DOI: 10.1002/cbdv.201900276

FULL PAPER

CHEMISTRY & BIODIVERSITY

Sequences of Acretocins, Peptaibiotics Containing the Rare 1-Aminocyclopropanecarboxylic Acid, from *Acremonium crotocinigenum* CBS 217.70

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Dedicated to the memory of *Walter Gams* (1934–2017), a great mycologist who isolated strain CBS 217.70 and described the species *Acremonium crotocinigenum*

Seven non-ribosomal polypeptide antibiotics, named acretocins (ACRs), were obtained from *Acremonium crotocinigenum* strain CBS 217.70. The microheterogeneous peptide mixture was isolated from the culture broth by column chromatography on *XAD-2* and *Sephadex LH-20*. Sequences were determined by ESI-MS^n and amino acid composition by GC/MS. Besides Gly, Leu, and Ala, the peptides contain the non-proteinogenic amino acids Acc (1-aminocyclopropane-1-carboxylic acid), Aib (α-aminoisobutyric acid), Iva (isovaline), Pip (pipecolic acid), β-Ala (β-alanine), and a C-terminal heterocyclic residue *N*-peptido-1-isobutyl-2-(2,3,4,6,7,8-hexahydro-1-pyrrolo[1,2-*a*]pyrimidinio)ethylamine (abbreviated X). After vigorous acidic total hydrolysis, release of L-Iva besides D-Iva was established by chiral GC/MS. ACRs show in part sequence identity with neoefrapeptins. Two sequences, ACR-5(6), are new: Ac-L-Pip-Aib-L-Pip-D-Iva-D-Iva(Aib)-L-Leu-β-Ala-Gly-Acc-Aib-L-Pip-Aib-L-Ala-L-Leu-L-Iva-X. The taxonomy of fungal producers of acretocins and neoefrapeptins is compared.

Introduction

Peptaibiotics – Fungal Peptide Antibiotics Containing α-Aminoisobutyric Acid

About 30 genera of cosmopolitan filamentous fungi, mostly belonging to the order of Hypocreales, have been recognized as prolific sources of a particular group of non-ribosomally synthesized **bio**active **pep**tides containing the non-proteinogenic, eponymous α-aminoisobutyric acid (2-methylalanine, **Aib**). For this group of peptides, the acronym **peptaibiotics** became established. Peptaibiotics include the large sub-group of **peptaibols** that are defined as linear, N-acetylated **pept**ides containing **Aib** and a C-terminal 1,2-amino alcoh**ol**. In peptaibiotics, the C-terminal amino alcohol is formally replaced by a structurally diverse substituent, including α-amino acids, polyamines, sugar alcohols, or heterocyclic residues. Besides Aib, this group of unique peptides frequently contains additional non-proteinogenic amino acids including those with quaternary C-atoms, in particular isovaline (2-ethylalanine, Iva), or, as shown here for two groups of peptaibiotics, 1-aminocyclopropane-1-carboxylic acid (Acc).

In the *Peptaibol Database*, first released in 1997 by Birkbeck College, School of Crystallography, London, UK,[1] 317 peptaibol sequences are compiled.[2] Due to a shortage of external resources, this database has not been updated for more than 13 years. In view of the constantly growing number of peptaibiotics reported in literature, the open domain *Comprehensive Peptaibiotics Database* became established in May 2013, which comprised the sequences of approximately 1000 peptaibiotics known by December 2012. Based on Microsoft (MS) Access, it could easily be installed and operated on any computer offering a Windows XP/7 environment. Finally, the *Peptaibiotics Database* was launched in May 2015 as a comprehensive online resource.[3,4] At the time of release, 1297 sequences of peptaibiotics were included. Due to basic infrastructural changes in spring of 2018, this database, which hosted between 1400 and 1500 individual sequences of peptaibiotics in 2017, is currently not available

online. Considering the recent literature published in this field, it seems reasonable to predict a total of 1800 peptaibiotics, the number of which is steadily increasing.[5–9] Notably, in this context, a special issue of the *Journal of Peptide Science*,[10] two topical issues of *Chemistry & Biodiversity*,[11,12] and a monograph,[13] exclusively devoted to peptaibiotics, have been published.

The Species Acremonium crotocinigenum

In the course of our extensive screening program for peptaibiotic metabolites, we scrutinized the filamentous fungus ***Acre****monium* *croto****cin****igenum* (Schol–Schwarz) W. Gams CBS 217.70 and detected abundant production of Aib-containing peptides that were named **acretocins** (ACRs).[14–16] Strain *A. crotocinigenum* CBS 217.70 was isolated by *W. Gams* in March 1967 from decaying wood in the 'Schüttbrehm', a small forest of about 0.76 km^2 size; located near Kiel-Kitzeberg, Schleswig-Holstein, Germany (latitude 54.3601272, longitude 10.2439581; corresponding to 54°21′36.458″, 10°14′38.249″). Specimens of *A. crotocinigenum*, deposited with CBS, are compiled in the classical monograph by *W. Gams*.[17]

The Genus Acremonium *– A Prolific Source of Peptaibiotics*

Notably, the genus *Acremonium* has been recognized as a prolific source of structurally highly diverse peptaibiotics: From *Acremonium persicinum* X21488, the peptaibiotic XR586 was obtained, which carries a C-terminal free Gly residue.[18] Recently, XR586 was reisolated from *A. persicinum* SC0105, along with four structurally closely related peptaibiotics named acremotins A–D.[19] Cephaibols P and Q from *A. tubakii* terminate in free Ser, whereas the remaining cephaibols carry a C-terminal Pheol residue.[20] The lipopeptaibiotic lipohexin, which carries an N-terminal Pro residue that is protected by a β-keto-2-methyltetradecanoyl (MOTDA) residue, was isolated from *Acremonium* (syn. *Moeszia*) *lindtneri* DSM 11119.[21] The lipoaminopeptide leucinostatin A was obtained from *Acremonium* sp. Tbp-5, an endophyte of *Taxus baccata*.[22] Notably, three modified lipoaminopeptides, acremostatins A–C, have been isolated from dual plate cultures of *Acremonium* sp. Tbp-5 with the roseoferin-producing mycoparasite *Mycogone rosea* DSM 12973.[23] Phoenistatin from *Acremonium fusigerum* (syn. *Gliomastix fusigera*) QN 5320 was the first Iva-containing[1] cyclotetrapeptaibiotic,[24] whereas the Iva-residue in FR235222 from *Acremonium* cf. *murorum* No. 27082 (= *Acremonium* sp. FERM BP-6539) was assigned the L-configuration.[25] Later on, the same strain was shown to produce the cyclotetrapeptaibiotic AS1387392, which also contains one L-Iva residue.[26] The atypical, sponge-associated marine fungus *Acremonium* sp. strain 021172cKZ was a prolific producer of five efrapeptins E, F, G, Eα, and H.[27,28]

In addition to those strains discussed in the last paragraph, several other species and strains of *Acremonium* have been found to produce the marker amino acid Aib.[14] Therefore, it is reasonable to assume that further prolific producers of peptaibiotics will be found in this particular genus. It should be kept in mind, however, that the taxon *Acremonium* is nowadays rather considered as a generic concept that is still under evaluation using state-of-the-art phylogenetic approaches (see *Discussion* section).

Acretocin-Type Peptaibiotics and Their Producers – Structural Considerations

Previous analyses of ACR revealed, besides Gly, Ala, and Leu, the presence of several residues of the eponymous Aib and its homolog Iva, Acc, pipecolic acid (Pip), β-Ala, and an unusual alkylated C-terminal 1,5-diazabicylo[4:3:0]non-5-ene (DBN) residue, known as *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 (**PIHPPE**). In the literature, this residue is mostly referred to as **X** despite its meanwhile known configuration.[29,30] The presence of this unusual residue of initially unknown structure was first recognized in a peptaibiotic named efrapeptin, which has been isolated from *Tolypocladium inflatum* (syn. *T. niveum*).[31] From an acidic partial hydrolysate (6 M HCl, 12 h, 110 °C) of efrapeptin E, low yields of the C-terminal peptide Leu^{14}-Iva^{15}-X^{16} could be isolated, but no smaller fragments.[29,30] From X-ray analysis of the trifluoroacetate of *N*-trifluoroacetyl-Leu^{14}-Iva^{15}-X^{16}, the L-configuration of C-terminal Iva as well as the configuration of residue X^{16} could be established for the first time. It appears that the L-configuration of Iva^4 in efrapeptins E and G, containing two Iva residues, was established by analogy.

Furthermore, production of efrapeptins by many species and strains of *Tolypocladium* was established as reported in the literature.[30,32–35] Notably, for the

[1]The configuration of the Iva residue has not been determined.

first time, peptaibiotic sequences distinguished from efrapeptins in particular by formal substitution of Aib[9] by the cyclic side-chain analog Acc, presence of D-Iva as well as L-Iva, and replacement of Pip by 3-methylproline (3-MePro) in another series were discovered and, therefore, named *neo*efrapeptins.[36] The fungal producer, however, was assigned as *Geotrichum candidum* (for taxonomic considerations, see *Discussion* section). To date, only two more peptaibiotics containing the rare residue X are known, namely adenopeptin from *Chrysosporium* sp.[2] PF1201[37] and acremopeptin from *Acremonium* sp. PF1450,[38] the N-terminus of which is substituted by AcPro instead of AcPip in both cases. A possible biosynthetic pathway of the residue X was proposed by *Uma et al.*,[39] providing evidence that spermidine serves as a linear precursor of the 1,5-diazabicylo[4:3:0]non-5-ene ring system.

