaction of Z-$(Aib)_4$-AibOx and H-$(Aib)_5$-O^{t}Bu in butyronitrile at 100° for 36 h provided Z-$(Aib)_{10}$-O^{t}Bu (**1**). Hydrogenolysis, followed by acetylation with Ac_2O/CH_2Cl_2 1:1 at ambient temp. for 4 h, provided Ac-$(Aib)_{10}$-O^{t}Bu (**26**) that, after precipitation from MeOH/CH_2Cl_2/petroleum ether, was obtained in *ca.* 65% purity as calculated from HPLC. Reaction of Z-$(Aib)_4$-AibOx and H-$(Aib)_2$-O^{t}Bu in butyl acetate/1,1,2,2-tetrachloroethane 10:1 at 100° for 18 h provided Z-$(Aib)_7$-O^{t}Bu (**27**). Protected peptides were characterized by MS, uniformity in TLC, and HPLC. For crystal structures of **1** and **27**, see [38][39]; the ESI-MS of **1** is depicted in *Fig. 2*.

Native Peptaibols. Native peptaibols were isolated in our laboratories as natural, microheterogeneous mixtures from the culture broths of the producing fungi. Alamethicin F50 containing the major componentent alamethicin F50/5 (**29**; see *Fig. 1*) was isolated from *Trichoderma viride* NRRL 3199 [40] (recently redescribed as *T. arundinaceum* [11]), crude paracelsins A–D were isolated from *Trichoderma reesei* QM 9414 [41], and the pure component paracelsin A (**28**) was isolated from the paracelsin mixture by HPLC; microheterogeneous antiamoebins were isolated from *Emericellopsis salmosynnemata* CBS 176.60 [42], and contained antiamoebin I as the major component **30** (see *Fig. 1*); trichotoxin A50/E (**17**) was synthesized by conventional soln.-phase synthesis [43] and also characterized by X-ray crystallography [44].

Trifluoroacetolysis of Peptides. TFA (100 µl) was added to 0.1 mg amounts of peptides in 1-ml screw-cap vials (size 25 mm × 10 mm ID). The tightly closed vials were immediately placed in a sand bath and incubated at 37 ± 1° for various periods of time.

After 0.5, 1, 3, 8, 15, 20, and 26 h treatment, TFA was immediately removed in a cold stream of N_2, CH_2Cl_2 was added to the dry residue, and vials were flushed again with N_2 in order to remove traces of TFA. The remaining residues were dissolved in MeOH (100 µl), and aliquots (10 µl) were subsequently analyzed by LC/ESI-CID-MS.

Instruments and Conditions for HPLC. For HPLC, a *HP 1100* series instrument comprising a model *G1322A* degasser, *G1312A* binary pump, *G1313A* autosampler, *G1316A* column thermostat, *G1314A* UV/VIS detector, and *HP ChemStation* software for LC (Rev. A.04.02) was used (all from *Agilent*, D-Waldbronn, or Palo Alto, CA, USA). The HPLC column used (150 mm × 4.6 mm ID) was filled with *Kromasil 100–3 C_8* material, particle size 3 μm. The column was kept at 35°. Elution profiles of peptides were recorded at a wavelength of 205 nm or as total ion currents (TIC) in on-line-ESI-MS. The following LC gradient was used: eluent *A:* MeOH/MeCN/H_2O 32 : 32 : 36; eluent *B:* MeOH/MeCN 50 : 50. TFA (0.1%) was added to both eluents.

Time [min]	Eluent [%]		Flow rate [ml/min]
	A	B	
0	100	0	0.8
5	100	0	0.8
45	50	50	0.8
65	0	100	0.8
75	0	100	1
76	100	0	1
85	100	0	1

Mass Spectrometry. For ESI-CID-MS, a calibrated *LCQ-MS*™ (*Thermo Quest*, Finnigan MAT, San Jose, CA, USA) was used. N_2 served as sheath and auxiliary gas. He (purity >99.9990%, *Messer-Griesheim*, D-Krefeld) was used as collision gas. The temp. of the heated capillary was 250°; sheath gas and auxiliary gas were set at 40 and 10 relative units, resp. Ion source collision-induced dissociation (CID)MS was performed at 0 and 45% relative collision energy. Sequence analyses were carried out in the positive ionization mode. The *m*/*z* values were recorded in centroid mode and have an accuracy of ±0.5 Da.

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Profiling of Arabidopsis Secondary Metabolites by Capillary Liquid Chromatography Coupled to Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry[1]

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Large-scale metabolic profiling is expected to develop into an integral part of functional genomics and systems biology. The metabolome of a cell or an organism is chemically highly complex. Therefore, comprehensive biochemical phenotyping requires a multitude of analytical techniques. Here, we describe a profiling approach that combines separation by capillary liquid chromatography with the high resolution, high sensitivity, and high mass accuracy of quadrupole time-of-flight mass spectrometry. About 2,000 different mass signals can be detected in extracts of Arabidopsis roots and leaves. Many of these originate from Arabidopsis secondary metabolites. Detection based on retention times and exact masses is robust and reproducible. The dynamic range is sufficient for the quantification of metabolites. Assessment of the reproducibility of the analysis showed that biological variability exceeds technical variability. Tools were optimized or established for the automatic data deconvolution and data processing. Subtle differences between samples can be detected as tested with the chalcone synthase deficient *tt4* mutant. The accuracy of time-of-flight mass analysis allows to calculate elemental compositions and to tentatively identify metabolites. In-source fragmentation and tandem mass spectrometry can be used to gain structural information. This approach has the potential to significantly contribute to establishing the metabolome of Arabidopsis and other model systems. The principles of separation and mass analysis of this technique, together with its sensitivity and resolving power, greatly expand the range of metabolic profiling.

Metabolomics, the comprehensive analysis of metabolites present in a biological sample, has emerged as the third major path of functional genomics beside mRNA profiling (transcriptomics) and proteomics (Fiehn, 2002; Sumner et al., 2003). Metabolomic approaches seek to profile metabolites in a nontargeted way, i.e. to reliably separate and detect as many metabolites as possible in a single analysis. Combined with information on transcript and protein abundance, this would ideally lead to a nearly complete molecular picture of the state of a particular biological system at a given time.

A characteristic of plant life is the production of a vast number of natural compounds, often called secondary metabolites. Secondary metabolites have crucial roles in plant development as well as in the interaction of a plant with its biotic and abiotic environment (Kutchan, 2001). They are "secondary" only in the sense that in different plant species, different sets of metabolites occur (Pichersky and Gang, 2000). Ample evidence has been obtained in the past three decades for a wide range of functions of secondary metabolites. Arrays of antimicrobial compounds help defend against pathogens (Dixon, 2001; Hahlbrock et al., 2003) and deter herbivores. Secondary metabolites can function as signals internally or in communication with a symbiont (Peters et al., 1986). They provide protection against abiotic stresses such as UV light, drought, or high salt concentrations (Jin et al., 2000).

The majority of steps in the biosynthesis of secondary metabolites is assumed to be catalyzed by specific enzymes (Pichersky and Gang, 2000). Appropriately, completion of the Arabidopsis genome sequence revealed that a significant proportion of the approximately 25,000 predicted genes encode proteins assumed to function in secondary metabolism. The Arabidopsis genome contains >250 cytochrome P450 genes (The Arabidopsis Genome Initiative, 2000), >100 acyl transferase genes, >300 glycosyl transferase genes, >300 glycoside hydrolase genes (http://www.Arabidopsis.org/info/genefamily/genefamily.html), and a large number of genes encoding enzymes such as dioxygenases, *O*-methyl transferases, terpene synthases, or polyketide synthases. There is a huge discrepancy between the number of these genes and the number of known reactions catalyzed by these types of enzymes in

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Arabidopsis, leading to the conclusion that a large number of metabolites have yet to be identified. Some of them might even belong to compound classes that so far have not been known to occur in Arabidopsis at all (The Arabidopsis Genome Initiative, 2000). Thus, understanding a significant part of Arabidopsis biology requires methods allowing the sensitive detection and quantification as well as the identification of secondary metabolites. Applying such techniques to various genetic backgrounds, and to different environmental and developmental conditions then would help elucidate the function of such compounds and of the genes involved in their biosynthesis.

