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FULL PAPER

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Sequences of Tolypins, Insecticidal Efrapeptin-Type Peptaibiotics from Species of the Fungal Genus *Tolypocladium*

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A peptide mixture named tolypin, originally isolated from species of the fungal genus *Tolypocladium*, was structurally characterised and sequences compared to those reported for efrapeptins isolated from strains of *Tolypocladium inflatum*. Chiral amino acid analysis, direct infusion, and online HPLC electrospray ionization tandem mass spectrometry provided composition, molecular weights of peptides, and series of diagnostic fragment ions. Sequences deduced from ESI-MS revealed that tolypins C–G are identical to efrapeptins C–G. The results were corroborated by ESI-MS and HPLC of an authentic efrapeptin sample from *Eli Lilly Research Laboratories* (USA). Comparison of the HPLC elution profiles of efrapeptin and tolypin indicated a pronounced microheterogeneity of the former. A high-resolution HPLC of authentic efrapeptin has not been published before. Close relationship and partial identity of sequences of tolypins and efrapeptins, which had previously been postulated, were definitely proven. The geographical origin of the two most important *T. inflatum* strains used for sequencing of efrapeptins/tolypins could unambiguously be clarified. A new minor compound, designated tolypin H1, was sequenced. High proportions of helicogenic Aib (α-aminoisobutyric acid) and L-isovaline, *N*-terminal acetyl-L-pipecolic acid and the unusual, amide-bound *C*-terminal residue, named (*S*)-2-amino-1-(1,5-diazabicyclo[4.3.0]non-5-ene-5-ylium)-4-methylpentane corresponding to 1-[(2*S*)-2-amino-4-methyl-pentyl]-2,3,4,6,7,8-hexahydropyrrolo[1,2-*a*]pyrimidin-1-ium, define these peptides as linear, cationic peptaibiotics.

Keywords: peptide antibiotics, antiviral agent, uncoupling of oxidative phosphorylation, *Tolypocladium inflatum*, acretocin, Aib, L-isovaline, amino acids.

Introduction

Survey of the History of Efrapeptins

The peptide antibiotic named efrapeptin[1] (previously known as A23871, or under its incorrect name 'efrastatin')[2] was first isolated from cultures of a filamentous fungus by *R. L. Hamill* and *J. G. Whitney*. This fungus was investigated in the course of a screening programme for antibiotics of *Eli Lilly and Company*, (Indianapolis, IN, USA).[3,4] It had been isolated from soil in Indianapolis before 1974 and was deposited in 1976 by *C. A. Powell* with the then *Commonwealth Mycological Institute* (*CMI*)[2]. According to *Jackson et al.*[4] who performed purification and preliminary structural analysis of efrapeptin, the producing fungus has been identified by *B. L. Brady*, *Commonwealth Mycological Institute*, Kew, UK, and has been deposited as *Tolypocladium inflatum* W. Gams with the accession number IMI 202309.[2]

[1] The name efrapeptin was chosen by *Henry A. Lardy*[1] (1917–2010) in honour of *Efraim Racker* (1913–1991)[2]

Despite its initially unknown structure, efrapeptin had already attracted considerable attention in the mid-1970s as a result of its ability for uncoupling oxidative phosphorylation, for example, in rat liver mitochondria[5] and photophosphorylation in spinach chloroplasts.[6] This particular bioactivity of efrapeptins are of continuous interest because they became established as potent inhibitors of the F_1 catalytic domain of the F_1F_o-ATP synthase assembly.[7,8] This is a complex protein nano-motor composed of a water-soluble F_1-head and a membrane-embedded F_o-subcomplex,[3] which synthesises nearly 90% of the adenosine triphosphate (ATP) made during cellular respiration. For example, in a study on cell cultures, *Papathanassiu et al.*[9] reported on the inhibition of F_1F_o-ATP synthase, which acts as a co-chaperone of Hsp90-substrate protein complexes, by efrapeptins. Such treatment leads to downregulation of Hsp70 in parallel with depletion of Hsp90. From these results, new approaches for breast cancer therapy *in vitro* and *in vivo* were concluded.[9]

Sequencing and Fungal Producers of Efrapeptins

From the culture broth of a submerged fermentation of *Tolypocladium inflatum* IMI 202309[4] in a complex medium, *Jackson et al.*[4] could isolate and preliminarily characterise crude efrapeptin in extracts with $CHCl_3$. HPLC of the efrapeptin mixture revealed the presence of components designated A–H. In a fundamental contribution, *Bullough et al.*[10] could determine the amino acid sequence and stereochemistry of preparatively isolated efrapeptin D by positive and negative ion FAB-MS, HR-MS and chiral gas chromatography. Although these authors could determine the amino acid sequence of their major compound, efrapeptin D, almost correctly, the structure of an unknown *C*-terminal capping group, therefore terminated 'X', could not been elucidated.

In extension of this work, *Gupta et al.*[11,12] isolated and purified efrapeptins from the culture broth of *Tolypocladium inflatum* ARSEF #616. Notably, this particular strain was originally isolated from a soil sample collected in the Bohemian-Moravian Highlands in 1966 and deposited as *Beauveria bassiana* CCF 962 by *Olga Fassatiová*, the former curator of CCF (for abbreviations of culture collections, see footnote 2). This strain is currently deposited as *T. inflatum*[13] ARSEF #616 and ATCC 18981 in the respective culture collections. It should be emphasised here that the Czech strain *T. inflatum* ARSEF #616[5] is *not* identical with the previously reported efrapeptin producer *T. inflatum* IMI 202309, the latter being a soil-borne North American isolate, as pointed out above. In spite of their different geographical origin, both strains are prolific producers of sequentially identical efrapeptin-type peptaibiotics.

In their pioneering contributions, *Gupta et al.*[11,12] could determine the complete sequences and stereochemistry of individual efrapeptin peptides C, D, E, F, and G, isolated from '*Tolypocladium niveum*' ARSEF #616 (see footnote 6). These authors used contemporary peptide sequencing techniques, including FAB-MS, HR-MS, ^{1}H- and ^{13}C-NMR, amino acid analysis by TLC, HPLC, achiral and chiral GC/MS and, in part, X-ray crystallography.

In particular, the structure of the enigmatic *C*-terminal blocking group 'X' could unambiguously be elucidated by *Gupta et al.*[11,12] These authors performed X-ray analysis of the *bis*-trifluoroacetate of *C*-terminal H-L-Leu-L-Iva-X that could be obtained from an acidic efrapeptin partial hydrolysate. The structure of 'X' was established as an alkylated 1,5-**d**iaza**b**icylo [4:3:0]**n**on-5-ene (**DBN**) moiety, originally named *N*-**p**eptido-1-**i**sobutyl-2-(2,3,4,6,7,8-**h**exahydro-1-**p**yrrolo-[1,2-α]**p**yrimidinio)**e**thylamine,[12] (**PIHPPE**), corre-

who was a 'distinguished and enthusiastic investigator of oxidative phosphorylation'.[1]

[2] Abbreviations of culture collections: **IMI**; formerly: ***I**mperial **M**ycological **I**nstitute*, from 1948–1986: ***C**ommonwealth **M**ycological **I**nstitute* (**CMI**), from 1986–1998: ***I**nternational **M**ycological **I**nstitute* (**IMI**) of the ***C**ommonwealth **A**gricultural **B**ureaux* (**CAB**). Currently: ***C**entre for **A**griculture and **B**ioscience **I**nternational* (**CABI**), Egham, Surrey, UK. **USDA-ARSEF**; ***US D**epartment of **A**griculture, **A**gricultural **R**esearch **S**ervice Collection of **E**ntomopathogenic **F**ungal Cultures*, Ithaca, NY, USA. **ATCC**; ***A**merican **T**ype **C**ulture **C**ollection*, Manassas, VA, USA. **CCF**; ***C**ulture **C**ollection of **F**ungi*, Charles University, Prague, CZ.