Natural Occurrence of Acc in Peptides and Other Natural Products

Acc in the free and conjugated state occurs in small amounts in fruit such as pears and apples and serves as a biosynthetic precursor of the plant hormone ethylene.[40] It is rarely found as a constituent of microbial metabolites. Cytotrienins A and B are two triene-ansamycins from a taxonomically unidentified *Streptomyces* sp. RK95-74 (= FERM P-15904), in which a hydroxy group of a 21-membered cyclolactam ring is esterified with N-acylated Acc.[41] From *Streptomyces* sp. TC 1190, two other triene-ansamycins were obtained, which are structurally closely related to cytotrienins.[42] A third, closely related mixture of triene-ansamycins, UCF116, contains one minor component A, which carries an N-acylated Acc residue.[43]

Examples from the fungal kingdom include the two cycloheptapeptaibiotics serinocyclins A and B produced by the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* ARSEF #2575 (Hypocreales, Clavicipitaceae).[44] The nonadepsipeptide BZR-cotoxin II is biosynthesized by the ascomycetous fungus *Bipolaris zeicola* (Pleosporales, Pleosporaceae) race 3, the northern corn leaf spot pathogen.[45] The alkaloid cottoquinazoline A was obtained from a marine strain of *Aspergillus versicolor* (Eurotiales, Aspergillaceae) MST-MF495.[46]

Synthetic Approaches towards Neoefrapeptins and Analogs

The first total synthesis of efrapeptin C, notably distinguished from neoefrapeptin N only by Aib[9] in place of Acc[9], has been described. Notably, the challenging synthesis of the C-terminal X[16] residue has also been achieved.[47] Continuation of this approach using combined solid- and solution-phase synthesis of peptide segments provided efrapeptins D–G and analogs, which also contain L-Iva.[48–50]

In a previous contribution, we briefly reported on the components and sequence determination of ACRs and assigned the configuration of Iva by chiral GC-MS in hydrolysates (6 M HCl, 110 °C, 24 h) .[16] Here, we would like to give a detailed account on the laboratory-scale fermentation, isolation, and determination of the amino acid sequences of ACRs using LC/ESI-MS and chiral GC/MS. The chiral sequences of ACRs determined in this work are compared with those of neoefrapeptins, and taxonomic relationships of their fungal producers are discussed.

Results and Discussion

Isolation and Purification of ACRs from the Culture Broth of Fermentations

Fermentation of strain CBS 217.70 was carried out in malt extract medium in mechanically agitated *Erlenmeyer* flasks. The optimum of the production of peptaibols was monitored by TLC. After 8 days of large-scale fermentation (see *Experimental Section*), intensive production of ACRs was observed. ACR provided one single spot of R_f 0.69 on TLC as revealed by spraying with water and TDM-reagent (*Figures 1a–d*). The mixture of peptides was isolated from the culture broth using *XAD-* and *Sephadex LH-20* chromatography as described in the *Experimental Section.*

Composition and Chirality of ACR Constituents

The *N*-trifluoroacetyl-*O*-2-propyl esters, which are prepared in a two-step derivatization of hydrolysates,were analyzed on *Chirasil*-L-Val[TM] (see *Experimental Section*, GC/MS, *Instrument A*). This analysis revealed the presence of Gly, L-Ala, and L-Leu as well as L-Pip, β-Ala, Aib, Acc, and DL-Iva (not resolved on this column). The

[2] From a chemotaxonomic point of view, it appears that the genus *Chrysosporium* (Onygenales, Onygenaceae) is not related to any of the 'classical' peptaibiotic-producing genera of fungi. Unfortunately, *Hayakawa et al.*[37] did not comment on the procedure how strain PF1201 was identified.

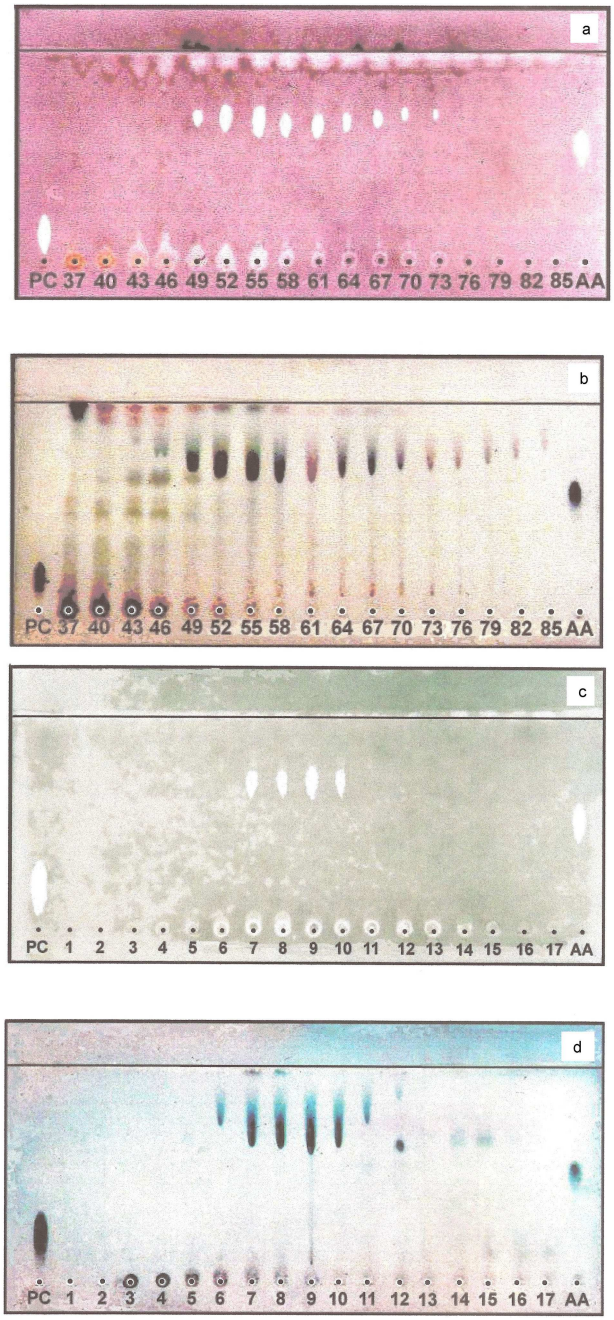

Figure 1. TLC monitoring of the acretocin isolation after 8 days of fermentation. Peptide-containing fractions eluting from the *XAD* column were visualized by subsequent spraying (a) with water and (b) with TDM reagent. Fractions eluting from the *Sephadex LH-20* column were visualized by subsequent spraying (c) with water and (d) with TDM reagent. Ten µl of the respective fractions were applied onto the TLC plates. TLC mobile phase: $CHCl_3$/MeOH 8:2 (v/v). The abbreviations PC and AA refer to paracelsin and antiamoebin, respectively, serving as reference peptaibols.

absence of Ile in the isolated peptide mixture was also proven by GC/MS. The presence of Acc in ACR hydrolysates was confirmed by GC/MS and diagnostic ions by comparison with an Acc standard (*Figure 2*). Release of the C-terminal X-residue was not recognized by GC/MS but its presence and chemical nature could be deduced from ESI-MS by the diagnostic fragment ion *m/z* 325.4 ($[y_2+2H]^+$, see *Table 1*) and, in particular, from a neutral loss of 101 Da from the y_6 ion *m/z* 703.9 and the y_{14} ion *m/z* 1422.6, respectively (see *Figure 7* and *Discussion* section). Notably, only D-Iva was found when the ACR peptide mixture was hydrolyzed under standard conditions (6 M HCl, 110 °C, 16–24 h) and analyzed as *N*-acetyl-*O*-2-propyl ester on *Chirasil*-L-Val. Total hydrolyses under more vigorous conditions (32% and 37% HCl, respectively, 135 °C, 72 h) revealed the release of about 20% L-Iva besides abundant D-Iva (*Figure 3*). No L-Iva was formed when enantiomerically pure D-Iva was heated (32% and 37% HCl, 135 °C, 72 h) in separate experiments and analyzed by GC on a chiral cyclodextrin column.[51]

HPLC and ESI-MS of ACRs

The analytical HPLC elution profile of the mixture of ACRs is shown in *Figure 4*; the total ion current of the isolated ACR peptide mixture in positive ion mode ESI-MS is displayed in *Figure 5*. Relative quantities of peptides in the ACR mixture were calculated from peak heights at $\lambda = 205$ nm and included in *Table 2*. Retention times of peptides increase with increasing molecular weight. The increasing hydrophobicity of peptides of the same molecular weight in dependence of positions of their amino acid methylene homologs (±14 Da) is also reflected in the HPLC elution profile. In peptides of identical molecular weight, which are distinguished by the positions of Aib and Iva (*m/z* 1618 and *m/z* 1632), a C-terminal Iva residue increases the hydrophobicity more in comparison to C-terminal Aib (ACR-3 *vice* ACR1a, and ACR-4 *vice* ACR-2). Replacement of Gly^{13} by Ala^{13} increases hydrophobicity of otherwise identical sequences (ACR-5 *vice* ACR-3 and ACR-6 *vice* ACR-4). Furthermore, formal replacement of Iva^5 and Gly^{13} by Aib^5 and Ala^{13} increases the hydrophobicity of the latter despite identical molecular weights of *m/z* 1646 (ACR-5 *vice* ACR-4). Finally, replacement of Aib^5 by Iva^5 results in the most hydrophobic peptide of the series (ACR-6 *vice* ACR-5); for sequences see *Table 2*.