Profiling schemes for Arabidopsis and other plants have been developed in recent years (Roessner et al., 2000, 2001; Fiehn et al., 2000; Wagner et al., 2003). The main focus of these mostly gas chromatography (GC)-mass spectrometry (MS)-based approaches have been metabolites of the primary metabolism such as sugars, amino acids, organic acids, or sugar alcohols. Several hundred compounds can be robustly and reliably detected. However, these first pioneering reports already emphasize the need for complementary liquid chromatography (LC)-MS-based approaches to allow a more comprehensive profiling of metabolites (Roessner et al., 2000). The coupling of electrospray ionization (ESI) MS with capillary (Cap) electrophoresis (Soga et al., 2002) and hydrophilic interaction chromatography (Tolstikov and Fiehn, 2002) has been successfully applied to metabolomics problems. Every analytical procedure is necessarily limited as to what type of compounds can be separated and detected. GC-MS is predominantly applied to very polar or unpolar substances, and the main application range of LC-MS is more related to compounds of medium polarity. Furthermore, LC coupled to an MS technique providing soft ionization and high mass accuracy has the potential to generate information useful for the identification of unknown compounds because molecular ions and characteristic fragments can be detected (De Hoffmann, 1996; Niessen, 1999).

We initiated a project aiming at exploiting the potential of hybrid mass spectrometers developed in the past few years for the profiling of Arabidopsis metabolites. Here, we introduce an experimental system based on Cap LC coupled to ESI quadrupole time-of-flight MS (CapLC-ESI-QqTOF-MS). Using this approach, we were able to detect more than 800 mass signals (*m/z* values) in root extracts and more than 1,400 mass signals (*m/z* values) in leaf extracts of Arabidopsis. Electrospray as a soft ionization method combined with the enhanced resolution and mass accuracy of the TOF instrument and tandem MS make the identification of unknown compounds feasible, and examples are presented.

RESULTS

The major objective of the work described here was to develop a metabolite profiling scheme using the robust and well-established separation of extracts by LC on reversed-phase material in combination with state-of-the-art MS. The basic assumption was that such an approach would very well complement the pioneering GC-MS-based schemes by extending the range of metabolome analysis to those compound classes not amenable to GC analysis. These would include a significant fraction of the plant secondary metabolism.

A few years ago, QqTOF mass spectrometers were introduced. They combine TOF mass analysis with the established technique of ESI, resulting in high sensitivity, high mass resolution and high mass accuracy (Chernushevich et al., 2001), and are therefore principally well suited for comprehensive metabolite profiling. However, to our knowledge, no reports of such an application of hybrid mass spectrometers have yet been published.

Plant Growth, Extraction, and Cap LC

To be able to grow Arabidopsis with high reproducibility and to have easy access to leaf and root material, we established an aseptic hydroponic growth system. This more laborious approach limits throughput to some extent. The major benefit in addition to a more complete analysis of plant biology is that this system allows us to also biochemically profile the responses in a plant organ not directly exposed to a certain change in the environment.

Plant tissues were routinely harvested after 6 weeks and were frozen in liquid nitrogen. Freeze-drying was omitted to minimize loss of potentially susceptible compounds, such as volatiles, glucosinolates, and glycosides. Frozen plant material was homogenized in liquid nitrogen and was extracted twice with 80% (v/v) aqueous methanol. A third extraction did not produce any considerable gain in yield. Extracts were separated by Cap LC using C18 material. Flow rate and composition of the gradient had to be optimized to obtain good separation and maximum stability of the electrospray. A flow rate of 5.5 μL min^{-1} and a gradient starting from 5% (v/v) acetonitrile were found to produce the best results.

Manual Data Analysis and Reproducibility

Cap LC was coupled to ESI-QqTOF-MS analysis operated in positive ion mode. Instrument parameters were set in such a way that the mass range from 106 to 1,000 D was monitored simultaneously. The signals generated by ions arriving at the detector were routinely summarized in a spectrum every 2 s for a total of 1,590 spectra during a 53-min run. The resulting total ion chromatogram did not resolve distinct peaks (Fig. 1A). Extensive data deconvolution

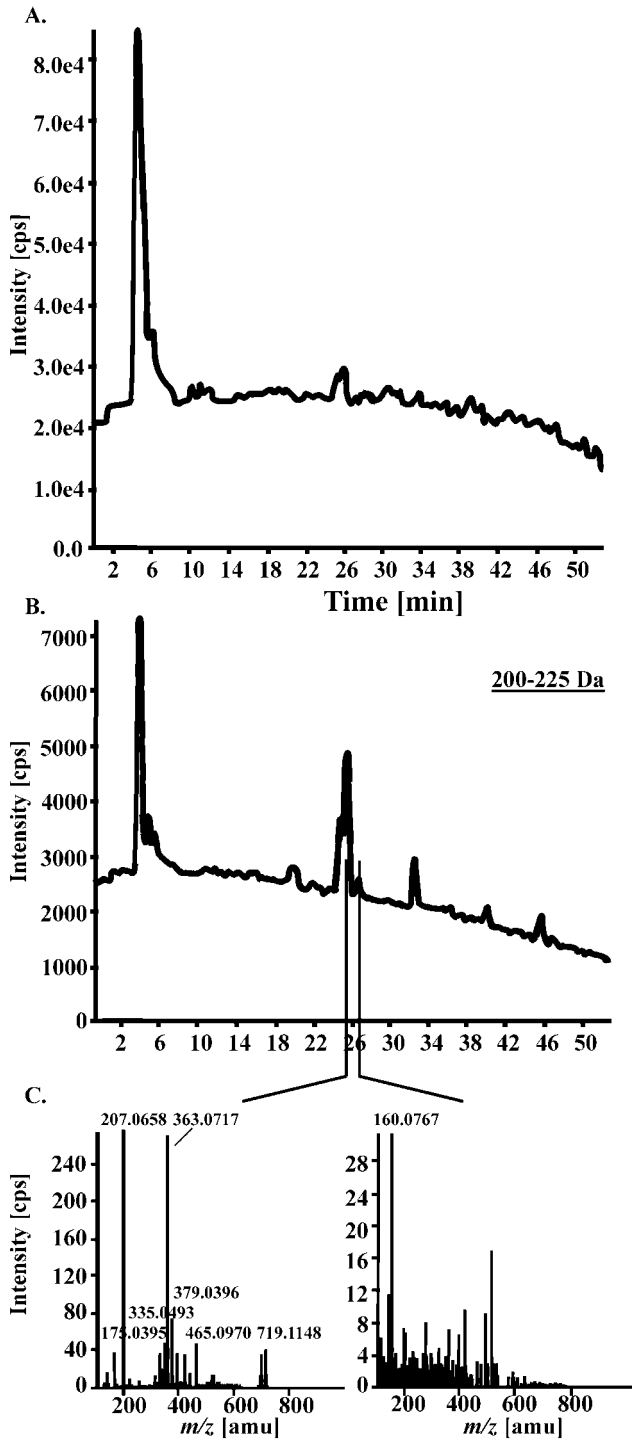

Figure 1. Manual deconvolution of data generated by CapLC-ESI-QqTOF-MS. A 53-min LC-MS analysis generated a total ion chromatogram consisting of 1,590 single MS spectra. A, The total ion chromatogram was manually deconvoluted in 25-D slices resulting in single extracted ion chromatograms (XICs) covering, for example, a mass range from 200 to 225 D. B, Mass spectra were then extracted from peaks apparent within a particular time frame of the XIC. The extracted peak at t_R 26.0 min (C, left) represents sinapoyl malate ($[M+Na]^+$ *m/z* 363.0717;[2 sinapoyl malate+K]$^+$ *m/z* 719.1148; in-source fragment at *m/z* 207.0658). The extracted XIC valley at 26.6 min (C, right) represents a typical background mass spectrum.

was required to extract defined mass spectra. This was done manually by dividing the mass range of 106 to 1,000 D into mass windows of 25 D (Fig. 1B). Mass spectra were then extracted from peaks apparent within a particular mass window (Fig. 1C). Several hundred mass signals are extractable in this way from a single run.