[3] The index '**o**' refers to a factor *F*, which selectively binds the antibiotic '**o**ligomycin'. Thus, the letter '**o**' must not be confused with a '0' (zero)[2] as frequently observed in the literature.

[4] Viable cultures of IMI 202309 are publicly available from *CABI*, Egham, Surrey, UK. A voucher specimen is stored as HIMI 202309 in the herbarium of *Kew Gardens*, Richmond, London, UK.

[5] In contributions published prior to 1994, the *Krasnoff* group preferably used the species name '*T. niveum*' (Rostrup) Bissett[14] that has been eventually proposed for rejection.[15] Later on, *T. inflatum* W. Gams was obligatorily

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sponding to (*S*)-2-amino-1-(1,5-diazabicyclo[4.3.0]non-5-ene-5-ylium)-4-methylpentane. Consequently, efrapeptins (and tolypins) are cationic peptides.

These structural details were further confirmed by *Abrahams et al.*[17] who co-crystallised bovine F_1-ATPase using the microheterogeneous efrapeptin mixture provided by *Eli Lilly and Company*[6] (see also *Figure 2,A* below). Notably, the crystallographic data suggest that the complexed species is efrapeptin C, the least hydrophobic sequence among efrapeptins, which accounts for only 2% of the peptide mixture. This noteworthy phenomenon of selective binding according to hydrophobicity (see *Experimental Section*) has never been discussed before in terms of the mode of action of microheterogeneous efrapeptins mixtures.

Tolypocladium *Species Are Recognised as Prolific Producers of Efrapeptin-Type Peptaibiotics*

In continuation of their sequential work, the *Krasnoff* group investigated the antifungal and insecticidal properties[18] of efrapeptins isolated from '*T. niveum*' #616 and compared the HPLC elution profiles of efrapeptins from this strain to those of *Tolypocladium geodes* ARSEF #2684.[19] Furthermore, the HPLC elution profiles of CH_2Cl_2 extracts of liquid cultures of nine *Tolypocladium* species were compared, comprising 44 strains from world-wide habitats covering different climate zones.[20] Strains of *T. cylindrosporum*, *T. geodes*, and '*T. niveum*' (=*T. inflatum*) were recognised as prolific producers of efrapeptin peptides. Some strains of *T. nubicola* and *T. tundrense* also produced efrapeptins. This study revealed that ratios of efrapeptin components C–H vary to a certain extent, efrapeptin F exceeding efrapeptin D in *T. geodes* and *T. cylindrosporum*, and D exceeding F in '*T. niveum*'.[20]

Muroi et al. isolated efrapeptins D, F, and G from 'a culture of an unidentified fungus' and observed suppression of syncytium formation (SF) and cytopathic effects (CPE) in baby hamster kidney (BHK) cells that had been infected by Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV), respectively. Efrapeptins selectively blocked the expression of NDV-HN and VSV–G glycoproteins on the cell surface. Only exocytotic protein trafficking was inhibited without affecting endocytic pathways and without altering the concentration of intracellular ATP.[21]

A marine *Tolypocladium* strain from sea mud yielded a new component named efrapeptin J, together with efrapeptins F and G; however, no HPLC profile or relative quantities were reported by *Hayakawa et al.*[22] It should be mentioned in this context that efrapeptin J has not been described for any of the previously analysed efrapeptin mixtures. The relatively large quantities of efrapeptin J that could be isolated (50 mg peptide from 4 L of culture broth) might be characteristic for the particular *Tolypocladium* sp. isolated and the fermentation conditions applied.

From an atypical *Acremonium* sp. growing on a marine sponge *Teichaxinella sp.*, efrapeptins C–G could be isolated by *Boot et al.*[23,24] and sequenced together with two new efrapeptins, designated E_α and H (see *Table 1*). Relative quantities of minor peptides E_α and H were not provided. Notably, efrapeptins were produced together with linear *N*-methylated octapeptides and two known cycloheptapeptaibiotics, scytalidamides A and B.

A hitherto unidentified *Tolypocladium* species, which is most likely different from *T. inflatum*, was isolated by *Du et al.*[25] from a sediment sample of Lake Superior, Michigan (USA). Besides non-peptidic metabolites, different subfamilies of peptaibiotics could be isolated from $NaNO_3$-supplemented potato-dextrose broth. Besides the two known efrapeptins F and G, seven new 21- and 22-residue peptaibols, named gichigamins, as well as six new 11-residue lipopeptaibols, named dakwaabakains A–E, were obtained.[25] The latter are structurally related to lipopeptaibiotics LP237-F5, -F7, and -F8 isolated from the culture broth of *Tolypocladium geodes*.[26] Depending on the fermentation conditions, these peptaibols/peptaibiotics are produced together with 1-alaninechlamydocin and other, non-peptidic low-molecular weight secondary metabolites.

Efrapeptins, together with the immunosuppressive cyclopeptide cyclosporin A, were also isolated by *Kebede et al.*[27] from a marine strain MF458 defined as *Tolypocladium geodes*. Five clusters of M^+ ions (*m/z* 1607, 1621, 1635, 1649 and 1663) typical for efrapeptins C–G, were detected. The major component of this mixture was assigned as EFR–D, based on its ^1H spectrum in $CDCl_3$. Together with these cyclic and linear peptides, low-molecular-weight secondary metabolites were biosynthesised, the production and ratio of which was strongly dependent on varying culture conditions.[27]

accepted as the legitimate name of this species, thus making *T. niveum* a '*nomen rejiciendum*'.[16]

[6]Efrapeptins had also been offered by *Sigma–Aldrich Comp.* According to specifications, peptide mixtures were isolated from *T. geodes* (article number E2396; *ca.* 60% F, 15% G) and '*T. niveum*' (article number E1646; *ca.* 50% D,

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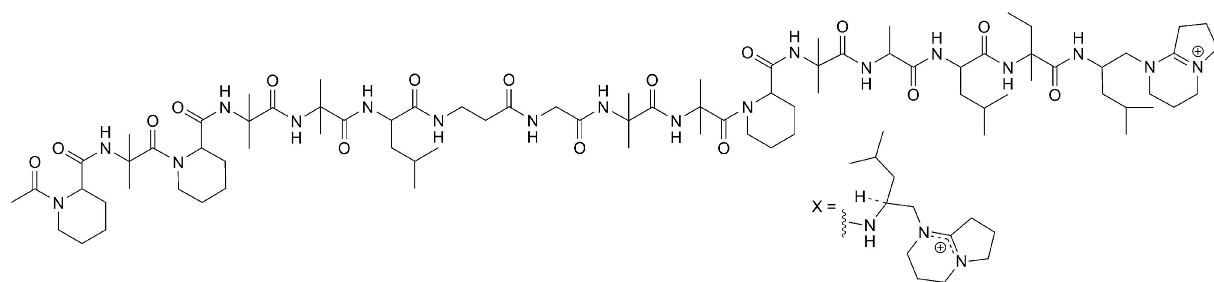
Table 1. Sequences, relative quantities [%] and molecular ions M^+ of tolypin (TOLY) and efrapeptin (EFRA) peptides.[a]