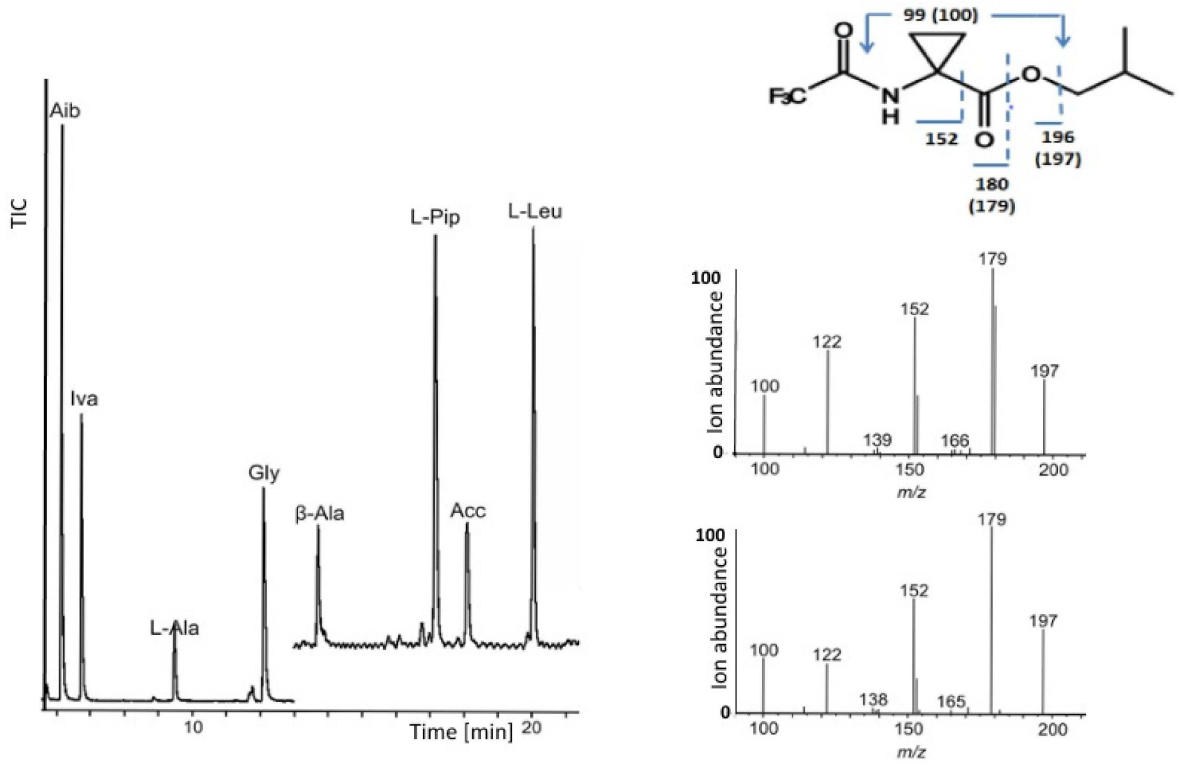

Figure 2. Sections of the GC/MS of TFA-*O*-2-propyl esters of an ACR total hydrolysate on *Chirasil*-L-Val^{TM} (left) and EI-fragmentation pattern (right, above) of a standard of Acc (right, middle) in comparison to Acc at retention time 18.2 min (right, bottom). D,L-Iva was not resolved under these conditions on this *Chirasil*-L-Val^{TM} column.

Sequencing of ACR Peptides

In the following, we comprehensively discuss molecular and fragment ions compiled in *Table 1*, and sequences derived thereof that are presented in *Table 2*. Diagnostic differences (Da) between fragment ions of the same series are attributed to amino acid residues as follows: Ac (43), Pip (111), Iva (99), Aib (85), Leu (113), β-Ala/Ala (71), Gly (57), Acc (83), and X (224). Note that exchange of β-Ala by Ala in the same position has not yet been reported in peptaibiotics, and it is not likely to occur in the non-ribosomal biosynthesis pathways of this class of peptides. Consequently, this particular exchange has not been taken into account. Because Ile was not detected by GC/MS in ACR hydrolysates, exchange of Leu/Ile was not considered here.

A series of $^{+}$ESI-MS infusion experiments of the ACR peptide mixture provided the $[M]^{+}$ ions of ACR peptides **1a**, **1b** (*m/z* 1618), **2** and **3** (*m/z* 1632), **4** and **5** (*m/z* 1646), as well as **6** (*m/z* 1660, see TIC in *Figure 6*).

The corresponding $[M+Na]^{2+}$ ions were also observed. Notably, the intensive ion *m/z* 325.4 is in agreement with $[y_2+2H]^{+}$ fragment ions expected to be generated from the C-terminal sequences Iva^{15}-X^{16}. The corresponding ion *m/z* 309, expected to be generated from the C-terminal fragment Aib^{15}-X^{16}, was not recorded. The fragment ion *m/z* 281.3 observed in the HPLC-ESI-MS spectrum might result from the b_3 fragment ion (*m/z* 350.9) that loses acetyl (43 Da) and carbonyl (28 Da; see *Figure 6*).

All peptides provided identical series of b_1 to b_3 fragment ions. Consequently, the N-terminal sequence Ac-Pip^1-Aib^2-Pip^3 was deduced. A difference of 14 Da between fragment ions b_4 and b_5 established the presence of either Iva or Aib in position 4 and 5 of the ACR **2**–**6**. Sequences of the isobaric peptides ACR **1a** and **1b** were deduced from the difference of b_5-b_3, corresponding to 183.4 (Iva-Aib) or 169.6 (Aib-Aib), respectively. These positions were corroborated by y_{12} and y_{13} fragment ions, also differing by 14 Da. Notably, almost complete regular series of y_7-y_{15} fragment ions were observed. Release of fragment ions y_7-y_{11} also confirmed the identical sequence Leu^6-β-Ala^7-Gly^8-Acc^9-Aib^{10} for all ACR peptides.

As a general rule, intensive y_6 and y_{14} fragment ions were observed, resulting from cleavage of the Aib^2-

Table 1. Diagnostic ions of ACR peptides resulting from infusion ESI-MS and HPLC-ESI-MS^{n}[a]

	1a	**1b**	**2**	**3**	**4**	**5**	**6**
t_R [min]	15.94 (15.82–16.82)	15.94 (15.82–16.82)	18.54 (18.33–19.33)	19.99 (19.58–20.35)	20.86 (20.75–21.32)	22.14 (21.95–23.12)	24.16 (23.93–24.34)
$[M]^+$	1618.9[a,d]	1618.9[a,d]	1632.2[a,d]	1632.8[a,d]	1646.0[a,d]	1646.0[a,d]	1660.2[a,d]
$[M+H]^{2+}$	809.6[a]	809.6[a]	816.8[a]	817.1[a]	823.7[a]	823.8[a]	830.8[a]
$[M+Na]^{2+}$	821.0[a]	821.0[a]	828.2[a]	828.0[a]	835.1[a]	835.1[a]	842.1[a]
b_1	154.2[a]	154.2[a]	154.1[a]	154.3[a]	154.1[a]	154.2[a]	154.2[a]
b_2	239.5[a]	239.5[a]	239.2[a]	239.5[a]	239.2[a]	239.4[a]	239.1[a]
b_3	350.7[a]	350.7[a]	350.3[a]	350.3[a]	350.3[a]	350.7[a]	350.3[a]
b_4	n.d.	n.d.	449.4[a]	449.3[c]	449.2[c]	449.2[c]	449.8[c]
b_5	534.1[d]	520.3[d]	548.4[a]	534.9[c]	548.3[a,d]	534.3[a,d]	548.3[a,d]
b_6	n.d.	n.d.	661.7[c]	n.d.	661.2[a,d]	n.d.	661.4[c,d]
b_7	718.5[e]	n.d.	n.d.	(718.5[d])	n.d.	n.d.	732.3[c]
b_8	n.d.	761.9[d]	n.d.	775.5[d]	789.5[a,d]	775.5[d]	789.5[d]
b_9	n.d.	n.d.	872.1[d]	857.4[d]	873.4[d]	858.2[d]	872.2[d]
b_{10}	943.5[a]	943.5[a]	957.6[a]	943.5[a]	957.3[a]	943.5[a]	957.5[a,d]
b_{11}	n.d.	n.d.	1068.9[d]	n.d.	n.d.	n.d.	1068.8[a,d]
b_{12}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
b_{13}	n.d.	n.d.	n.d.	1195.8[d]	1209.0[d]	1209.0[d]	1224.5[d]
b_{14}	n.d.	n.d.	n.d.	1309.5[d]	n.d.	n.d.	1337.4[d]
b_{15}	n.d.	n.d.	n.d.	n.d.	1421.9[d]	1421.9[d]	1435.5[d]
y_{16}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
y_{15}	1464.9[a]	1464.9[a,d]	1479.9[a,d]	1479.0[b,d]	1493.0[b,d]	1493.0[d]	1507.7[b,d]
y_{14}	1379.9[b]	1379.9[a,d]	1394.2[a,d]	1394.0[a,d]	1408.0[a,d]	1408.0[d]	1422.3[a,d]
y_{13}	1268.9[a]	1268.8[a,d]	1283.0[a,d]	1282.5[a,d]	1296.9[a,d]	1296.9[d]	1311.1[a,d]
y_{12}	1170.2[b]	1183.9[a,d]	1184.0[c,d]	1184.0[a,d]	1197.9[a,d]	1197.9[d]	1212.0[a,e]
y_{11}	1085.2[b]	1098.9[a,d]	1085.3[c,d]	1099.7[a,d]	1098.9[b,d]	1112.8[a,d]	1113.2[a,d]
y_{10}	971.6[c,d]	985.7[a,d]	971.9[b]	986.4[c,d]	985.8[a,d]	999.9[d]	1000.0[b,d]
y_9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
y_8	843.7[d]	857.7[d]	844.3[d]	858.4[b,d]	857.8[b,d]	872.0[d]	871.2[d]
y_7	760.8[b,d]	774.7[d]	760.8[d]	774.8[b,d]	774.8[d]	788.7[e]	788.9[d]
y_6	675.6[a,d]	689.7[d]	675.8[d]	689.7[a,d]	689.8[a,d]	703.7[d]	703.9[d]
y_5	n.d.	n.d.	564.5[f]	578.6[e]	578.5[f]	592.5[d]	592.5[d]
y_4	479.3[e]	493.6[e]	479.1[e]	493.8[e]	493.2[f]	507.5[e]	507.6[d]
y_3	422.5[f]	436.5[f]	422.5[e]	436.4[e]	436.4[e]	436.4[e]	436.4[f]
$[y_2+2H]^+$	n.d.	325.4[a]	n.d.	325.4[a]	325.4[a]	325.4[a]	325.4[a]
y_1 (X)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$y_{14}-101$	1278.8[b,d]	1278.8[b,d]	1293.0[b,d]	1293.0[b,d]	1307.0[b,d]	1307.0[d]	1321.0[b,d]
y_6-101	574.5[b,d]	588.5[b,d]	574.9[b,d]	588.6[b,d]	588.7[b,d]	602.7[d]	602.9[b,d]