At this stage of manual data deconvolution, quantifiability of mass signals, as well as the reproducibility of the mass spectrometric analysis, of the extraction and of the biological material were assessed. The isoflavone biochanin A was selected as an internal standard. A calibration curve was recorded, and 5 pmol was determined to lie within the range of linearity between amount and signal intensity. When added to a plant extract, the recovery rate of biochanin A was practically 100%. Twenty-one and 24 mass signals in root and leaf extracts, respectively, across the Cap LC run and representing a wide variety of intensities and masses were then randomly chosen (Tables I and II). Analysis of different dilutions of extracts and inspection of resulting calibration curves showed that, for most signals, the linear range covered almost two orders of magnitude. Up to 50-fold dilution resulted in a linear decrease in intensity. Untreated wild-type Arabidopsis Columbia (Col-0) plants were analyzed in four independent experiments. To obtain maximum mass accuracy, each run was recalibrated against five characteristic fragment or adduct ions of sinapoylmalate (Fig. 1C, left) in the case of leaf samples, and five characteristic fragment or adduct ions of hirsutin in the case of root samples. The selected signals were then quantified by integrating the peak areas using AnalystQS and by normalizing them to the fresh weight of the plant material extracted.

The reproducibility of the CapLC-ESI-QqTOF analysis was determined by extracting the same material three times and by running three times the same extract. The cumulative SD for repeated analysis of the same extract was 11.1% (Fig. 2, A and C). When different extracts of the same material were compared, an average variability of 25% ± 17.9% was found for leaves, and 21.6% ± 13.4% for roots. The numbers indicate that the technical reproducibility varies considerably, indicating a dependence on the mass signal in question. When four independent experiments were analyzed, an average biological variation was measured of 35.5% ± 14.0% for leaf signals and 55.9% ± 26.0% for root signals (Fig. 2, B and D). Again, the degree of variation was dependent on the mass signal.

Signals were identified based on t_R and mass. The stability of t_R was determined for six mass peaks eluting between 25 and 51 min. Seventeen analyses of six different extracts were performed over a period of several days. The maximum SD was 0.3 min and the average SD was 0.25 min, indicating the stability of the Cap LC separation. The mass accuracy was eval-

Table I. *Mass signals from root extracts used for assessing the variance of the CapLC-ESI-QqTOF-MS analysis*

A total of 21 signals and the internal standard biochanin A were chosen. Listed here are their high-resolution (HR) masses and retention times (t_R) as identifiers. Based on the mass, elemental compositions were calculated and the corresponding compounds were tentatively identified.

HR Mass	t_R	Elemental Composition (if available)	Compound	Substance Class
m/z	*min*			
138.1277	46.5	$C_9H_{16}N$	9-Methylthiononanenitrile $[M + H\text{-}CH_3SH]^+$	Nitrile
138.1277	26.5	$C_9H_{16}N$	9-Methylsulphinylnonanenitrile $[M + H\text{-}CH_4SO]^+$	Nitrile
160.0756	38.8	$C_{10}H_{10}NO$	Methoxy-substituted indole	Indole
160.0756	30.1	$C_{10}H_{10}NO$	Methoxy-substituted indole	Indole
179.0722	24.2			
202.1251	26.5	$C_{10}H_{20}NOS$	9-Methylsulphinylnonanenitrile $[M + H]^+$	Nitrile
234.0986	35.9	$C_{10}H_{20}NOS_2$	8-Methylsulphinyloctyl ITC (hirsutin $[M + H]^+$)	Isothiocyanate
256.0798	35.9	$C_{10}H_{19}NOS_2Na$	8-Methylsulphinyloctyl ITC (hirsutin $[M + Na]^+$)	Isothiocyanate
277.1416	43.4			
285.0756	**40.7**	**$C_{16}H_{13}O_5$**	**Biochanin A (internal standard)**	**Isoflavone**
317.1200	4.6			
381.0858	4.7			
429.2454	4.7			
438.1396	24.0			
441.1396	24.2	$C_{20}H_{25}O_{11}$	Substituted ferulic acid glucoside?	
457.1134	24.2	$C_{23}H_{21}O_{10}$	Substuituted phenylpropane glucoside?	
489.1708	35.9	$C_{20}H_{38}N_2O_2S_4Na$	8-Methylsulphinyloctyl ITC (hirsutin. $[2M + Na]^+$)	Isothiocyanate
537.3151	43.4			
553.2743	43.4			
699.3608	38.8			
810.2553	27.9			
844.2019	28.0			

uated by averaging the mass difference for 11 signals from known compounds. Signal intensities covered a range of about two orders of magnitude. The average mass difference through 18 runs was 9.0 ± 3.3 ppm.

Thus, we concluded at this stage that CapLC-ESI-QqTOF-MS can be a valuable tool for the robust and reproducible simultaneous detection and quantification of several hundred mass signals.

Identification of Known Compounds

As mentioned above, it has to be assumed that most of the Arabidopsis metabolites are as yet unknown. Moreover, unlike in the analysis of the major primary metabolites, standards are available for only very few compounds monitored by LC-MS and, in contrast to GC-MS, extensive mass spectral libraries do not exist for LC-MS. Therefore, being able to directly gain structural information on metabolites detected in profiling experiments is a key requirement for metabolomics. The QqTOF-MS technology has the potential to provide this information because the HR mass of a single ion can be used for calculation of a number of elemental compositions corresponding to those within a window of less than 30 ppm, usually 5 to 15 ppm. As shown, this mass accuracy is routinely achievable in profiling experiments with complex samples. A first evaluation of the potential of QqTOF-MS was carried out with the list of mass signals selected for the assessment of reproducibility. Elemental compositions were calculated. To further restrict the number of possible elemental compositions, the isotopic patterns of the ions of interest were checked for the presence of characteristic heteroatoms, such as sulfur, by comparing the calculated with the measured isotopic distribution. The possible presence of nitrogen can be deduced from even-mass numbered $[M+H]^+$ and/or $[M+Na]^+$ ions, respectively. With this information, a literature search for known natural products could now be performed using commercially available sources such as SciFinder, the Chapman & Hall Dictionary of Natural Products on CD-ROM, the NIST database, etc. Tables I and II list the information obtained on these peaks based on the exact mass and literature data. In leaf extracts, nine out of 24 signals could tentatively be identified, and in root extracts,

Table II. *Mass signals from leaf extracts used for assessing the variance of the CapLC-ESI-QqTOF-MS analysis*

A total of 24 signals and the internal standard biochanin A were chosen. Listed here are their HR masses and t_R as identifiers. Based on the mass, elemental compositions were calculated and the corresponding compounds tentatively were identified.