TOLY[b]	$y_{15} \leftarrow y_1$	[%][c]	M^{+}[d]
	y_{15} y_{14} y_{13} y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1		
C	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Aib^{4}-Aib^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Aib^{10}-Pip^{11}-Aib^{12}-Gly^{13}-Leu^{14}-Aib^{15}-X^{16}	6.3	1606
D	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Aib^{4}-Aib^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Aib^{10}-Pip^{11}-Aib^{12}-Gly^{13}-Leu^{14}-Iva^{15}-X^{16}	16.4	1620
E	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Iva^{4}-Aib^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Aib^{10}-Pip^{11}-Aib^{12}-Gly^{13}-Leu^{14}-Iva^{15}-X^{16}	3.3	1634
F	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Aib^{4}-Aib^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Aib^{10}-Pip^{11}-Aib^{12}-Ala^{13}-Leu^{14}-Iva^{15}-X^{16}	44.8	1634
G	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Iva^{4}-Aib^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Aib^{10}-Pip^{11}-Aib^{12}-Ala^{13}-Leu^{14}-Iva^{15}-X^{16}	20.1	1648
H1	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Iva^{4}-Aib^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Iva^{10}-Pip^{11}-Aib^{12}-Ala^{13}-Leu^{14}-Iva^{15}-X^{16}	2.2	1662
	b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11} b_{12} b_{13} b_{14} b_{15}		
	$b_1 \rightarrow b_{15}$		
	Total yield 93.1		
EFRA[e]			
H	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Iva^{4}-Iva^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Aib^{10}-Pip^{11}-Aib^{12}-Ala^{13}-Leu^{14}-Iva^{15}-X^{16}	n.r.[f]	1662
E_{α}	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Iva^{4}-Aib^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Aib^{10}-Pip^{11}-Aib^{12}-Ala^{13}-Leu^{14}-Aib^{15}-X^{16}	n.r.	1634
J	Ac-Pip^{1}-Aib^{2}-Pip^{3}-Aib^{4}-Aib^{5}-Leu^{6}-β-Ala^{7}-Gly^{8}-Aib^{9}-Aib^{10}-Pip^{11}-Aib^{12}-Ala^{13}-Leu^{14}-Aib^{15}-X^{16}	n.r.	1620

[a] The peptide sequence represents tolypin F. For *b*- and *y*-type fragment ions see *Table 2*. Chiral amino acids are of the L-configuration, including L-Iva (see text). Abbreviations: Ac (acetyl), Pip (L-pipecolic acid), Aib (α-aminoisobutyric acid), Iva (L-isovaline), β-Ala (β-alanine), Leu (L-leucine), Gly (glycine); X = (*S*)-2-amino-1-(1,5-diazabicyclo[4.3.0]non-5-ene-5-ylium)-4-methyl-pentane. [b] Tolypin (TOLY) sequences C–G correspond to efrapeptin (EFRA) sequences C–G.[11,12] TOLY H1 is new. [c] % = relative quantities of tolypins estimated from HPLC peaks (see *Figure 1*). [d] M^+ = nominal molecular mass. [e] For comparison, new efrapeptin (EFRA) sequences from the literature designated H and E_{α}[24] and J[22] are included. [f] n.r., not recorded.

Finally, the *Sewald* group confirmed structure and stereochemistry of efrapeptins C–G by performing the first total syntheses and conformational analyses of a number of synthetic analogues.[28–31]

Tolypins as Insecticidal Peptides

Early biochemical work on efrapeptins isolated from *T. inflatum* mainly focussed on bioassays related to uncoupling of oxidative phosphorylation in mitochondria. In contrast, the group of *Weiser et al.*[32,33] investigated the entomopathogenic action of spores of *T. cylindrosporum* and *T. inflatum* in the 1980s. It was realised that aqueous or organic solvent extracts of cultures of *T. cylindrosporum, T. inflatum* and *T. geodes* grown on agar slopes kill mosquito and black-fly larvae. The latter two fungal species were found to be prolific producers of an insecticidal metabolite that was named tolypin.[34,35]

The structural similarity of tolypins and efrapeptins was indicated by fast atom bombardment (FAB) mass spectrometry, which provided molecular masses (M^+) of 1620, 1634, 1648 and 1662 Da for tolypin peptides, corresponding to efrapeptins D, E, F, G, and H,[36,37]

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with efrapeptin F as the major compound. A total hydrolysate of crude tolypin was derivatised using *o*-phthaldialdehyde (OPA) together with 1-thio-β-D-mannose for chiral amino acid analysis by HPLC. This method revealed the presence of L-Iva exclusively, together with abundant Aib, β-Ala, L-Ala, L-Leu and Gly whereas Pip was not detectable using this approach.[36] From those preliminary data, it was concluded that tolypin represents a mixture of peptides closely related or almost identical to efrapeptins isolated and sequenced from '*Tolypocladium niveum*' as outlined above.[11,12]

Quantities of tolypins[7] could be isolated using organic solvent extracts of the culture broth of surface cultures of *Tolypocladium inflatum*[37] and *Tolypocladium geodes*.[38] Notably, the HPLC elution profile of the tolypin analysed in this work corresponds to the profile of efrapeptins isolated from *Tolypocladium geodes* ARSEF #2684,[19] with both isolates providing peptide F as the major compound. Efrapeptins isolated from *T. inflatum* ARSEF #616 that had been used for sequencing by the *Krasnoff* group, were reported to contain peptide D as the major component.[11,12,19]

Different isolates of 'tolypins' were investigated in particular with emphasis on their *in vitro* action on larvae of mosquitoes (*Culex pipiens*, *Culex molestus*, *Aedes aegypti*, *Anopheles maculipennis*),[34,35,37,38] black fly species (*Simulium noelleri*, *Odagmia ornata*),[35] last instar larvae of greater wax moth (*Galleria mellonella*),[34,35,39,40] blowfly (*Calliphora vomitoria*) and meal worm beetle (*Tenebrio molitor*)[39,40] as well as adults of green peach aphid (*Myzus persicae*) and American cockroach (*Periplaneta americana*).[39]

Dumas et al.[41] investigated the cytopathological effects of a tolypin sample isolated from *T. inflatum*[37,38] on different types of epithelial cells of *Galleria mellonella* larvae by scanning (SEM) and transmission (TEM) electron microscopy, considering efrapeptin and tolypin peptides as structurally identical. In agreement with *Mat'ha et al.*,[37] alteration and lysis of mitochondria were observed. *Nagaraj et al.*[42] tested the antimalarial activities of their isolation of efrapeptins from *T. inflatum* ARSEF #616 (='*T. niveum*' ATCC 18981) against the protozoan parasite *Plasmodium falciparum* in comparison to peptaibol antibiotics zervamicins and antiamoebins.[43] All three peptides killed *P. falciparum in vitro* with IC_{50} in the low micromolar range.[42]

Although the name tolypin became established in the literature and its close structural relationship to efrapeptins was assumed as outlined before, no detailed information on sequences and ratio of constituents have ever been published. In the following, we would like to demonstrate that the major sequences of tolypins are identical to those reported for efrapeptins. However, the microheterogeneity of the latter is much more pronounced.

20% F). Use of these commercial preparations had been reported by various research groups.[8,9]

[7] Regrettably, the tolypin producers *T. inflatum* strain 1897,[35] *T. inflatum* MW 104,[37,38] *T. cylindrosporum* strain

Results and Discussion

Composition, Stereochemistry, and Sequencing of Tolypin Peptides

Chiral GC/MS. *N*-TFA-*O*-2-propyl esters, obtained after derivatisation of total hydrolysates of tolypin, were analysed by GC/MS on *Chirasil*-L-Val. Comparison with standards revealed the presence of Aib, Gly, β-Ala, L-Pip, L-Ala, and L-Leu (*Figure 1*). Since the TFA-derivatives of standards of D/L-Iva were unsatisfactorily resolved on the commercial *Chirasil*-L-Val capillary column (length: 25 m) employed, the stereochemistry of L-Iva in tolypin was determined in separate analyses as *N*-acetyl-Iva-*O*-2-propyl ester (see inset in *Figure 1*). Since in tolypin hydrolysates neither Ile nor Val were detected, these amino acids had not to be considered. The heterocyclic residue, designated 'X' in sequences, could not be analysed by GC/MS but its presence was unequivocally established by ESI-MS (see below).