[a] The origin of fragment ions is indicated by superscript.[a–f] [a]HPLC/$^+$ESI-MS of the ACR mixture; [b] HPLC/$^+$ESI-MS/MS of $[M]^+$; [c]HPLC/$^+$ESI-MS of the respective peptides **1a**, **1b** and **2**–**6**, obtained by micro-preparative isolation; [d]infusion $^+$ESI-MS/MS of $[M]^+$; [e] infusion $^+$ESI-MS^3 of y_{14}; [f]infusion $^+$ESI-MS^3 of y_6.

Pip^3 and Aib^7-Pip^{11} peptide bonds, together with complementary b_{10} and b_2 fragment ions. Notably, for all peptides, diagnostic fragment ions $y_{14}-101$ Da (*m/z* 1321.4) and y_6-101 Da (*m/z* 602.8) were observed in $^+$ESI-MS^2 spectra (see *Table 1* and *Figure 7*). The neutral loss of 101 Da corresponds to the molecular formula $C_6H_{15}N$ that is attributed to the release of the corresponding dialkylamine residue (ethylbutylamine or dipropylamine) from the C-terminal residue X (*m/z* 224) (see *Figure 8*). This observation definitely proves the structure of the C-terminal residue X.

For all ACR peptides, almost regular series of y_3-y_{15} fragment ions could be observed, with the exceptions of y_9 (resistance of β-Ala^7-Gly^8 cleavage) and y_5 in minor peptides ACR **1a** and **1b**. Accordingly, cleavage of the β-Ala^7-Gly^8 bond, yielding the b_7 fragment ions, was either not detected or provided ions of very low intensity (ACR-**1a** and ACR-**3**).

Fragment ions of the series y_3-y_{11} provided identical sequence domains Leu^6-β-Ala^7-Gly^8-Acc^9-Aib^{10}-Pip^{11}-Aib^{12}-Gly^{13} for ACR **1**–**4** peptides and presence of Ala^{14} in ACR peptides **5** and **6**. The

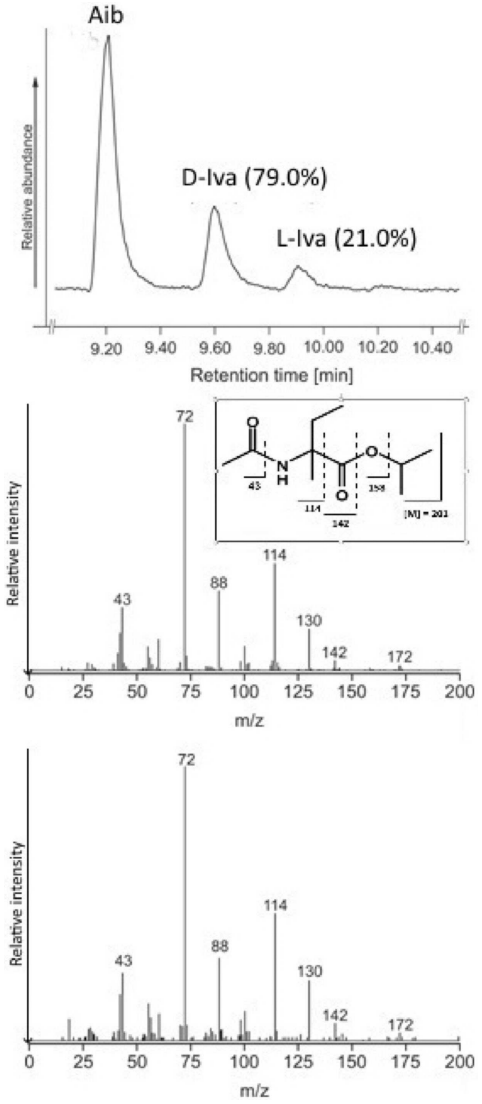

Figure 3. Section of (above) GC-TIC/MS of Ac-*O*-2-propyl esters of Aib and D- and L-Iva released from the mixture of ACR peptides on vigorous hydrolysis (135 °C, 37% HCl, 72 h) and (middle) resulting MS of derivatives of D-Iva and (bottom) L-Iva. Insert shows EI-fragmentation pattern of derivatives.

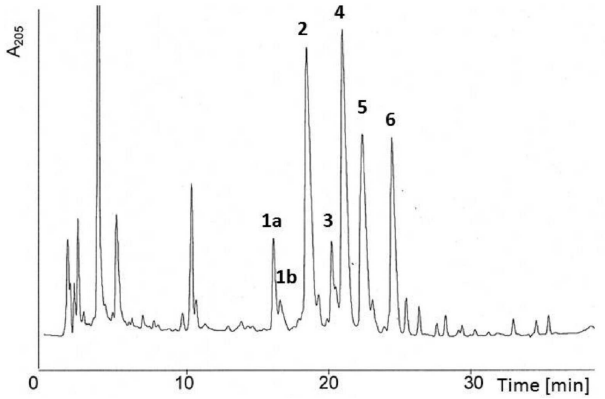

Figure 4. HPLC elution profile of the isolated ACR peptide mixture ($\lambda = 205$ nm). Numbers assigned to ACR-peptides are indicated and correspond to those in *Figure 5*. Peaks eluting prior to ACR **1a** are no peptides.

positions of Aib and Iva in the C-terminal sequence Leu^{14}-Aib^{15}/Iva^{15}-X^{16} were deduced from y_2 and y_3 fragment ions. Exchange of Gly^{13} by Ala^{13} in ACR-**5** and ACR-**6** was concluded from the series of y_2–y_6 fragment ions.

To summarize, all peptide sequences start with N-terminal Ac-Pip^1-Aib^2-Pip^3 and terminate in the C-terminal Leu^{14}-Aib^{15}/Iva^{15}-X^{16}. All peptides have common domains Leu^6-β-Ala^7-Gly^8-Acc^9-Aib^{10}-Pip^{11}-Aib^{12}. Amino acid exchange in ACR peptides is restricted to N-terminal Aib^4/Iva^4 or Aib^5/Iva^5, C-terminal Aib^{15}/Iva^{15}, and C-terminal Gly^{13}/Ala^{13} in two peptides.

Detailed sequencing of ACR peptides is illustrated in the following with the new acretocin peptide **6**. The $^+$ESI-MS total ion current (TIC) of the isolated ACR peptide mixture is depicted in *Figure 5*, with ACR-**6** eluting at 23.93–24.7 min. Diagnostic fragment ions b_1–b_{11} and y_6–y_{15} are assigned in a CID-MS, depicted in *Figure 9*. ESI-CID-MS and ESI-MS^2 using *m/z* 1660 as parent ion provided the series b_5–b_{15} and y_4–y_{15} fragment ions as well as diagnostic fragment ions at y_6–101 Da and y_{14}–101 Da (*Figures 7* and 9).

Owing to the presence of Ala^{13} in ACR-**5** and ACR-**6**, these peptides represent new sequences. The sequences of ACR-**1b**, **3**, and **4** correspond to neoefrapeptins (NEF)-D, A, and B, respectively (see *Table 2*). Since about 20% L-Iva – besides abundant D-Iva – is released under vigorous hydrolytic conditions (see *Figure 3*), the presence of L-Iva^{15} in ACR-peptides is assigned by analogy with neoefrapeptins.