HR Mass	t_R	Elemental Composition (if available)	Compound	Substance Class
m/z	*min*			
114.0371	23.8	C_5H_8NS	4-Methylsulphinylbutyl-ITC (sulforaphan, $[M + H\text{-}CH_4SO]^+$	Isothiocyanate
116.0706	4.9	$C_5H_{10}NO_2$	Proline $[M + H]^+$	Amino acid
170.0997	35.9	$C_9H_{16}NS$	8-Methylsulphinyloctyl ITC (hirsutin, $[M + H\text{-}CH_4SO]^+$	Isothiocyanate
200.0180	23.8	$C_6H_{12}NOS_2Na$	4-Methylsulphinylbutyl-ITC (sulforaphan, $[M + Na]^+$	Isothiocyanate
207.0652	25.4	$C_{11}H_{11}O_4$	Sinapoyl malate [CVF: sinapic acid + $H\text{-}H_2O]^+$	Phenylpropanoid
285.0756	**40.7**	**$C_{16}H_{13}O_5$**	**Biochanin A (internal standard)**	**Isoflavone**
321.1960	30.3		$[M + H]^+$ or $[M + H\text{-}H_2O]^+$	
349.2379	31.2			
363.0687	25.4	$C_{15}H_{16}O_9Na$	Sinapoyl malate $[M + Na]^+$	Phenylpropanoid
365.1536	31.2		$[M + H]^+/2$	Glycoside
423.2202	49.9		$[M + H]^+/2$	
431.2107	50.4		$[M + H]^+/2$	
494.2567	45.0		$[M + H]^+/2$	Glycoside
502.2501	45.0		$[M + H]^+/2$	Glycoside
510.2358	45.0		$[M + H]^+/2$	Glycoside
579.1708	23.7	$C_{27}H_{31}O_{14}$	Kaempferol-3-*O*-a-L-rhamnopyranoside-7-*O*-a-L-rhamnopyranoside $[M + H]^+$	Flavone glycoside
675.2524	31.4			
703.1481	25.4	$C_{30}H_{32}O_{18}Na$	[2 Sinapoyl malate + $Na]^+$	Phenylpropanoid
719.1220	25.4	$C_{30}H_{32}O_{18}K$	[2 Sinapoyl malate + $K]^+$	Phenylpropanoid
735.0755	25.3			
802.4232	47.6			
815.4580	46.6			
829.4661	50.3			
965.5394	45.0		$[M + H]^+$	Glycoside
984.5030	45.0		$[M + H]^+$	Glycoside

10 out of 21 signals could tentatively be identified. For six additional peaks in leaf extracts, it was found that they represent glycosides.

Automatic Data Deconvolution and Analysis

Using manual data deconvolution, we were able to demonstrate the potential of CapLC-QqTOF-MS for metabolomics with respect to sensitivity and robustness, as well as the ability to quantify and identify compounds. However, manual data deconvolution is time consuming and therefore obviously not acceptable for any type of metabolomic analysis that is aiming at a considerable throughput. To overcome this limitation, we tested and optimized MetaboliteID, a metabolite processing software (Applied Biosystems, Foster City, CA). MetaboliteID allows us to automatically extract mass spectra from the total ion chromatogram and to generate peak lists displaying t_R, and mass and intensity of a peak. We first evaluated MetaboliteID by comparing its output with the results of manual data deconvolution. Following the optimization of data extraction parameters such as minimum signal strength (50 counts s^{-1}, 2.5-fold higher than background) and XIC window width (0.35), we analyzed the same set of mass signals as before in the reproducibility experiments. The average variability calculated from the same data sets with the unsupervised method was only slightly higher than that determined manually. The technical variability changed from 25.0% to 34.1% for leaf extracts and from 21.6% to 26.9% for root extracts. Values for the biological variability changed from 35.5% to 40.7% and from 55.9% to 67.5% for leaves and roots, respectively. Thus, the cumbersome manual data deconvolution could be replaced by unsupervised automatic deconvolution without significant losses in reproducibility.

Next, the number of detectable mass signals was determined using the optimized MetaboliteID parameter settings. Through 72 runs of 24 extracts from four independent experiments, on average, 1,415 sig-

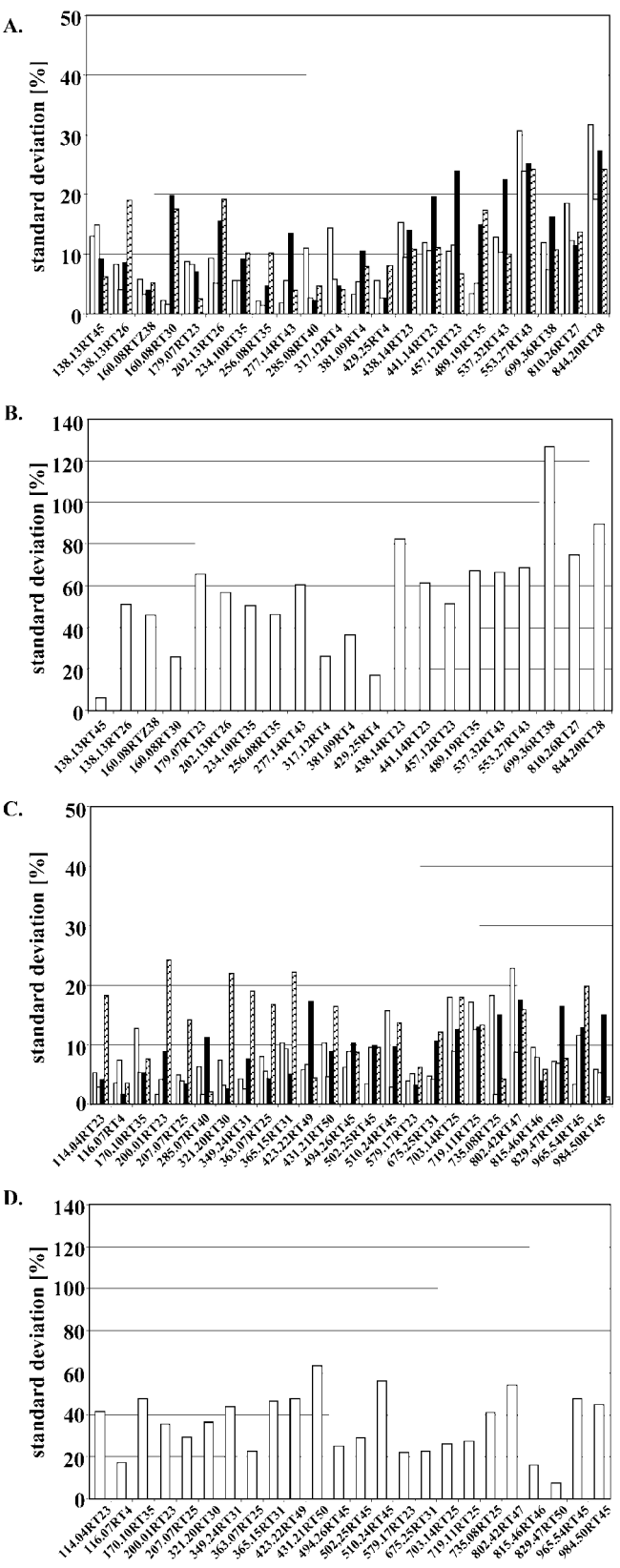

Figure 2. Technical and biological variance of the CapLC-ESI-QqTOF-MS analysis assessed by analyzing 25 mass signals from leaf extracts and 22 mass signals from root extracts. Signals are identified by mass and t_R. Technical reproducibility of the LC-MS analysis of methanolic root (A) and leaf extracts (C) was determined by extracting the same material three times as well as performing three repetitions of the LC-MS measurements. Quantification of 22 selective ion traces in root extracts and 25 ion traces in leaf extracts was carried out. The SD of the three independent LC-MS analyses of a single extract is represented in the white, gray, and black bars of the charts. The cumulative SD for repeated analysis of the same extract showed a value of 11.1%. Comparison of the three different extracts of the same material (striped bar) resulted in an average variability of 25% ± 17.9% for leaves and 21.6% ± 13.4% for roots. When four independent experiments were analyzed, an average biological variation was measured of 35.5% ± 14.0% for leaf signals (D) and 55.9% ± 26.0% for root signals (B).