Mass Spectrometry and HPLC Fingerprinting. The full scan infusion ESI-CID-MS of an authentic efrapeptin

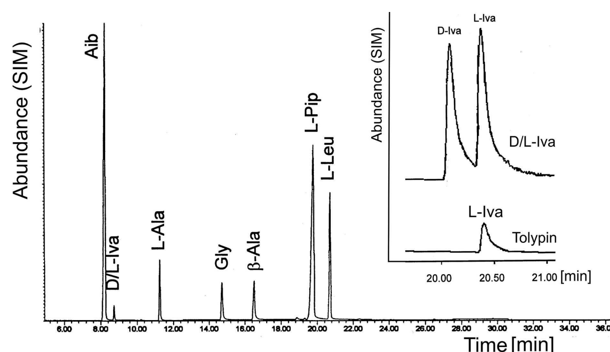

Figure 1. GC-SIM-MS (*N*-trifluoroacetyl-*O*-2-propyl esters; *Chirasil*-L-Val) of a total hydrolysate of the tolypin mixture and (inset) of a standard of *N*-acetyl-D/L-Iva-*O*-2-propyl esters in comparison to *N*-acetyl-L-Iva-*O*-2-propyl ester resulting from tolypin hydrolysis. For chromatographic conditions, see *Experimental Section*.

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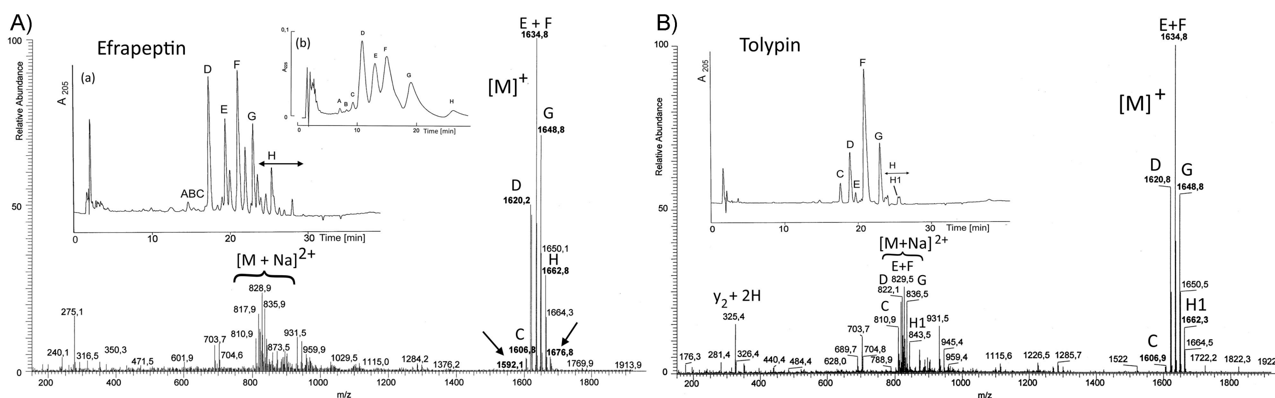

Figure 2. A) CID-ESI-MS (infusion) of an original efrapeptin sample from *Eli Lilly Research Laboratories* (see *Experimental Section*), displaying M^+ and $[M+\mathrm{Na}]^{2+}$ ions of efrapeptins C–D. Arrows indicate the presence of minor efrapeptins of *m/z* 1592.1 and *m/z* 1676.8 (M^+) in the mixture. Inset (a) shows a high-resolution HPLC of this efrapeptin sample and inset (b) the HPLC of an isolate of efrapeptin from *Tolypocladium inflatum* IMI 202309 at lower resolution (adapted from *Jackson et al.*[3] with permission from *Portland Press Ltd.*, London). Assignment of efrapeptins is in accordance with the literature. B) CID-ESI-MS (infusion) of the tolypin mixture with assignment of H^+ and $[M+\mathrm{Na}]^{2+}$ ions of peptides C–H1; the fragment ion *m/z* 325.4 $[y_2+2\,\mathrm{H}]$ is assumed to result from cleavage of the *C*-terminal Iva^{15}-X^{16} residue of tolypins D–H1.

sample is presented in *Figure 2,A*. Molecular ions M^+ and $[M+\mathrm{Na}]^{2+}$ of efrapeptins C–H are annotated. Arrows indicate the presence of two minor clusters of efrapeptins not yet sequenced. The presence of these peptides (M^+: 1592 Da and 1676 Da, respectively) has previously been recognised by *Nagaraj et al.*[42] in efrapeptins isolated from the fermentation broth of '*Tolypocladium niveum*' ATCC 18981 (= ARSEF #616).

Figure 2,A(a) shows a high-resolution HPLC elution profile of the efrapeptin sample distributed by *Eli Lilly Research Laboratories*. *Figure 2,A(b)* shows a HPLC elution profile resulting from a separate laboratory-scale fermentation conducted by *Jackson et al.* in 1979, taken from reference.[3] Minor peaks denoted A and B, eluting prior to peptide C, have not yet been characterised. Sharp, well-resolved peaks of the HPLC in *Figure 2,A(a)* result from gradient elution. Notably, the TFA-acidified eluents used suppress the ionization equilibrium of the *C*-terminal amidinium cation. In contrast, the broad HPLC peaks in *Figure 2,A(b)* were caused by elution of peptides using a neutral eluent (acetonitrile/aq. 12.5 mM $(NH_4)_2SO_4$) under isocratic conditions. The HPLC of authentic efrapeptin from *Eli Lilly Research Laboratories* is in principle agreement with that of tolypin (see inset in *Figure 2,B*) but clearly indicates the presence of additional peptides. It also reveals that peptide H, eluting as broad peak after peptide G in *Figure 2,A(b)* consists of about eight individual peptides as indicted in *Figure 2,A(a)*. To the best of our knowledge, the well-resolved HPLC presented in *Figure 2,A(a)*, is the only one published for those efrapeptins fermented and distributed by *Eli Lilly Research Laboratories*. This is of interest because donation of this particular material is acknowledged in many research publications.

The ESI-CID-MS of tolypin shown in *Figure 2,B* is in excellent agreement with that one of efrapeptins, providing the same M^+ and $[M+\mathrm{Na}]^{2+}$ ions of peptides C–H. The two additional minor peptides, indicated by arrows in the ESI-MS of *Figure 2,A*, cannot be seen. The inset shows the HPLC analysis of tolypin conducted under identical chromatographic conditions as efrapeptins shown in *Figure 2,A(a)*. Peptides C–H are assigned accordingly. Peptide F is displayed as the major compound of the tolypin mixture. Notably, the HPLC elution profile displayed in *Figure 2,B* matches very well that one of efrapeptins C–H reported for *T. geodes* ARSEF #2684 by *Krasnoff* and *Gupta*.[19] After tolypin G, four minor peptides are eluting that could only be resolved partially. For the peptide designated H1, a sequence has been determined (see below).

To summarise, the microheterogeneity of the efrapeptin sample obtained from *Eli Lilly Research Laboratories* is much more pronounced in comparison to the tolypin mixture. In the latter, peptide F is the major compound, whereas the former contains about equal amounts of peptides D and F.

Sequencing of Tolypins. Tolypin sequences were analysed using infusion and online HPLC-ESI tandem MS^n ($n=1-3$). Nominal masses (*m/z*) of diagnostic ions and methods used for sequence determination are

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Table 2. Retention times t_R [min] and nominal masses of diagnostic ions [*m/z*] of tolypin peptides C–H1 compiled from HPLC/ESI-MS^n and infusion ESI-MS.