Discussion

Configuration of Amino Acids and Unusual Hydrolytic Stability of the C-Terminal Iva^{15}-X^{16} Bond in Acretocins and Structurally Related Peptides

Presence and configuration of chiral amino acids in ACRs were determined by GC/MS on *Chirasil*-L-Val column. Since only D-Iva was detected in acidic standard hydrolysates, exclusively D-Iva was assigned to positions 4 and 5. Based on analytical data, sequence identity of ACR **1b**, **3**, and **4** with neoefrapeptins D, A, and B, respectively, was recognized, the major difference being detection of exclusively D-Iva in ACR hydrolysates. However, in the course of a

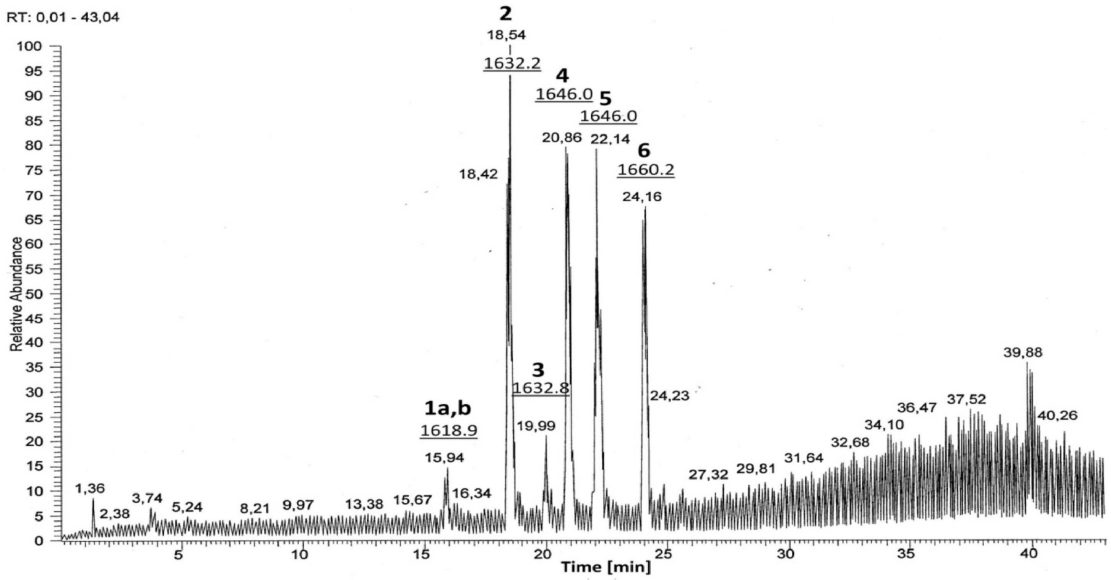

Figure 5. HPLC-$^{+}$ESI-MS (total ion current *vice* retention time) of the mixture of ACR peptides **1a**,**b**–**6**. The corresponding molecular ions ($[M]^{+}$) are underlined. This figure is complementary to *Figure 4*.

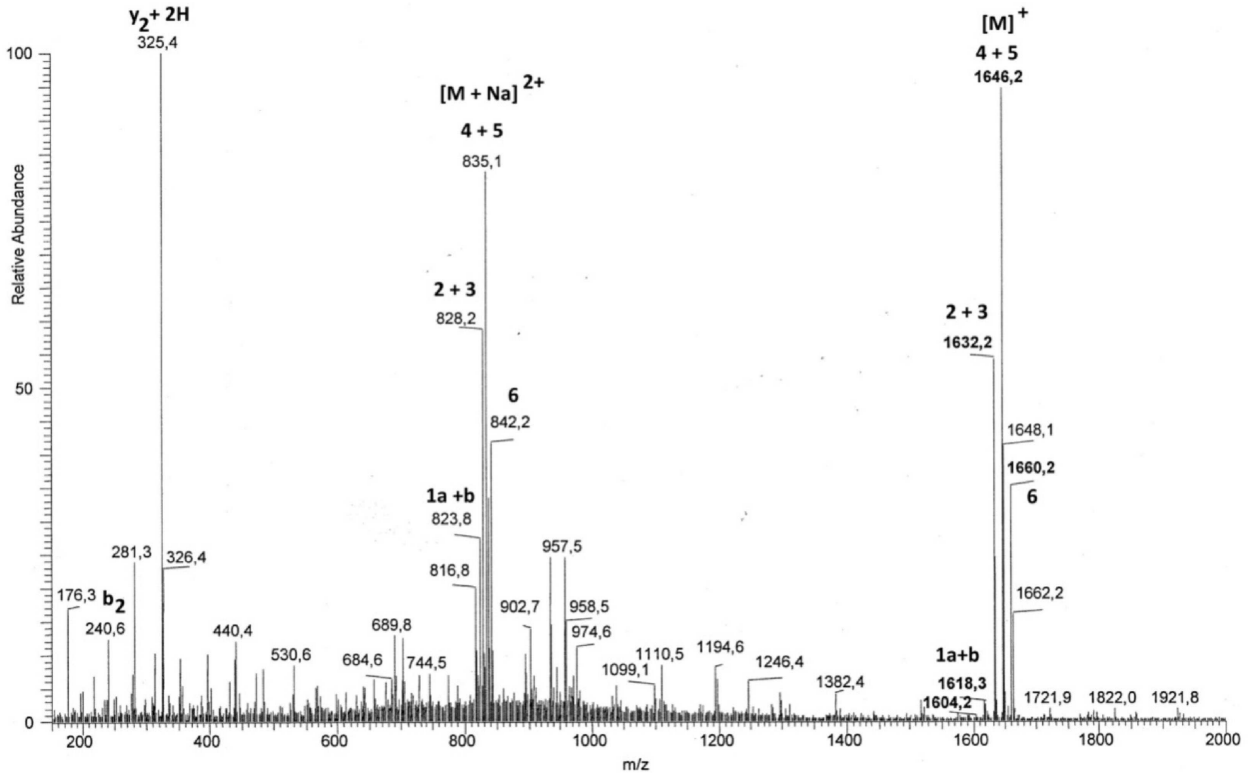

Figure 6. ESI-MS (infusion) of the mixture of ACR peptides **1**–**6** showing the $[M]^{+}$ and $[M+\mathrm{Na}]^{2+}$ ions, C-terminal $[y_2+2\mathrm{H}]^{+}$ ions at *m/z* 325.4, and N-terminal ions b_2 at *m/z* 240.6.

discussion on neoefrapeptins we became informed[52] that in acidic total hydrolysates of the intact neoefrapeptin A under standard conditions (6 M HCl, 110 °C, 24 or 48 h, respectively) the ratio D/L was not 50:50, as expected, but about 85:15. In order to clarify this ambiguity, enzymatic cleavage of neoefrapeptins

Table 2. Sequences and relative quantities [%] of acretocin (ACR) peptides **1a**, **1b**, **2**–**6**. Neoefrapeptin (NEF) sequences, which are positionally isomeric with ACRs, were given in parentheses. Exchange positions are highlighted in bold.

ACR	Residue															$[M]^+$	[%]
	1	2	3	***4***	***5***	6	7	8	9	10	11	12	***13***	14	***15***	16	
1a	Ac	L-Pip	Aib	L-Pip	**D-Iva**	**Aib**	L-Leu	*β*-Ala	Gly	Acc	Aib	L-Pip	Aib	**Gly**	L-Leu	**Aib**	X	1618.9	7.6
1b (**NEF–D**)	Ac	L-Pip	Aib	L-Pip	***Aib***	**Aib**	L-Leu	*β*-Ala	Gly	Acc	Aib	L-Pip	Aib	**Gly**	L-Leu	**L-Iva**	X	1618.9	2.4
2	Ac	L-Pip	Aib	L-Pip	**D-Iva**	**D-Iva**	L-Leu	*β*-Ala	Gly	Acc	Aib	L-Pip	Aib	**Gly**	L-Leu	**Aib**	X	1632.2	23.4
3 (**NEF–A**)	Ac	L-Pip	Aib	L-Pip	**D-Iva**	**Aib**	L-Leu	*β*-Ala	Gly	Acc	Aib	L-Pip	Aib	**Gly**	L-Leu	**L-Iva**	X	1632.8	8.7
4 (**NEF–B**)	Ac	L-Pip	Aib	L-Pip	**D-Iva**	**D-Iva**	L-Leu	*β*-Ala	Gly	Acc	Aib	L-Pip	Aib	**Gly**	L-Leu	**L-Iva**	X	1646.0	24.8
5	Ac	L-Pip	Aib	L-Pip	**D-Iva**	**Aib**	L-Leu	*β*-Ala	Gly	Acc	Aib	L-Pip	Aib	**L-Ala**	L-Leu	**L-Iva**	X	1646.0	16.3
6	Ac	L-Pip	Aib	L-Pip	**D-Iva**	**D-Iva**	L-Leu	*β*-Ala	Gly	Acc	Aib	L-Pip	Aib	**L-Ala**	L-Leu	**L-Iva**	X	1660.2	18.4

using papain was performed providing peptides which could be separated by HPLC. On total hydrolysis of isolated peptide exclusively L-Iva (if present) was released from the isolated peptides Leu[13]-Iva[14]-X[15] and *β*-Ala[7]-Gly[8]-Acc[9]-L-Iva[10]-Pip[11]-Aib[12]-Gly[13], but only D-Iva from N-terminal peptides AcPip[1]-Aib[2]-Pip[3]-Iva[4]-Iva[5]-Leu[6]. Thus, in a laborious approach, the location of Iva-enantiomers in neoefrapeptins could ultimately be assigned. The decelerated release of C-terminal L-Iva from the intact peptide was explained by the positively charged X[16] residue.[36] *Bullough et al.*,[31] however, determined L-Iva[15] in hydrolysates of efrapeptin D (6 M HCl, 105 °C, 48 h) using GC/MS on a 'chiral column' without providing further specifications. Since only a single C-terminal Iva occurs in efrapeptin D, even partial release would provide evidence for L-Iva. Resistance of the 14-residue peptaibiotic adenopeptin, which also carries the C-terminal dipeptide Iva[13]-X[14], to acidic hydrolysis was briefly mentioned whilst commenting on amino acid analysis.[37] These authors confirmed previous observations by *Gupta et al.*[30] who noted that the C-terminal capping group of efrapeptins was rather difficult to hydrolyze.