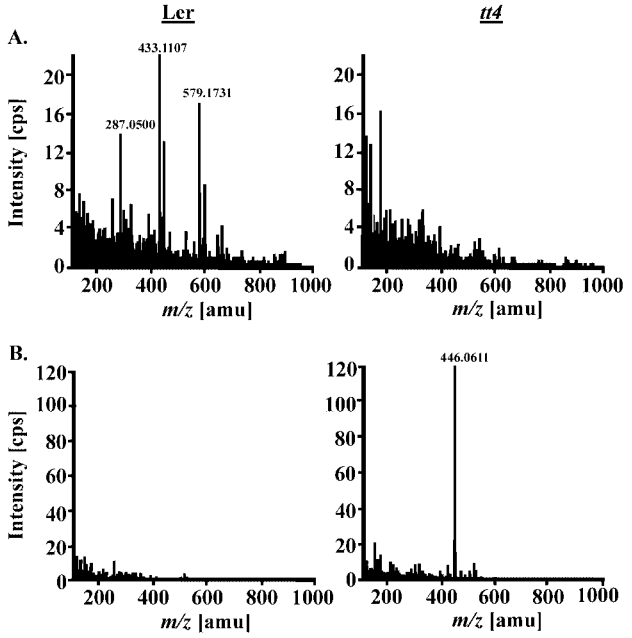

Figure 3. Mass spectra of two metabolites differentially accumulating in Arabidopsis wild-type (Ler WT) and mutant plants (Ler *tt4* mutant). The flavonoid glycoside Rha-Rha-kaempferide ($[M+H]^+$ at *m/z* 579.1731, in-source fragments at *m/z* 433.1107 and 287.0500) was not detected in leaves of the *tt4* mutant line (A), whereas the glucosinolate gluconasturtiin ($[M+Na]^+$ at *m/z* 446.0611) accumulated to much higher amounts in the roots of *tt4* plants (B).

nals were detected in leaf extracts and 827 in root extracts. The SD was ±204 for the number of leaf signals and was ±180 for the number of root signals. When the same extract was analyzed three times, the numbers varied on average by only 5.8% for leaves and 5.5% for roots.

Tools are required for the processing of such large data sets. We decided to use Excel macros and programming in Visual Basic to establish automatic analysis of peak lists generated by MetaboliteID. As a first step, a procedure for establishing master lists for each analysis was developed. This was necessary to summarize ions that were detected more than once; for instance, because of problematic peak shape. The correct assignment of corresponding signals is essential for comparing different CapLC-ESI-QqTOF-MS

Table III. *List of the most abundant mass signals from root (top) and leaf (bottom) extracts incremented in 100-Da steps*

From left to right and indicated by the shading, the five strongest signals are listed. HR mass (*m/z*) and t_R (min) are used as identifiers. Intensities (Int.) are given as area per milligram of fresh weight. Doubly charged ions are marked with the charge state in brackets (2).

	HR mass [*m/z*]	t_R	Int.	HR mass [*m/z*]	t_R	Int.	HR mass [*m/z*]	t_R	Int.	HR mass [*m/z*]	t_R	Int.	HR mass [*m/z*]	t_R	Int.
100-200	160.08	40	1853	145.05	40	1343	117.06	40	1193	129.06	40	924	128.05	40	445
200-300	234.10	37	683	228.23	52	572	202.13	27	517	288.29	32	328	281.17	51	257
300-400	317.12	5	477	315.16	5	384	381.08	5	358	301.14	52	255	353.21	5	144
400-500	429.24	5	276	441.14	24	238	415.12(2)	29	113	438.13(2)	24	81	437.37	47	65
500-600	535.18	29	62	592.16	22	60	557.10	5	40	532.06	22	27	525.18	32	27
600-700	623.06	41	36	607.41	52	22	699.36[a,b]	39	4	634.44[b]	51	3	614.17[a,b]	39	3
700-800	764.50[a]	54	19	705.13[b]	5	3	797.33[a,b]	41	1	781.36[a,b]	41	1			
800-900	813.25	29	30	808.29[b]	28	7	810.25(2)[b]	28	6	829.23[b]	28	4	844.20[b]	28	2
900-1000	938.71[a,b]	54	1												

	HR mass [*m/z*]	t_R	Int.	HR mass [*m/z*]	t_R	Int.	HR mass [*m/z*]	t_R	Int.	HR mass [*m/z*]	t_R	Int.	HR mass [*m/z*]	t_R	Int.
100-200	110.01	4	593	114.09	25	349	128.02	4	338	145.01	5	175	175.04	26	160
200-300	207.07	26	1193	228.23	53	515	281.17	51	280	288.29	32	252	275.12	4	233
300-400	363.07	26	703	317.12	5	511	315.16	5	380	379.04	26	308	301.14	52	235
400-500	407.21(2)	48	455	418.23(2)	47	351	429.24	5	342	426.22	47	128	494.26(2)	46	96
500-600	502.25(2)	46	349	513.27(2)	46	86	516.07	42	98	509.27	40	75	502.05	36	55
600-700	613.41	48	297	675.26	32	130	698.19	26	60	641.44	52	47	661.25	31	43
700-800	797.45	48	169	792.49	48	157	775.46	48	150	703.15	26	115	794.94(2)	49	76
800-900	825.47	52	57	802.93(2)	49	56	814.52	47	55	802.43	49	55	829.48	51	52
900-1000	987.53	46	33	982.57	46	30	965.54	46	29	984.54	46	22	900.52[b]	51	5

[a] Mass signals detected in <75% of the runs. [b] Signal manually integrated because it was below the 50 cps threshold normally used for MetaboliteID analysis.

runs. Identifiers of signals are the mass and the t_R. The demonstrated accuracy and robustness of the analysis allowed us to define narrow mass windows of 0.02 to 0.06 *m/z*. Because the mass error increases slightly with increasing mass, we divided the mass range into three areas: *m/z* = 106 to 300 (mass window 0.02), *m/z* = 301 to 600 (mass window 0.03), and *m/z* = 601 to 1,000 (mass window 0.06). An adjustment of t_R was introduced based on the simplifying assumption of a linear shift across a particular LC run. A routinely used t_R window for signal identification is 0.4 min. When these windows were applied, it was possible to correctly assign corresponding signals from different CapLC-ESI-QqTOF-MS runs. Based on this, signal intensities can be averaged and compared between different experiments.

To provide a reference list for future use of this technology, we listed in Table III the five strongest signals in root and leaf extracts for each 100-D mass window between 100 and 1,000 D. Thirty-six MetaboliteID analyses of the reproducibility experiments were scanned for the mass signals that showed highest intensity (given as area per milligram) and were present in at least 75% of the LC-MS runs. For the mass ranges above 600 D, few signals are detectable in root extracts. In these cases, we included manually integrated signals that were below the routinely used MetaboliteID threshold of 50 counts s^{-1}.

Example for Detection of Metabolic Differences between Samples

Having established rapid unsupervised automatic data extraction and processing, we assessed the applicability of our profiling scheme by analyzing a well-characterized mutant with a metabolic defect. Landsberg erecta (Ler) *tt4* plants lack a functional chalcone synthase (Shirley et al., 1995) and are therefore deficient in flavonoid biosynthesis. Kaempferol glycosides are the main flavonoids biosynthesized by Arabidopsis plants under normal laboratory conditions (Veit and Pauli, 1999). When Ler and *tt4* mutant plants were compared by CapLC-ESI-QqTOF-MS, the expected lack of kaempferol and its glycosides rha-rha-kaempferide and rha-kaempferide in leaves of *tt4* plants was detected (Fig. 3A; Table IV). Addi-

tionally, two methoxy-substituted indole carboxaldehydes, probably the 1- and 4-methoxy isomers, 8-methylthiooctyl amine (*m/z* 176.1472; Kawabata et al., 1989), and probably a methoxy-substituted indole-glutathione conjugate, such as L-γ-glutamyl-*S*-[(1-methoxy-1H-indol-3-yl) methyl]-L-cysteinyl-Gly (*m/z* 467.1600; Bjergegaard et al., 2000), were found to be reduced in amount. However, both substances have not been obtained from natural sources. To also test the feasibility of handling very diverse data sets, we performed a comparison of metabolite composition of root and leaf tissue. Through nine samples each, the average overlap was only 8.7% (±1.1%).