	C	D	E	F	G	H1
t_R [min]	13.90–14.25	16.55–17.00	18.77–18.88	19.23–20.55	21.65–22.18	24.39–25.00
M^+	1606^a	1620^a	1634^a	1634	1648^a	1662^a
$[M+H]^{2+}$	803^a	810^a	817^a	817^a	824^a	831^a
$[M+Na]^{2+}$	814^a	822^a	829^a	829^a	836^a	843^a
b_1	154^a	154^a	154^a	154^a	154^a	154^a
b_2	239^b	239^a	239^a	239^a	239^a	239^a
b_3	350^a	350^a	350^a	350^a	350^a	350^a
b_4	n.d.[a]	435^a	449^a	435^a	449^a	449^a
b_5	520^a	520^a	534^a	520^a	534^a	534^a
b_6	633^a	633^a	646^b	633^a	647^a	647^a
b_7	704^b	704^a	n.d.	704^b	n.d.	717^a
b_8	761^b	761^a	775^a	761^a	775^b	775^b
b_9	846^b	846^a	861^a	846^b	861^a	n.d.
b_{10}	931^a	931^a	945^a	931^a	945^a	959^a
b_{11}	1042^b	n.d.	1056^a	1043^b	1058^b	1070^b
b_{12}	1127^a	n.d.	n.d.	n.d.	n.d.	n.d.
b_{13}	n.d.	1186^a	1199^a	1199^b	n.d.	n.d.
b_{14}	n.d.	1298^a	n.d.	n.d.	n.d.	n.d.
b_{15}	1383^b	1397^b	1410^a	n.d.	n.d.	n.d.
y_{15}	1453^d	1467^a	1481^b	1481^a	1496^a	1509^a
y_{14}	1368^b	1382^a	1396^a	1396^a	1409^b	1424^a
y_{13}	1256^a	1272^a	1285^a	1285^a	1299^a	1313^a
y_{12}	1171^b	1187^a	1186^a	1200^a	1200^a	1214^a
y_{11}	1087^a	1101^a	1100^a	1115^a	1115^a	1129^a
y_{10}	973^b	988^a	987^b	1002^a	1000^b	1015^a
y_9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
y_8	846^c	860^b	860^a	875^b	873^d	887^d
y_7	761^c	774^c	775^a	789^a	789^d	802^d
y_6	675^a	689^a	689^a	703^a	703^a	703^a
y_5	564^d	578^d	579^a	593^a	593^c	592^c
y_4	n.d.	n.d.	n.d.	506^b	n.d.	n.d.
$[y_2+2H]^+$	n.d.	325^e	325^e	323^a	325^e	325^e
$y_{14}-101$	1266^a	1280^a	1296^a	1296^b	1296^a	1296^a
y_6-101	574^a	588^a	588^a	602^b	602^a	602^a

The origin of the respective diagnostic ions is indicated by superscript $^{a-e}$: ^{a} HPLC/ESI-MS at retention time t_R. ^{b} HPLC/ESI-MS^2 from precursor ion M^+. ^{c} HPLC/ESI-MS^3 from precursor ion y_{14}. ^{d} Infusion ESI-MS^2 from precursor M^+. ^{e} Deduced from infusion ESI-MS of the tolypin mixture. [a] n.d. = not detected.

compiled in *Table 2*. From the mass differences of the respective fragment ions of the *b*- and *y*-type series, the increment masses of the corresponding amino acid residues can be calculated, thus providing the sequences of the peptides. The relevant mass differences (Δ *m/z*) to be considered are: Ac-Pip (154), Pip (111), Aib (85), Iva (99), Leu (113), β-Ala/Ala (71), Gly (58), and residue X–H (224).

Almost complete series of diagnostic ions resulted from HPLC-ESI-MS that could be completed and confirmed by infusion ESI-MS^2 and, in a few cases, ESI-MS^3 using selected precursor ions (see *Table 2*). For all peptides, intensive fragment ions resulted from cleavage of the Pip^3-Aib^4 bond, thus leading to the generation of b_3 and complementary y_{13} fragment ions. Cleavage of the Aib^{10}-Pip^{11} bond provided intensive b_{10} and y_6 fragment ions. From fragment ions b_1–b_3, the identical *N*-terminal sequence AcPip^1-Aib^2-Pip^3 could be deduced for all peptides. Fragment ions b_4 and b_5, differing by 14 Da, indicated an Aib^4/Iva^4 exchange in the respective peptides. The presence of the *N*-terminal acetyl group in all peptides was deduced from the b_1 ion *m/z* 154, which is composed of acetyl (43 Da) and Pip (111 Da). The y_9 fragment ion that should result from cleavage of the β-Ala^7-Gly^8 bond was not observed. Despite this, the conserved domain Leu^6-β-Ala^7-Gly^8-Aib^9 could be deduced from the consecutive b_6–b_9 and corresponding y_{10}–y_7 frag-

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ment ions in all tolypins. The *C*-terminal Iva^{15}-X^{16} sequence in tolypins D–H1 was deduced from the intensive $[y_2+2\ \text{H}]^+$ fragment ion *m/z* 323.2 as displayed in the infusion ESI-MS of tolypin (see *Figure 2,B*).

Tandem MS (MS^2) of the y_6 and y_{14} fragment ions of all tolypins were accompanied by characteristic, intensive ions [y_6−101 Da] and [y_{14}−101 Da], tentatively assigned to the loss of C-terminal $C_6H_{15}N$ from the respective *y*-ions. This leaving group is assumed to represent an *N*-di- or trialkylamine released from the 1,5-diazabicylo[4 : 3:0]non-5-ene moiety of the *C*-terminal residue X.[44] This also corroborates the structure of 'X' in tolypins.

HPLC of the tolypin minor component designated H1 shows that actually two peptides of the same molecular mass (1662 Da) are almost co-eluting (see inset, *Figure 2,B*). Fragment ions b_1–b_8 represent those of tolypin G, whereas the b_{10} and y_7 in H1 are increased by 14 Da. The fact that fragment ions y_5 and y_6 of tolypin G and H1 are identical, establishes amino acid positions Iva^4, Aib^5, and Iva^{10} (see *Table 2*). Notably, the occurrence of Iva^{10} in efrapeptin sequences has not yet been described.

The total ion current of the HPLC-separated tolypin mixture is displayed in *Figure 3,A.* In this figure, retention times and molecular weights (M^+) of components C–H are assigned. Sequencing of the major compound, tolypin F, based on specific fragment ions of the *b*- and *y*-series, is illustrated in *Figures 3,B* and *3,C* (*cf. Tables 1* and *2*). HPLC-ESI-MS at retention time 19.04–21.03 min provided the molecular ion (M^+) together with diagnostic fragment ions b_1–b_6, b_8 and b_{10}, as well as y_2, y_5–y_7 and y_{10}–y_{15} (*Figure 3,B*). The hitherto missing fragment ions b_7, b_{11} and b_{13} and complementary fragments y_4 and y_8 could be obtained from MS^2 of *m/z* 1634 (M^+) after direct injection (*Figure 3,C*). The y_9 fragment ion has not been observed owing to the pronounced stability of the β-Ala^7-Gly^8 bond. Despite this, the positions of these amino acids were localised by the corresponding b_7 ion. Sequences of all tolypins, including the new minor compound H1, are compiled in the *Figure 2*, which clearly indicates that tolypins C–G correspond to efrapeptins C–G. For comparison, sequences of efrapeptins E$_\alpha$, H, and J, are included.