Such an unexpected resistance to standard hydrolysis conditions (6 M HCl, 110 °C, 24 or 48 h), preventing release of the C-terminal 1,2-diamino-4-methylpentane unit from Ile or Leu, was also recognized in cicadapeptins I and II, peptaibiotics from the entomopathogenic fungus *Cordyceps heteropoda* ARSEF #1880 (≡*Ophiocordyceps heteropoda*: Hypocreales, Cordycipitaceae).[35] Taking all data together, this indicates – at least for peptaibiotics – general resistance towards acidic hydrolysis of C-terminal residues from peptides of the structure -Aaa-X (Aaa, *α*-amino acid including Aib and Iva; X, amide-bound, positively charged capping group), preventing or hampering release of the preceding C-terminal amino acid.

This information caused us to subject a sample of the ACR mixture to severe hydrolysis conditions (37% HCl, 135 °C, 72 h). Indeed, L-Iva was released as revealed by analyzing the *N*-acetyl-Iva-*O*-2-propyl esters on *Chirasil*-L-Val (see *Figure 3*). Notably, release of 20% L-Iva from the ACR mixture was observed. From that, the presence of terminal L-Iva[15] residues is concluded for the corresponding sequences. Our observation is in analogy to neoefrapeptins and the closely related efrapeptins.[29,36] An acid-catalyzed partial enantiomerization of D-Iva, located in positions 4 and 5 of ACRs, is excluded because treatment of enantiomerically pure D-Iva under these drastic con-

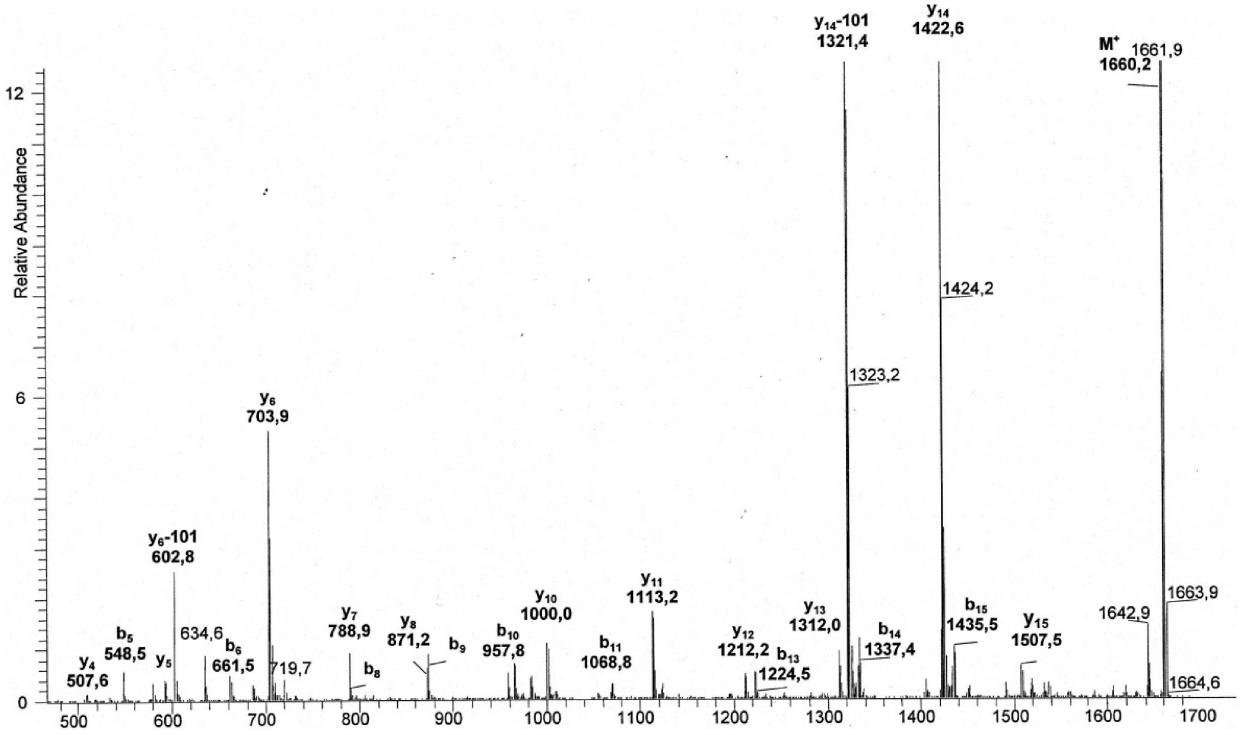

Figure 7. $^{+}$ESI-MS^{2} of *m/z* 1660 (infusion) of ACR-**6** peptide. Intense diagnostic fragment ions y_{14} and $y_{14}-101$ Da dominate the spectrum; the diagnostic pair of fragment ions y_6 and y_6-101 Da is also visible.

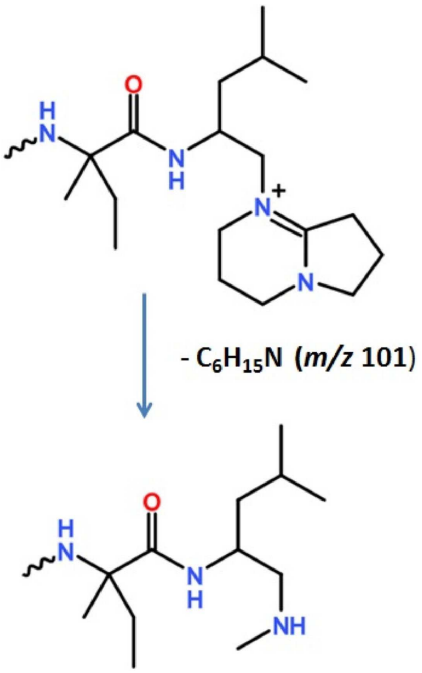

Figure 8. Proposed neutral loss of $C_6H_{15}N$ (101 Da) from C-terminal y_6 and y_{14} fragment ions.

ditions did not result in the formation of L-Iva. This is in agreement with classical experiments demonstrating that heating of dextrorotatory (+)-Iva (corresponding to L-Iva) in a bomb tube according to *Carius* in 1 M or conc. HCl (about 24% by weight) at 160–180 °C for 5 h did not change the optical rotation. Even heating in $Ba(OH)_2$ (4 d, 150–160 °C) did not change the optical rotation of L-Iva.[53] Chiral resistance of Iva against alkaline hydrolysis (2 N NaOH, 16 h, 100 °C) has already been reported by *Fischer* and *von Grävenitz*.[54]

To summarize, based on *i*) the release of L-Iva from ACR on vigorous acidic hydrolysis, *ii*) analogy of sequences of some ACR peptides with neoefrapeptins, and *iii*) presence of a C-terminal L-Iva also in efrapeptins, the configuration of D-Iva[4,5] and L-Iva[15] as depicted in *Figure 3* and *Table 2* is assumed. The new acretocins **5** and **6** contain Ala in positions 13, whereas this position is occupied by Gly in all neoefrapeptins. In contrast to the neoefrapeptin producer DSM 15068, the ACR producer CBS 217.70 does not biosynthesize peptides containing 3-MePro. Thus, the architecture of the nonribosomal peptide synthetase of the acretocin producer differs to some extent from that one of the neoefrapeptin producing strain.

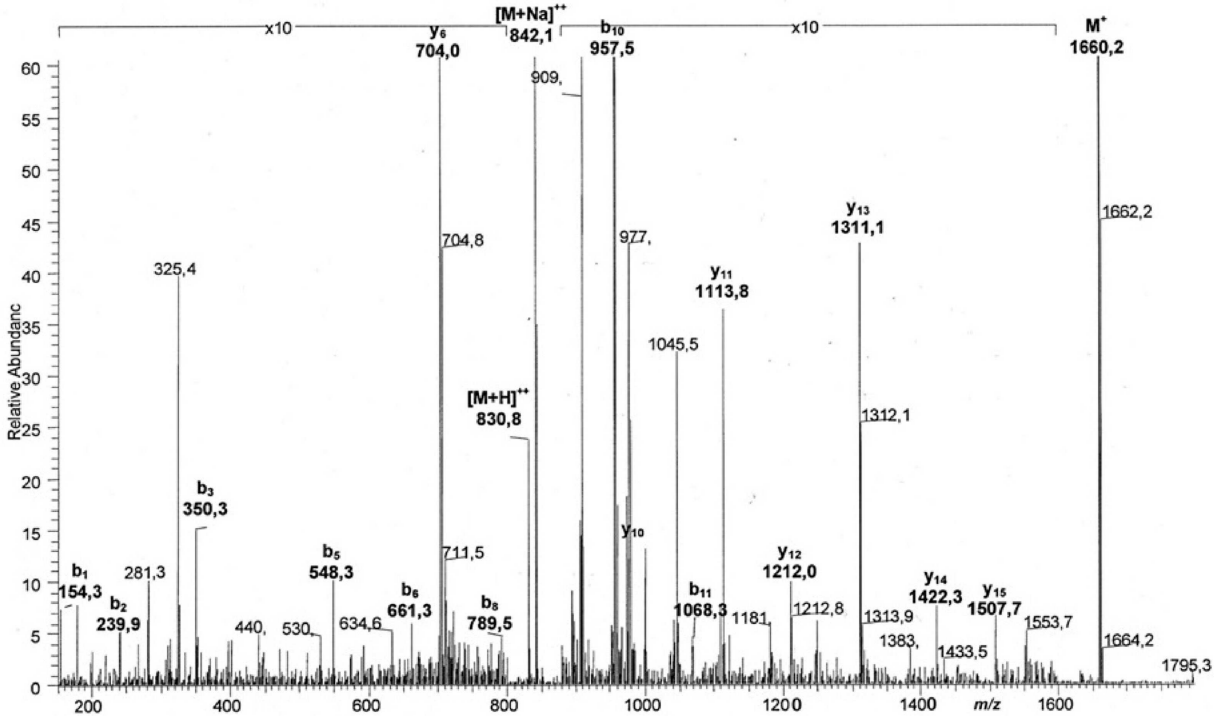

Figure 9. ESI-CID-MS of *m/z* 1660 (infusion) of peptide ACR-**6** displaying diagnostic fragment ions b_1–b_{11} and y_6–y_{15} (see *Table 1*).