Identification of Compounds by Tandem MS

Frequently, specific metabolites display changes correlated to a particular genetic background or elicited by an environmental or developmental stimulus. After detection of such changes by nontargeted metabolomic analysis, the identification of the respective metabolites is of key importance for biochemical phenotyping and gene function analysis. Additionally, identification is desirable to extend the catalog of known metabolites for Arabidopsis or any other plant species under study.

In-source fragmentation of compounds can already provide valuable structural information sufficient for identification. This was demonstrated above for the mass signals used in the reproducibility experiments (see Tables I and II). Tandem MS represents an additional powerful option. The first quadrupole of the mass spectrometer can be used as a mass filter and the second quadrupole can be used as a collision cell. Collision-induced dissociation (CID) yields fragments that can be used for elucidation of structures. In the following three examples from the method evaluation experiments are documented.

Roots of *tt4* mutants were found to accumulate high amounts of gluconasturtiin, whereas only minor amounts were found in the corresponding root extracts of the Ler plants (Fig. 3B). The isotopic pattern of the observed ion at *m/z* 446.0623 ($[M+Na]^+$) clearly indicates the presence of two sulfur atoms (see Fig. 4A). The CID spectrum is dominated by fragment ions at *m/z* 284 {$[M+Na\text{-}162(glucosyl)]^+$}, *m/z* 266 ($[m/z\ 284\text{-}H_2O]^+$), whereas *m/z* 186

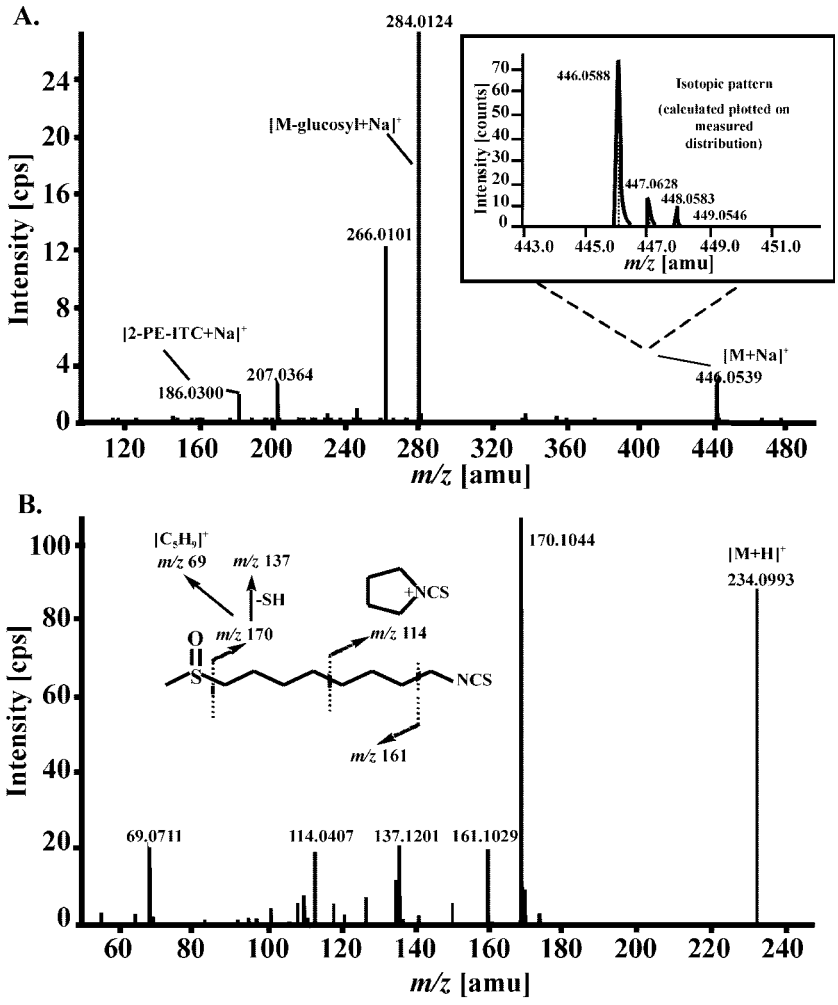

Figure 4. Identification of significant metabolites of Arabidopsis by HR-ESI-MS/MS (product ion scan). A, Positive ion CID-MS of gluconasturtiin (in the box: calculated and measured isotopic pattern of the $[M+Na]^+$ ion at *m/z* 446). B, Positive ion ESI-CID-MS of hirsutin.

Table IV. *Mass signals differentially expressed between Ler wild-type and tt4 mutant plants Tentative identification is listed whenever possible.*

	HR Mass	t_R	Average Fold Change	Molecular Formula (if available)	Substance Class	Compound
	m/z	*min*				
Leaf, reduced	287.0550	23.7	17 (+/−7)	$C_{15}H_{11}O_6$	Flavone Glycoside	Kaempferol $[M + H]^+$ [*m/z* 579−2x146(Rha)]
	579.1708	23.8	88 (+/−12)	$C_{27}H_{31}O_{14}$	Flavone Glycoside	Rrha-rha-kaempferide $[M + H]^+$
	433.1129	24.0	11 (+/−7)	$C_{21}H_{21}O_{10}$	Flavone Glycoside	Rha-kaempferide $[M + H]^+$ [(*m/z* 579−146 (Rha)]
Leaf, higher	661.3178	38.8	4.2 (+/−1.2)			
	789.4299	49.4	4.2 (+/−1.5)			
Root, reduced	116.0500	33.6	3 (+/−0.1)	C_8H_6N	Methoxy-subst. indole	Methoxyindolecarboxaldehyde
	133.0521	33.6	3.4 (+/−0.4)	C_8H_7NO	Methoxy-subst. indole	Methoxyindolecarboxaldehyde
	144.0443	33.6	4.2 (+/−0.9)	C_9H_6NO	Methoxy-subst. indole	Methoxyindolecarboxaldehyde
	176.0711	33.6	3.2 (+/−0.1)		Methoxy-subst. indole	Methoxyindolecarboxaldehyde
	176.1472	22.9	3.5 (+/−0.9)	$C_9H_{22}NS$	Amine	8-Methylthiooctylamine
	136.0579	5.3	4.7 (+/−0.1)			
	467.1600	23.7	5.1 (+/−0.9)	$C_{20}H_{27}N_4O_7S$	Methoxy-subst. indolyl-GSH conjugate	L-γ-glutamyl-*S*-[(1-methoxy-1H-indol-3-yl) methyl]-L-cysteinyl-glycine $[M + H]^+$
Root, higher	419.2765	52.1	11.8 (+/−3.2)			
	423.0106	6.1	7.8 (+/−3.2)		S-Containing compound	
	446.0555	26.6	581.5 (+/−418)	$C_{15}H_{21}NO_9S_2Na$		2-Phenylethyl glucosinolate (gluconasturtiin $[M + Na]^+$)

([phenylethylisothiocyanate+Na]$^+$) represents the phenylethyl moiety. Hirsutin was used for our root analysis as a stably occurring reference compound. It is known to arise as a myrosinase-catalyzed degradation product from its parent compound, 8-methylsulphinyloctyl glucosinolate (glucohirsutin). Both metabolites were originally obtained from the seeds of rock cress (*Arabis hirsuta*) by Kjær and Christensen (1958). EI-MS spectra were given by Kjær et al. (1963) as well as by Spencer and Daxenbichler (1980). The ESI-TOF mass spectrum displays a $[M+H]^+$ ion at *m/z* 234 (see Fig. 4B). The CID spectrum displays a prominent key ion at *m/z* 170 ($[M+H-CH_4SO]^+$), also appearing as an intense in-source fragment, which is a group-characteristic ion of methylsulphinyl isothiocyanates (ITCs). Additional typical ions are *m/z* 161 ($[M+H-CH_3-NCS]^+$), which is a key fragment of mustard oils, and the stable cyclic immonium ion at *m/z* 114.