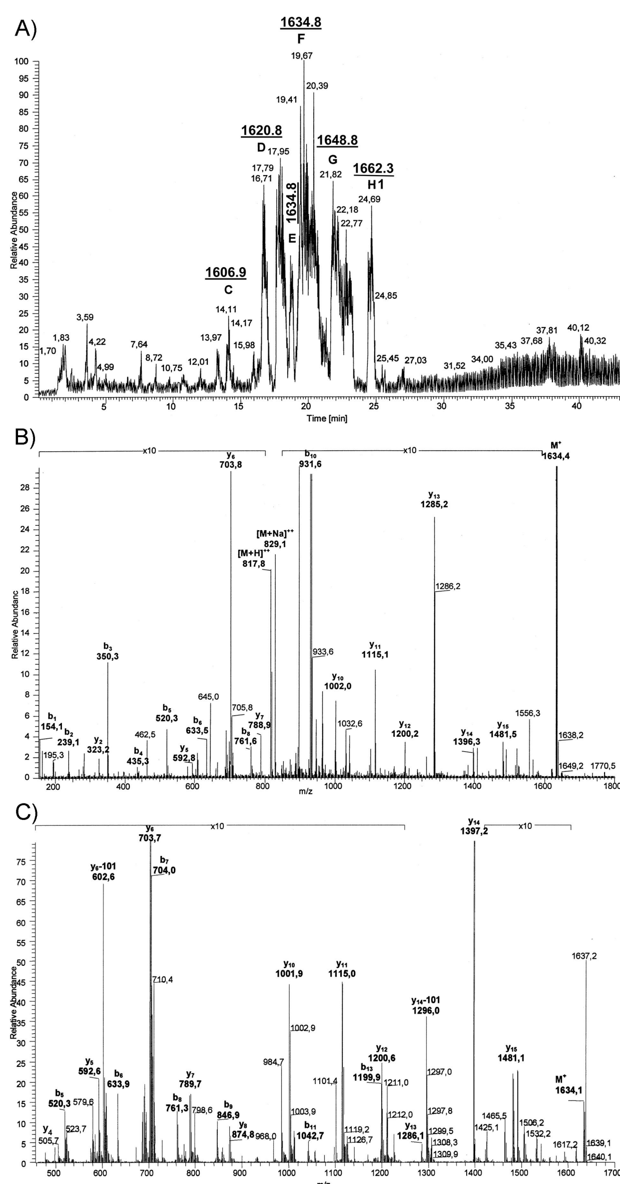

Figure 3. A) HPLC-ESI-MS (total ion current) of the mixture of tolypins with assignment of tolypin components C–H1 (molecular weights, taken from *Figure 2,B*, are underlined). b) HPLC-ESI-MS (full scan) of tolypin F at retention time 19.04–21.03 min. Series of b_1–b_{10} and y_2–y_{15} fragment ions are indicated. Missing fragment ions in series are indicated in *Figure 3,C.* c) ESI-MS^2 (infusion) of precursor ion *m/z* 1634.0 of tolypin F. Here, fragment ions missing in *Figure 3,B*, that is, b_7, b_9, b_{11}, b_{13}, b_{14} and y_4 and y_8, are recorded as well as diagnostic ions y_{14}−101 Da and y_6−101 Da.

HPLC Elution Profiles of Efrapeptin and Tolypin Peptides

Peptides are eluted from reversed-phase columns according to increasing hydrophobicities and molecular weights, that is, peptide C elutes first and H last in the series. In isobaric peptides, replacement of Aib by the more hydrophobic Iva, or of Gly by the more hydrophobic Ala, results in an increase of the retention time.

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The group of peaks eluting after peptide G and designated 'H' is a mixture of minor peptides (see insets of *Figure 2,A* and *2,B*). Tolypin H1 is the most hydrophobic peptide in the series owing to the presence of $Iva^{4,10,15}$ and Ala^{13} residues. The peptide designated efrapeptin H by *Boot et al.*[24] is expected to elute close to isobaric tolypin H1, because both peptides display the same amino acid stoichiometry (see *Table 1*). The peptide designated efrapeptin E_{α} is distinguished from isobaric efrapeptin E by the presence of Ala^{13}/Aib^{15} in place of Gly^{13}/Iva^{15}.[24] Consequently, it should elute close to compound E. Efrapeptin J, displaying a molecular weight of 1620 (M^{+}), is distinguished from efrapeptin C only by exchange of Gly^{13} by a more hydrophobic Ala^{13} residue and from isobaric efrapeptin D only by exchange of Iva^{15} by less a hydrophobic Aib^{15} residue.[22] Therefore, if present, it is least hydrophobic of all efrapeptins and expected to elute close to efrapeptin C under the chromatographic conditions considered here.

Conclusions and Perspectives

Sequences of tolypins isolated from various strains of *T. geodes* and *T. inflatum* are identical to those established for efrapeptins. However, ratios of efrapeptin peptides, in particular those of the major compounds D and F, differ as revealed by HPLC. Thus, tolypins are now synonymised with efrapeptins although 'fingerprint' HPLC reveals pronounced microheterogeneity of the latter (see *Figure 2*). This agrees with observations and statements that efrapeptin biosynthesis and ratio of peptides is influenced by the nutrients available and the intrinsic physiological attributes of the strains.[20,39] The structurally closely related acretocins[44] and neoefrapeptins[45] are distinguished in particular by characteristic exchange of an Aib^{9} residue by 1-aminocyclopropane-1-carboxylic acid (Acc^{9}). Furthermore, Iva residues in efrapeptins and tolypins are of the L-configuration whereas Iva in neoefrapeptins and acretocins occurs of both D- and L-configuration.

Production of varying mixtures of linear, 16-residue *N*-acetylated peptaibiotics with a *C*-terminal heterocyclic residues 'X' is fairly common in terrestrial and aquatic species of *Tolypocladium*, some of which parasitise on insects. Therefore, the genus *Tolypocladium* is a rich source of species and strains producing efrapeptin-type peptaibiotics. So far, *T. inflatum*, *T. geodes, T. cylindrosporum*, and *T. tundrense* have been recognised as prolific producers of peptaibiotics, whereas *T. parasiticum* was found to produce efrapeptin E, only.[39] Thus, ratios of individual peptides produced are dependent on species and individual strains of *Tolypocladium* investigated.[20,39] Insecticidal activity *in vivo* appears to result from synergistic action with other pathogenicity-determining factors.[39]

There is clear proof that certain fungal producers of efrapeptins simultaneously biosynthesise structurally different peptides and/or low-molecular weight compounds, at least under highly defined laboratory culture conditions.[24,25,27]

Further investigations of this unique group of peptaibiotics, which mostly originate from soil-borne or entomopathogenic fungi, may lead to the development of environmentally friendly insecticides.[18,40] The detection of efrapeptin-type peptaibiotics in a remarkable number of fungal species and strains, as pointed out above, further corroborates the importance of peptide-bound α,α-dialkylamino acids in the biosphere.[46,47] Consequently, this structurally unique family of peptaibiotics may help the producers to conquer and defend[48–51] their particular ecological niches[8]. Last but not least, efrapeptins/tolypins may also serve as new lead compounds in medical research.[29,53]

Experimental Section

Characterization and sequencing of tolypins was performed in parallel to that of acretocins previously described in detail.[44] Accordingly, methods and instrument settings as well as chemicals used were the same as described before. They are briefly compiled in the following:

Authentic **efrapeptin** (Lot No. 361–872B-130-5 A from the *Eli Lilly Research Laboratories*, a *Division of Eli Lilly* and *Company*, Indianapolis, Indiana, USA) isolated from *T. inflatum* IMI 202309, was a gift from *Robert L. Hamill* to *H. B.* in October 1984. It was stored in our reference collection of peptaibiotics.

Tolypin[37,38] was provided by *Alexandr Jegorov* (formerly *Galena*, *Research and Development Unit*, České Budějovice, Czech Republic).

134,[35] and *T. geodes* strain D 141[38] have never been deposited in public culture collections, including CCF.

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Chiral Gas Chromatography. Total hydrolysis of the tolypin mixture was performed using standard conditions (6 M HCl, 24 h, 110 °C). Amino acids released were converted into *N*-trifluroroacetyl-*O*-2-propyl esters (Iva: *N*-acetyl-D/L-Iva-*O*-2-propyl esters) and analysed on a *Chirasil*-L-Val capillary column using a *Hewlett Packard HP 6890* GC/MS instrument as described in reference[44] (see *Figure 1*). The stereochemistry of amino acids was determined by comparison with retention times of standards and diagnostic fragment ions (*m/z*) resulting from selected ion monitoring (*m/z* in parentheses): Pip (180), Aib (154), Leu (182), β-Ala (140), Gly (126), Ala (140), Iva (114).