Taxonomic Considerations of the Neoefrapeptin and Acretocin Producers

Acremonium crotocinigenum has originally been described as *Cephalosporium crotocinigenum*,[55] which was later transferred to the genus *Acremonium*.[17] Based on morphological characteristics, the species *A. crotocinigenum* was considered as an intermediate between the genera *Acremonium* and *Cylindrocarpon*. In 2011, *Summerbell et al.*[56] published a phylogenetic revision of *Acremonium* and the genera closely related to it, *i.e.*, *Gliomastix*, *Sarocladium*, and *Trichothecium*. Molecular sequencing of the nuclear ribosomal large subunit (nucLSU) and second largest subunit (SLU) revealed a close relationship of *A. crotocinigenum* CBS 129.64 (=type culture of *A. crotocinigenum* isolated from the white rot fungus *Trametes versicolor*) with '*Trichothecium indicum*' CBS 123.78, *T. roseum* DAOM 208997, and *T. sympodiale* ATCC 36477, which formed a separate 'Trichothecium clade'. Based on these results, the authors recombined *A. crotocinigenum* and *Spicellum roseum* in *Trichothecium*, irrespective of different morphological characters and different modes of conidiogenesis. The question as to whether the producer of acretocins, *A. crotocinigenum* CBS 217.70, has to be included in the genus *Trichothecium* cannot be resolved for the time being because no detailed phylogenetic analysis of this particular strain has been performed up to now.

To continue, neoefrapeptins A–N have been reported from a filamentous fungus originally identified and deposited as *Geotrichum candidum* SID 22780 (=DSM 15068). Given that this strain would have been correctly identified, this would have made it the first peptaibiotic-producing yeast-like organism (Saccharomycetales, Dipodascaceae) reported in literature. Careful light-microscopic examination of plate cultures of DSM 15068 revealed that no yeast-like organism was present (see *Experimental Section*). The strain formed a thin white mycelium, rich in septations, but no reproductive structures such as conidiophores and conidia could be observed. Consequently, a taxonomic reinvestigation based on marker genes of DSM 15068 was ordered by us (H.B. and T.D.) at *DSMZ* (Braunschweig, Germany). Sequencing of the large subunit rRNA gene (LSU rRNA=nucLSU) revealed that DSM 15068 is, in fact, closely related to *Trichothecium indicum* (syn. *Leucosphaerina indica*) and *Trichothecium crotocinigenum* (syn. *Acremonium crotocinigenum*, type strain CBS 129.64). In contrast, little similarity was observed in

internal transcribed spacer (ITS)[3] sequences of *T. crotocinigenum* type strain CBS 129.64 and DSM 15068; and no ITS sequences are publicly available for *T. indicum*. Consequently, there is strong evidence that the neoefrapeptin-producer '*Geotrichum candidum*' DSM 15068 has been misidentified and does not belong to the genus *Geotrichum*. Like the acretocin producer *A. crotocinigenum* CBS 217.70, it may also represent a new species. In conclusion, since efrapeptins biosynthesized by numerous species of the fungal genus *Tolypocladium* (Hypocreales, Ophiocordycipitaceae), comparison of the respective peptide sequences with those of neoefrapeptins and acretocins from a chemotaxonomic point of view is of limited value. Non-ribosomal biosynthesis of acretocins and neoefrapeptins is, of course, independent of changing taxonomic considerations of their fungal producers.

Experimental Section

Chemicals and Materials

MeOH, 2-PrOH, MeCN, CH_2Cl_2, $CHCl_3$, AcOH, acetic acid anhydride (Ac_2O), acetyl chloride (AcCl), CF_3COOH (TFA), trifluoroacetic anhydride (TFAA), and H_2SO_4 were purchased from *Sigma* (Deisenhofen, Germany). Potassium iodide and 4,4′-bis(dimethylamino)diphenylmethane (TDM; *Arnold*'s base, a substitute for the toxic *o*-tolidine[57]) were obtained from *Fluka* (Buchs, Switzerland). Preparation of TDM-reagent: (I) 7.5 g TDM in 30 ml AcOH were made up to 150 ml by addition of distilled water and filtered by means of a fluted filter paper; (II) 15 g KI were dissolved in 150 ml distilled water, then (I) and (II) were mixed to provide the TDM-reagent. Acc and other amino acids used as reference compounds were purchased from *Sigma* or *Fluka*; L-Iva and D-Iva were from *Acros* (Geel, Belgium). Fuming HCl (37%) and conc. HCl (32%) were from *Carl Roth* (Karlsruhe, Germany). Acidic hydrolyses were performed in 1-ml *Wheaton® ReactiVials* (tightly closed with *Teflon*-lined screw caps (*MAGV Laborbedarf*, Rabenau, Germany). H_2O used was from a *Milli-Q*^{R} reversed osmosis system (*Millipore*, Schwalbach am Taunus, Germany) or double distilled water from a quartz glass distillation unit (*Destamat Bi18E*®, *Heraeus*, Hanau, Germany). *Servachrom Amberlite XAD-2* polystyrene adsorber resin type 7, particle size 0.3–1 mm, was obtained from *Serva* (Heidelberg, Germany). It was conditioned before use by subsequent washing with several bed volumes (bv) of 1% aq. H_2SO_4 in MeOH (4 bv), Me_2CO/MeOH/water 10:45:45 (v/v/v; 4 bv) and distilled water at 50 °C (10 bv). *Sephadex LH-20*, particle size 25–100 μm, was obtained from *Pharmacia-LKB* (Freiburg i. Br., Germany), and was conditioned with MeOH before use.

TLC. For analytical TLC, glass plates pre-coated with silica gel *60* F_{254} (10×20 cm, 0.25 mm layer thickness, *Merck*, Darmstadt, Germany) were used. TLC was performed in glass chambers (*Desaga*, Wiesloch, Germany) coated with filter paper to saturate the atmosphere. The distance from start to solvent front was usually 7.5–8 cm. The mobile phase was composed of $CHCl_3$/MeOH 8:2 (v/v). ACRs provided white spots of R_f 0.69 by spraying with water (see *Figure 1a* and *1c*). After nearly drying the plates, treatment with chlorine for 20 min (generated in a desiccator from $KMnO_4$ and conc. HCl) was performed, followed by cold blow-drying to remove excess of chlorine and spraying with the TDM-reagent, dark-blue spots were furnished indicating peptides (see *Figure 1b* and *1d*).

Cultivation, Laboratory-Scale Fermentation, and Product Monitoring of Strain CBS 217.70

The fungus was obtained as a freeze-dried culture. This strain is still available under this accession number from *Westerdijk* Fungal Biodiversity Institute, CBS-KNAW, Fungal Culture Collection, Utrecht, The Netherlands. The herbarium specimen of this strain is deposited at CBS with accession number CBS H-8135. After conditioning of the inoculum in sterile 0.9% NaCl solution, it was grown in *Petri* dishes on malt extract agar (MEA), consisting of [g/l] malt extract 30 (*Servabacter* light, No. 28397, *Serva*, Heidelberg, Germany); soy peptone 3 (*Oxoid Unipath*, Wesel, Germany), and agar-agar 15 g/l (No. 1614, *Merck*, Darmstadt, Germany). Liquid malt extract media (MEM) was prepared without agar and adjusted to pH 5.6+/−0.1 prior to sterilization. Vigorous growth of the fungus on MEA was observed after 12 days at 25 °C, and the color of the aerial mycelium changed from white to beige. Subcultures were prepared by inoculating two baffled 2-l *Erlenmeyer* flasks, each containing 400 ml of MEM, with sterile discs of 1.5 cm diameter. After 8 days of gentle shaking at room temperature, the production of ACRs was monitored daily by TLC as follows. For solid phase extraction (SPE) of culture broths, commercial *Sep-Pak C18* cartridges (*Waters/Millipore*, Eschborn, Germany) or laboratory-made cartridges (20×10 mm)

[3] Internationally recognized as the first DNA barcode for fungi.[58]

filled with *LiChroprep RP-8*, particle size 40–63 μm (*Merck*, Darmstadt, Germany) were used. Cartridges were connected to a syringe with *Luer-lock*™ tip and conditioned with Me_2CO, MeOH, and water (20 ml each). After that, 20 ml aliquots of culture filtrates resulting from fermentations were applied. After washing with 20 ml of water, the adsorbed peptides were eluted with 5 ml MeOH. After evaporation to dryness, the remaining residues were dissolved in 500 μl MeOH and aliquots of 1–10 μl analyzed by TLC or used for other analytical procedures.