We could confirm the expected reduction of kaempferol-3-*O*-α-L-rhamnopyranoside-7-*O*-α-L-rhamnopyranoside in *tt4* mutants compared with Ler control plants. Its ESI-CID mass spectrum showed a subsequent loss of the two rhamnosyl moieties from the $[M+H]^+$ ion at *m/z* 579 generating fragments at *m/z* 433 and 287, respectively, and was found to be identical with that of a standard sample (data not shown). The structure of the aglycon was established by comparing the CID-MS of *m/z* 287 with the $[M+H]^+$ ion of authentic kaempferol. Thus, characteristic aglycon fragments are formed by subsequent losses of water and carbon monoxide, respectively, or from the retro-Diels-Alder reaction.

DISCUSSION

The current status of metabolomics can be viewed as being in some ways equivalent to the situation of sequencing programs such as the Human Genome Project around 1990 (Sumner et al., 2003). The enormous potential of comprehensive biochemical phenotyping for the functional analysis of biological systems is realized and numerous projects have been initiated. However, major technological limitations need to be overcome. For instance, the chemical diversity of the metabolome necessitates the use of different analytical techniques to cover the wide range of polarities found among the metabolites occurring in a cell.

Metabolomic approaches aim at monitoring the biochemical status of an organism by simultaneously measuring as many metabolites as possible. A robust and reproducible analysis that provides qualitative and quantitative data and allows high sample throughput is desired. Maybe equally important at this stage is to contribute to cataloging the metabolome of an organism. No metabolome is completely known as of yet. Systematic identification of the metabolites occurring in a species is particularly relevant for plants, given the wealth of natural products they produce. Sequence data indicate that Arabidopsis expresses a large number of enzymes for which substrates and products are unknown (The Arabidopsis Genome Initiative, 2000).

LC coupled to HR-MS has great potential to play an important role in metabolomics as a complement

to GC-MS. However, reports of such a use are few, and thus far, most concern targeted profiling approaches (Lange et al., 2001; Huhman and Sumner, 2002). Our objective was to explore Cap LC coupled to state-of-the-art ESI-QqTOF-MS for the nontargeted profiling of Arabidopsis extracts. Reversed-phase LC on C18 was chosen because this was found to be suitable and highly reproducible for secondary metabolite profiling in a number of plant species including Arabidopsis (Graham, 1991). In principle, other techniques such as Cap electrophoresis could also be coupled to ESI-QqTOF-MS to further expand the range of metabolite profiling. Hallmarks of TOF-MS are the HR and high mass accuracy that make it an invaluable tool for the identification of compounds and its use for the sensitive simultaneous detection of large numbers of metabolites separable by LC on reversed-phase material. Major question marks concerning this approach were the robustness of the analysis, its reproducibility, and its suitability for high-throughput and quantitative analysis. Our results demonstrate that CapLC-ESI-QqTOF-MS principally meets all the necessary criteria for powerful metabolic analysis.

t_Rs of compounds eluting from the Cap LC column were found to show only little variation. Optimized flow rate and gradient composition produced a stable ion spray. Evaluation of the mass accuracy showed that the target value in the range of 5 ppm error (Chernushevich et al., 2001) can be reached for many signals even in a complex mixture of compounds, allowing, as discussed below, the calculation of elemental compositions and thereby the tentative identification of compounds. Obviously, the accuracy is correlated with signal strength and the error is higher for signals with intensities closer to background. Robustness of the analysis is illustrated by the fact that the average deviation in intensity for a range of mass signals was no higher than 11% when the same extract was run several times. Reproducibility of t_R and mass accuracy provided a sufficient basis for the unequivocal detection and identification of mass signals.

Quantification of metabolites is a serious hurdle for metabolomics (Sumner et al., 2003). There are inherent limitations to the dynamic range of MS and, as emphasized by Trethewey et al. (1999), for nontargeted approaches, compromises have to be made concerning the quantification of metabolites. Dilution series of Arabidopsis extracts indicated a satisfactory linear range of the CapLC-ESI-QqTOF-MS analysis for many of the signals tested. However, the linear range is dependent on the metabolite in question, and there is currently no feasible way of exactly assessing it for every signal. As predicted by Fiehn (2002), we therefore see the immediate application of CapLC-ESI-QqTOF-MS predominantly in the highly sensitive and rapid detection of qualitative and also pronounced quantitative metabolic differences.

The potential for such a use is very high because of the large number of mass signals that can be resolved. On average, about 1,400 signals were detected in leaf extracts and about 800 signals were detected in root extracts. Given the limited overlap between root and leaf metabolites, a total of about 2,000 different signals can be detected in extracts from Arabidopsis plants grown under control conditions. Most likely, the numbers will be significantly higher once the analysis is extended to other tissues such as flowers (Chen et al., 2003), to different developmental stages, and to plants exposed to environmental stimuli. Increases in the number of detectable mass signals can be achieved by extending the analysis to the negative mode, which is less effective yet allows the measuring of metabolites not seen in positive mode, such as the intact glucosinolates or salicylic acid (data not shown).

The robustness of the analysis is again indicated by the stability of mass signal numbers when the same extract was analyzed repeatedly. The average variation was no greater than 6%. Because of in-source fragmentation and the formation of adduct ions, the number of mass signals detected is definitely higher than the number of metabolites. It is impossible at this stage to reliably estimate the actual number of metabolites detected.

Technical variance (as the sum of variation introduced by the extraction and by chromatography and mass spectrometric analysis) was below 30% for all but two of the chosen mass signals. This provides a solid basis for the detection of differences between samples. The biological variability of around 40% is similar to what has been reported for other large-scale profiling techniques. Technical and biological variance are dependent on the mass signal in question. Possible causes are, for instance, a coelution of ions that affects quantification through ion suppression and high metabolic fluctuation. Processing of a large number of profiling experiments will be required to eventually define the range of variability for individual mass signals.

The efficient use of CapLC-ESI-QqTOF-MS for gene function analysis implies a considerable throughput of samples. Automatic unsupervised deconvolution of MS data and processing of the extracted information is a must. After extensive optimization, we found the MetaboliteID software able to function sufficiently well for deconvolution. A comparison with the results from manual stepwise extraction and integration of a range of mass signals showed only small differences. Peak lists generated by MetaboliteID are further processed using Excel macros and Visual Basic programs. These allow us to assign peaks correctly based on t_R and mass. The tools are tailored to discover qualitative and quantitative differences between samples and sets of samples. We tested the procedures by analyzing Ler

wild-type and *tt4* mutant plants. The expected difference in kaempferol glycosides due to the lack of a functional chalcone synthase was detected. In addition, we found several as yet unknown differences. Comparison between root and shoot samples demonstrated that very diverse data sets can also be handled. The limited overlap we found is further proof for the fundamental difference between root and leaf metabolism.

CapLC-QqTOF-MS is to be used for the detection and elucidation of metabolic changes elicited by environmental or developmental stimuli, as well as for the determination of metabolic differences attributable to a particular genetic background. Furthermore, substantial progress in establishing the metabolome of Arabidopsis and other model species is envisioned. All of these uses will rely on the potential to identify metabolites. We explored a nontargeted and a targeted way of gaining structural information. The highly accurate mass of the molecular ion and in-source fragments obtained during routine analysis can be used to tentatively identify metabolites with a good success rate. By systematically analyzing the mass data generated, we predict the ability to identify a large number of compounds that to date have not been known to occur in Arabidopsis. In selected cases of mass signals showing a change of immediate biological interest, CID can be used to generate fragments that, in many cases, provide a basis for an assignment of structure.