Analytical HPLC. For HPLC of tolypin and authentic efrapeptin, a *LaChrom*® instrument was equipped with a reversed-phase column (*Kromasil® 100-C8*, size 150 × 4.6 mm i.d., particle size 3.5 μm). Gradient elution provided the HPLC elution profiles of efrapeptin and tolypin presented in in the insets of *Figure 2,A* and *2,B*, respectively. Gradients were generated from eluents *A* and *B*, increasing from initially 40 % *B* (5 min) to 80 % *B* (30 min), isocratic elution (15 min) and return (10 min) to initial conditions. The flow rate was 1 ml/min at 40 °C. Eluent *A*: MeCN/MeOH/H_2O 25:25:30 (*v/v/v*) + 0.1 %TFA, eluent *B*: MeCN/MeOH 1:1, (*v/v*) + 0.1 % TFA. This gradient also holds for HPLC of acretocins.[44] The HPLC of efrapeptin isolated from IMI 202309 (*Figure 2,A*, inset (b)) was adapted from the article of *Jackson et al.*[3] and reproduced with permission of *Portland Press Ltd.*, London, UK. Therein, isocratic elution from a *LiChrosorb® RP C8* column (100 × 45 mm; particle size 5 μm) using a neutral eluent composed of a mixture of acetonitrile/aq. 12.5 mM $(NH_4)_2SO_4$ 31:18 (*v/v*) at 20 °C and flow rate 0.9 ml/min was applied.

Positive-Ion ESI-MS. An ion-trap electrospray-ionisation tandem mass spectrometer (ESI-MS) *LCQ Classic*™ from *Finnigan MAT*, Bremen, Germany was used in the positive-ion mode as previously described for acretocins.[44] For collision-induced dissociation (CID-ESI-MS) of the efrapeptin and tolypin mixtures, 0.1 % solutions of analytes in MeOH were diluted (1:10, *v/v*) with 1 % HCOOH in MeOH (1:1, *v/v*) and infused by the integrated syringe pump of the MS. A relative CID energy of 48 % was applied for all infusion CID-ESI-MS experiments. For HPLC/ESI-MS, a *HP1100* instrument was coupled to the mass spectrometer specified above using identical gradient elution conditions.[44]

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Author Contribution Statement

H. B. acquired the tolypin and efrapeptin sample, established the analytical procedures, organised and guided the research and supervised the persons acknowledged above. Both authors evaluated the analytical data, prepared figures and tables, and wrote the paper.

References

[1] H. A. Lardy, 'Antibiotic inhibitors of mitochondrial energy transfer', *Pharmacol. Ther.* **1980**, *11*, 649 – 660.

[2] N. Kresge, R. D. Simoni, R. L. Hill, 'Unraveling the Enzymology of Oxidative Phosphorylation: the Work of *Efraim Racker*', *J. Biol. Chem.* **2006**, *281*, e4 – e6.

[3] P. E. Linnet, R. B. Beechey, 'Inhibitors of the ATP synthetase systems', *Methods Enzymol.* **1979**, *55*, 472 – 518.

[4] C. G. Jackson, P. E. Linnett, R. B. Beechey, P. J. F. Henderson, 'Purification and Preliminary Structural Analysis of the Efrapeptins, a Group of Antibiotics that Inhibit the Mitochondrial Adenosine Triphosphatase', *Biochem. Soc. Trans.* **1979**, *7*, 224 – 226.