After 13 days, ACR production was observed in the pre-cultures, of which 20 ml aliquots were used for inoculating 12 2-l baffled *Erlenmeyer* flasks, each one containing 400 ml of MEM. Vigorous fungal growth was observed already after 24 h. Production of ACRs was monitored again by TLC. After eight days of fermentation, intensive production of ACRs was detected. Hence, the mycelium was removed by filtration using a nylon cloth followed by vacuum-assisted filtration using filter paper and final centrifugation at 3500 rpm. A total of 4.7 l of clear culture broth and 215 g of wet mycelium were obtained. The mycelium was not analyzed further for ACRs.

Cultivation and Light-Microscopic Examination of Strain DSM 15068

The fungus was obtained from *DSMZ* as a living culture on HA agar upon the rights granted by patent law.[59] Upon arrival, it was streaked out on HA agar plates and grown for five days at 25 °C. The actively growing strain was transferred to new HA agar plates and grown up to five weeks at 25 °C. The fungus was also cultivated on TSM-37 agar plates for up to 19 days at 25 °C.[59] Plate cultures growing on HA or TSM-37, respectively, were regularly (every seven days) examined under the light microscope after staining with lactic acid-cotton blue (glycerol, 20 ml; lactic acid, 20 ml; aqua dist., 20 ml; cotton blue, 50 mg).[60]

XAD-Medium Pressure Liquid Chromatography

A *Labomatic* MPLC System (Sinsheim, Germany) comprising pump, glass columns, and fraction collector was used. The filtrate (4.7 l) was pumped through a heavy-wall glass column (38×3.7 cm i.d.) filled with *XAD-2* resin at a flow rate 20 ml/min). Peptides were eluted with a gradient from 40% to 100% MeOH at a flow rate of 5 ml/min. Fractions of 25 ml were collected, and the elution of peptides was monitored by TLC, applying 10 μl samples (see *Figure 1a* and *1bd*). ACRs, displaying R_f 0.69, were abundant in fractions 50–59. They were combined, yielding 615 mg of solid material.

Sephadex LH-20 Chromatography

The crude peptide mixture (615 mg) obtained by *XAD* chromatography was dissolved in 10 ml MeOH and subjected to *Sephadex LH-20* gravity-flow column chromatography (glass column, 100 cm×3.1 cm i.d.). Two samples of 5 ml were applied onto the column. MeOH was used as an eluent at a flow rate of 2.5 ml/min. Elution of peptides was monitored by TLC. The first 150 ml of the eluate were discarded; afterwards 15 ml fractions were collected. Fractions 8–10 from first and fractions 7–10 from second run (see *Figure 1c* and *1d*) were combined. After evaporation to dryness, 184 mg of ACR peptides were obtained.

Instruments

GC/MS (*Instrument A*). For the determination of ACR amino acids released under standard conditions (6 M HCl, 16–24 h, 110 °C), a HP 6890 instrument with mass-selective detector *Series 5972* (*Agilent*, Waldbronn, Germany) was used. The instrument was equipped with a *Chirasil*-L-Val capillary column (25 m× 0.25 mm i.d., film thickness 0.12 μm), (from *Varian-Chrompack*, Darmstadt, Germany). Helium 5.0 (*Messer-Griesheim*, Krefeld, Germany) was used as carrier gas. The instrument was run in total-ion current (TIC) and in selected-ion monitoring mode (SIM), respectively, using electron impact (EI) ionization at 70 eV. The *N*-trifluoroacetyl-amino acid 2-propyl esters were prepared as follows: to the dry residue resulting from the total hydrolysate of 0.2 mg ACR peptide, 500 μl AcCl in 2-PrOH (8:2; v/v) were added and esterified in a closed reaction vessel for 1 h at 100 °C. After evaporation to dryness in a stream of nitrogen, 300 μl CH_2Cl_2 and 50 μl TFAA were added for trifluoroacetylation. This solution was heated for 20 min at 100 °C. After evaporation to dryness in a stream of cold N_2, 100 μl CH_2Cl_2 were added and 1 μl aliquots injected into the GC in split mode (ca. 1:30). The temperature of injector and interface was set at 250 °C. The temperature program was 70–100 °C (2.5 °C/min); 100–135 °C (3.5 °C/min); 135–150 °C (5 °C/min); 150–190 °C (20 °C/min). Inlet pressure of helium was 5 kPa for 1 min, increased at 0.2 kPa/min to 7 kPa, then 0.3 kPa/min to 11 kPa, then 1.6 kPa/min to 15.0 kPa. Diagnostic ions [*m/z*] resulting either from C^{α} or ester cleavage (the

latter in parentheses) of *N*-trifluoroacetyl-amino acid-*O*-2-propyl esters were: Aib (154); D/L Iva, not resolved (168); L-Ala 140; Gly 126 (154); β-Ala 140 (168); L-Pip (180); Acc 152 (179/180); L-Leu (182). For GC and MS fragmentation scheme of Acc used for identification see *Figure 2*.

GC/MS (*Instrument B*). The chirality of D- and L-Iva in ACR hydrolysates, which resulted from vigorous hydrolysis using fuming or concentrated HCl, was determined using a *6890N* gas chromatograph with mass-selective-detector *MSD 5973* injector 7673 (all from *Agilent Technologies*, Waldbronn, Germany). The GC was equipped with a *Chirasil*-L-Val capillary column (25 m×0.25 mm i.d., film thickness 0.12 μm), (from *Varian-Chrompack*, Darmstadt, Germany). The instrument was run in the electron impact ionization mode at 70 eV. The MSD was set at mass range *m/z* 35–350 using the full scan mode. Helium was used as carrier gas at 6.5 kPa inlet pressure, inlet temperature 220 °C, quadrupole temperature 150 °C, ion source temperature 230 °C. The *N*-acetyl-DL-Iva-2-propyl esters were prepared as follows: to the dry hydrolysate resulting from vigorous total hydrolysis was esterified as described before. For subsequent acetylation, 300 μl CH_2Cl_2 and 50 μl Ac_2O were added, and the mixture was heated for 20 min at 100 °C. After evaporation to dryness in a stream of nitrogen, 100 μl CH_2Cl_2 were added and 1 μl aliquots injected into the GC in split mode (ca. 1:30). The temperature of the oven was kept at 85 °C for 10 min, then ramped to 200 °C in 25 min, and held for 5 min. For GC/MS, see *Figure 3*.

Analytical HPLC. A *LaChrom*®-System comprising column oven (*L-3000*), UV-detector (*L-7420*), interface (*L-7000*), autosampler (*L-7250*), all from *Merck-Hitachi* (Darmstadt, Germany, and Kyoto, Japan), were used. For analytical HPLC, a *Kromasil® 100-C8* column, size 150×4.6 mm i.d., particle size 3.5 μm was used (*MZ-Analysentechnik*, Mainz, Germany). A gradient was generated from eluent A and B, increasing from 40% B to 80% B within 50 min at a flow rate of 1 ml/min and column temperature of 40 °C. Eluent A: MeCN/MeOH/water, 25:25:30; v/v/v+0.1% TFA; eluent B: MeCN/MeOH, 1:1; v/v+0.1% TFA. UV-detection was performed at 205 nm (see *Figure 4*). This column and chromatographic conditions were also used for HPLC-ESI-MS (see *Figure 5*). There, a *HP 1100* HPLC instrument (*Agilent*, Waldbronn, Germany) was coupled to an ion-trap mass spectrometer (*LCQ Classic*™ from *Finnigan MAT*, Bremen, Germany). Conditions for HPLC-ESI-MS in positive ion mode (data for direct infusion measurements in parentheses) were: sheath and auxiliary gas: N_2, set at 65 (50) arbitrary units (au) and 20 (5) au, respectively; collision gas: He 5.0; electrospray voltage +4.00 kV; photo multiplier 1400 V; dynode −1.5 kV; heated capillary temperature 250 (230) °C; capillary voltage +3.00 V; tube lens offset +30.00 V; relative CID (for CID-MS, if applicable): 48%. For $^+$ESI-MS in the infusion mode, 0.1% solutions of peptides in MeOH were diluted 1:10 with 1% HCOOH in MeOH/H_2O 1:1 (v/v), and ca. 10 pmol/μl solvents were infused at a flow rate of 1 μl/min.

Acknowledgements

Preliminary work by former students and co-workers on initial characterization and sequencing of ACRs at various stages of the work is acknowledged. In particular, the assistance of Heike tom Diek, Marketa Slavíčková, Jochen Kirschbaum, and Dieter Becker is acknowledged. H.B. and T.D. thank Andreas Fredenhagen for leading their attention to these stoichiometric problems, which resulted from the decelerated release of the C-terminal L-Iva in neoefrapeptins under standard hydrolysis conditions. We cordially thank Gerard Verkleij (*Westerdijk* Fungal Biodiversity Institute, CBS-KNAW Fungal Collection, Utrecht, The Netherlands) for providing photos of the herbarium specimen of CBS 217.70.

Author Contribution Statement

H.B. established the screening procedure, managed fermentation, supervised the co-workers mentioned in acknowledgments, and guided the experimental parts of the research. S.F. performed the vigorous hydrolysis and chiral analysis of the isolated peptide mixture. T.D. and H.B. interpreted the mass spectra and compiled the tables. T.D. and H.B. evaluated the taxonomy of microorganisms mentioned in the text. All authors contributed to and approved the final version of the manuscript for the reliability of data and conclusions.

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Received May 21, 2019
Accepted July 18, 2019