As is the case for other profiling schemes, aqueous methanol is used here for the extraction of Arabidopsis metabolites. Obviously, the choice of solvent limits the scope of the analysis, and the use of additional extraction methods is likely to result in the detection of further substance classes. A survey of the compounds tentatively identified in this study shows that the relevant known classes of Arabidopsis secondary metabolites such as indole-derived compounds (e.g. indole acetic acid derivatives), degradation products of glucosinolates (sulfinylnitriles and isothiocyanates), phenylpropanoids (sinapoylmalate), and flavonoids as well as their glycosides (e.g. kaempferol-3-*O*-α-*L*-rhamnopyranosid-7-*O*-α-*L*-rhamnopyranosid) can be detected by the method described. Indole derivatives, for instance, can be clearly classified by LC-ESI-MS/MS measurements according to their substitution pattern at the ring skeleton (e.g. methoxyindoles). The same is true for the ITCs (e.g. hirsutin). The example of gluconasturtiin, which contains two sulfur atoms, demonstrates the usefulness of the isotopic pattern as an additional feature to support the proposed elemental composition. Arabidopsis secondary metabolites not covered by this method are mono- and sesquiterpenoids, triterpenoid alcohols, phytosterols, waxes, and carotenoids.

MATERIALS AND METHODS

Plant Growth

Surface-sterilized seeds of the Arabidopsis ecotypes Col-O and Ler as well as of the *tt4* mutant line were sown on agarose plugs (0.5%, w/v) and were grown hydroponically in one-tenth Hoagland nutrient solution No. 2 (pH 5.3–5.5; Sigma, St. Louis). The medium was supplemented with iron, chelated by N,N′-di-(2-hydroxybenzoyl)-ethylenediamine-N,N′-diacetic acid to a final concentration of 5 μM Fe-N,N′-di-(2-hydroxybenzoyl)-ethylenediamine-N,N′-diacetic acid (Chaney, 1988). Seedlings were grown for 6 weeks in hydroponic greenhouse boxes containing 10 plantlets each. The nutrient solution in the boxes was changed on a weekly basis and was aerated through 0.2-μm filters. Light conditions in the growth cabinet were fixed to 230 to 240 $\mu E\ m^{-2}\ s^{-1}$ and a photoperiod of 8 h of light/16 h of dark at 23°C, day and night. Leaves and roots of plants were harvested separately approximately 1 h into the light period, pooled, and stored at −80°C.

Extraction and CapLC-ESI-QqTOF-MS

For the metabolite LC-MS analysis, freshly ground plant material (about 100 mg) was subjected twice to the following extraction procedure: mixing of the plant material with 200 μL of 80% (v/v) MeOH, sonication for 15 min (20°C–22°C), and centrifugation at 19,000*g* for 10 min. The extracts were combined, filtered (polytetrafluoroethylene filter, pore size of 0.2 μm), and analyzed by LC-MS. Positive LC-ESI-TOF mass spectra were recorded on an API QSTAR Pulsar Hybrid Quadrupole TOF instrument (Applied Biosystems) coupled to a capillary HPLC system (Ultimate; Dionex, Sunnyvale, CA). Typical MS instrument settings were an electrospray voltage of 5.5 kV with nebulizer gas being N_2 and collision gas being N_2, as well. The LC separation was performed on a Fusica C18 column (3 μm, 0.3 × 150 mm, PepMap; Dionex) applying a gradient system starting from 95% eluent A (0.1% [v/v] HCOOH/water) and 5% eluent B (0.1% [v/v] HCOOH/acetonitrile) to 95% eluent B in 45 min at a flow rate of 5.5 $\mu L\ min^{-1}$ (sample injection volume: 1 μL). Formic acid was used because of its compatibility with MS analysis.

Data Analysis

Peak finding and quantification of selective ion traces was accomplished using the instrument's AnalystQS software. Automatic raw mass data deconvolution was performed using the MetaboliteID software (Applied Biosystems). Peak lists generated by MetaboliteID were further analyzed using Excel and Visual Basic.

CID-MS

2-Phenylethyl glucosinolate (gluconasturtiin): t_R, 26.69 min; collision energy (CE), 20 eV; declustering potential (DP), 50 V; *m/z* (relative intensity, in percentages), 446.0556 (calculated for 446.0555: $C_{15}H_{21}NO_9S_2Na$, $[M+Na]^+$, 10); 284.0124 (calculated for 284.0021: $C_9H_{11}NO_4S_2Na$, [M+Na-162(glucosyl)]$^+$, 100); 266.0101 (calculated for 265.9922: $C_9H_9NO_3S_2Na$, [*m/z* 284-H_2O]$^+$, 45); 186.0300 (calculated for 186.0353: C_9H_9NSNa [2-phenylethyl ITC+Na]$^+$, 8). 8-Methylsulphinyloctyl isothiocyanate (hirsutin, 8-MSOO-ITC): t_R, 26.54 min; CE, 30 eV; DP, 50 V; *m/z*, 234.0993 (calculated for 234.0986: $C_{10}H_{20}NOS_2$, $[M+H]^+$, 80); 170.1044 (calculated for 170.0997: $C_9H_{16}NS$, $[M+H\text{-}CH_4SO]^+$, 100); 161.1029 (calculated for 161.0994: $C_8H_{17}OS$ $[M+H\text{-}CH_3NCS]^+$, 18); 137.1201 (calculated for 137.1198: $C_9H_{15}N$, [*m/z* 170-SH]$^+$, 20); 114.0407 (calculated for 114.0371: C_5H_8NS, 18); 69.0711 (calculated for 69.0698: C_5H_9, 19). Kaempferol-3-*O*-α-L-rhamnopyranoside-7-*O*-α-L-rhamnopyranoside: t_R, 23.54 min; CE, 20 eV; DP, 50 V; *m/z* (relative intensity, in percentages), 579.1697 (calculated for 579.1708: $C_{27}H_{31}O_{14}$, $[M+H]^+$, 3); 433.1197 (calculated for 433.1129: $C_{21}H_{21}O_{10}$, [M+H-146(rhamnosyl)]$^+$, 100); 287.0538 (calculated for 287.0550: $C_{15}H_{11}O_6$, [*m/z* 433 - 146]$^+$, 31). Kaempferol (reference compound): CE, 40 eV; DP, 75 V; *m/z* (relative intensity, in percentages), 287.0557 (calculated for 287.0550: $C_{15}H_{11}O_6$, $[M+H]^+$, 66); 258.0514 (calculated for 258.0528: $C_{14}H_{10}O_5$, $[M+H\text{-}CHO]^+$, 14); 241.0503 (calculated for 241.0500: $C_{14}H_9O_4$, $[M+H\text{-}H_2O\text{-}CO]^+$, 8); 213.0584 (calculated for 213.0547: $C_{13}H_9O_3$, $[M+H\text{-}H_2O\text{-}2CO]^+$, 21); 185.0602 (calculated for 185.0597: $C_{12}H_9O_2$, $[M+H\text{-}H_2O\text{-}3CO]^+$, 10);

165.0206 (calculated for 165.0176: $C_8H_5O_4$, [rings A&C-CO]$^+$, 56) 157.0653 (calculated for 157.0653: $C_{11}H_9O$, [M+H-H_2O-4CO]$^+$, 14); 153.0188 (calculated for $C_7H_5O_4$, [retro-Diels-Alder fragment from rings A & C]$^+$, 100); 147.0439 (calculated for 147.0446: $C_9H_7O_2$, 10); 137.0269 (calculated for 137.0238: $C_7H_5O_3$, 14); 121.0339 (calculated for 121.0289: $C_7H_5O_2$, $^+$OC-C_6H_4OH (ring B), 13).

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