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[5] H. Lardy, P. Reed, C.-H. Lin, 'Antibiotic Inhibitors of Mitochondrial ATP Synthesis', *Fed. Proc.* **1975**, *34*, 1707–1710.
[6] H. A. Lucero, R. A. Ravizzini, R. H. Vallejos, 'Inhibition of spinach chloroplasts photophosphorylation by the antibiotics leucinostatin and efrapeptin', *FEBS Lett.* **1976**, *68*, 141–144.
[7] J. E. Walker, 'ATP Synthesis by Rotary Catalysis (Nobel lecture)', *Angew. Chem. Int. Ed.* **1998**, *37*, 2308–2319; *Angew. Chem.* **1998**, *110*, 2438–2450.
[8] J. R. Gledhill, J. E. Walker, 'Inhibition sites in F_1-ATPase from bovine heart mitochondria', *Biochem. J.* **2005**, *386*, 591–598.
[9] A. E. Papathanassiu, N. J. MacDonald, A. Bencsura, H. A. Vu, 'F_1F_0-ATP synthase functions as a co-chaperone of Hsp90-substrate protein complexes', *Biochem. Biophys. Res. Commun.* **2006**, *345*, 419–429.
[10] D. A. Bullough, C. G. Jackson, P. J. F. Henderson, F. H. Cottee, R. B. Beechey, P. E. Linnett, 'The amino acid sequence of efrapeptin D', *Biochem. Int.* **1982**, *4*, 543–549.
[11] S. Gupta, S. B. Krasnoff, D. W. Roberts, J. A. A. Renwick, L. S. Brinen, J. Clardy, 'Structures of the efrapeptins: potent inhibitors of mitochondrial ATPase from the fungus *Tolypocladium niveum*', *J. Am. Chem. Soc.* **1991**, *113*, 707–709.
[12] S. Gupta, S. B. Krasnoff, D. W. Roberts, J. A. A. Renwick, L. S. Brinen, J. Clardy, 'Structure of efrapeptins from the fungus *Tolypocladium niveum*: peptide inhibitors of mitochondrial ATPase', *J. Org. Chem.* **1992**, *57*, 2306–2313.
[13] W. Gams, '*Tolypocladium*, eine Hyphomycetengattung mit geschwollenen Phialiden', *Persoonia* **1971**, *6*, 185–191.
[14] J. Bissett, 'Notes on *Tolypocladium* and related genera', *Can. J. Bot.* **1983**, *61*, 1311–1329.
[15] M. M. Dreyfuss, W. Gams, 'Proposal to reject *Pachybasium niveum* Rostr. in order to retain the name *Tolypocladium inflatum* W. Gams for the fungus that produces cyclosporin', *Taxon* **1994**, *43*, 660–661.
[16] W. Gams, 'Report of the Committee for Fungi: 6', *Taxon* **1996**, *45*, 309–311.
[17] J. P. Abrahams, S. K. Buchanan, M. J. van Raaij, I. M. Fearnley, A. G. Leslie, J. E. Walker, 'The structure of bovine F1-ATPase complexed with the peptide antibiotic efrapeptin', *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 9420–9424.
[18] S. B. Krasnoff, S. Gupta, R. J. St. Leger, J. A. A. Renwick, D. W. Roberts, 'Antifungal and insecticidal properties of the efrapeptins: metabolites of the fungus *Tolypocladium niveum*', *J. Invertebr. Pathol.* **1991**, *58*, 180–188.
[19] S. B. Krasnoff, S. Gupta, 'Identification and directed biosynthesis of efrapeptins in the fungus *Tolypocladium geodes* Gams (Deuteromycotina: Hyphomycetes)', *J. Chem. Ecol.* **1991**, *17*, 1953–1962.
[20] S. B. Krasnoff, S. Gupta, 'Efrapeptin production by *Tolypocladium* fungi (Deuteromycotina: Hyphomycetes): Intra- and interspecific variation', *J. Chem. Ecol.* **1992**, *18*, 1727–1740.
[21] M. Muroi, N. Kaneko, K. Suzuki, T. Nishio, T. Oku, T. Sato, A. Takatsuki, 'Efrapeptins Block Exocytic but Not Endocytic Trafficking of Proteins', *Biochem. Biophys. Res. Commun.* **1996**, *227*, 800–809.
[22] Y. Hayakawa, Y. Hattori, T. Kawasaki, K. Kanoh, K. Adachi, Y. Shizuri, K. Shin-ya, 'Efrapeptin J, a New Down-regulator of the Molecular Chaperone GRP78 from a Marine *Tolypocladium* sp.', *J. Antibiot.* **2008**, *61*, 365–371.
[23] C. M. Boot, K. Tenney, F. A. Valeriote, P. Crews, 'Highly *N*-Methylated Linear Peptides Produced by an Atypical Sponge-Derived *Acremonium* sp.', *J. Nat. Prod.* **2006**, *69*, 83–92.
[24] C. M. Boot, T. Amagata, K. Tenney, J. E. Compton, H. Pietraszkiewicz, F. A. Valeriote, P. Crews, 'Four classes of structurally unusual peptides from two marine-derived fungi: structures and bioactivities', *Tetrahedron* **2007**, *63*, 9903–9914.
[25] L. Du, A. L. Risinger, C. A. Mitchell, J. You, B. W. Stamps, N. Pan, J. B. King, J. C. Bopassa, S. I. V. Judge, Z. Yang, B. S. Stevenson, R. H. Cichewicz, 'Unique amalgamation of primary and secondary structural elements transform peptaibols into potent bioactive cell-penetrating peptides', *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E8957–E8966.
[26] Y. S. Tsantrizos, S. Pischos, F. Sauriol, P. Widden, 'Peptaibol metabolites of *Tolypocladium geodes*', *Can. J. Chem.* **1996**, *74*, 165–172.
[27] B. Kebede, S. K. Wrigley, A. Prashar, J. Rahlff, M. Wolf, J. Reinshagen, P. Gribbon, J. F. Imhoff, J. Silber, A. Labes, B. Ellinger, 'Establishing the Secondary Metabolite Profile of the Marine Fungus: *Tolypocladium geodes* MF458 and Subsequent Optimization of Bioactive Secondary Metabolite Production', *Mar. Drugs* **2017**, *15*, 84.
[28] M. Jost, J.-C. Greie, N. Stemmer, S. D. Wilking, K. Altendorf, N. Sewald, 'The First Total Synthesis of Efrapeptin C', *Angew. Chem. Int. Ed.* **2002**, *41*, 4267–4269; *Angew. Chem.* **2002**, *114*, 4438–4440.
[29] M. Jost, W. Weigelt, T. Huber, Z. Majer, J.-C. Greie, K. Altendorf, N. Sewald, 'Synthesis, and Structural and Biological Studies of Efrapeptin C Analogues', *Chem. Biodiversity* **2007**, *4*, 1170–1182.
[30] S. Weigelt, T. Huber, F. Hofmann, M. Jost, M. Ritzefeld, B. Luy, C. Freudenberger, Z. Majer, E. Vass, J.-C. Greie, L. Panella, B. Kaptein, Q. B. Broxterman, H. Kessler, K. Altendorf, M. Hollósi, N. Sewald, 'Synthesis and Conformational Analysis of Efrapeptins', *Chem. Eur. J.* **2012**, *18*, 478–487.
[31] A. D. Konar, E. Vass, M. Hollósi, Z. Majer, G. Grüber, K. Frese, N. Sewald, 'Conformational Properties of Secondary Amino Acids: Replacement of Pipecolic Acid by *N*-Methyl-L-alanine in Efrapeptin C', *Chem. Biodiversity* **2013**, *10*, 942–951.
[32] J. Weiser, J. S. Pillai, '*Tolypocladium cylindrosporum* [Deuteromycetes, *Moniliaceae*], a new pathogen of mosquito larvae', *Entomophaga* **1981**, *26*, 357–361.
[33] J. Weiser, 'Mosquito-killing activity of strains of *Tolypocladium cylindrosporum* and *T. niveum*', *Česká Mykol.* **1987**, *41*, 219–224.
[34] J. Weiser, V. Mat'ha, 'Tolypin, a new insecticidal metabolite of fungi of the genus *Tolypocladium*', *J. Invertebr. Pathol.* **1988**, *51*, 94–96.
[35] V. Mat'ha, J. Weiser, J. Olejníček, 'The effect of tolypin in *Tolypocladium niveum* crude extract against mosquito and blackfly larvae in the laboratory', *Folia Parasitol.* **1988**, *35*, 379–381.
[36] A. Jegorov, V. Mat'ha, T. Trnka, M. Černý, 'Enantiomeric separation of leucines', *J. High Resolut. Chromatogr.* **1990**, *13*, 718–720.

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[37] V. Mat'ha, A. Jegorov, M. Kiess, H. Brückner, 'Morphological alterations accompanying the effects of peptaibiotics, α-aminoisobutyric acid-rich secondary metabolites of filamentous fungi, on *Culex pipiens* larvae', *Tissue Cell* **1992**, *24*, 559–564.
[38] J. Weiser, V. Mat'ha, Z. Žižka, A. Jegorov, 'Ultrastructural changes in *Culex pipiens* larvae treated with tolypin, the insecticidal metabolite of *Tolypocladium inflatum* Gams (Deuteromycetes)', *Cytobios* **1992**, *69*, 179–186.
[39] A. R. Bandani, B. P. S. Khambay, J. L. Faull, R. Newton, M. Deadman, T. M. Butt, 'Production of efrapeptins by *Tolypocladium* species and evaluation of their insecticidal and antimicrobial properties', *Mycol. Res.* **2000**, *104*, 537–544.
[40] A. R. Bandani, B. Amiri, T. M. Butt, R. Gordon-Weeks, 'Effects of efrapeptin and destruxin, metabolites of entomogenous fungi, on the hydrolytic activity of a vacuolar type ATPase identified on the brush border membrane vesicles of *Galleria mellonella* midgut and on plant membrane bound hydrolytic enzymes', *Biochim. Biophys. Acta* **2001**, *1510*, 367–377.
[41] C. Dumas, M. Ravallec, V. Mat'ha, A. Vey, 'Comparative Study of the Cytological Aspects of the Mode of Action of Destruxins and Other Peptidic Fungal Metabolites on Target Epithelial Cells', *J. Invertebr. Pathol.* **1996**, *67*, 137–146.
[42] G. Nagaraj, M. V. Uma, M. D. Shivayogi, P. Balaram, 'Antimalarial Activities of Peptide Antibiotics Isolated from Fungi', *Antimicrob. Agents Chemother.* **2001**, *45*, 145–149.
[43] A. Jaworski, H. Brückner, 'New sequences and new fungal producers of peptaibol antibiotics antiamoebins', *J. Pept. Sci.* **2000**, *6*, 149–167.
[44] H. Brückner, S. Fox, T. Degenkolb, 'Sequences of Acretocins, Peptaibiotics Containing the Rare 1-Aminocyclopropanecarboxylic Acid, from *Acremonium crotocinigenum* CBS 217.70', *Chem. Biodiversity* **2019**, *16*, e1900276.
[45] A. Fredenhagen, L.-P. Molleyres, B. Böhlendorf, G. Laue, 'Structure Determination of Neoefrapeptins A to N: Peptides with Insecticidal Activity Produced by the Fungus *Geotrichum candidum*', *J. Antibiot.* **2006**, *59*, 267–280.
[46] H. Brückner, D. Becker, W. Gams, T. Degenkolb, 'Aib and Iva in the Biosphere: Neither Rare nor Necessarily Extraterrestrial', *Chem. Biodiversity* **2009**, *6*, 38–56.
[47] J. E. Elsila, M. P. Callahan, D. P. Glavin, J. P. Dworkin, H. Brückner, 'Distribution and Stable Isotope Composition of Amino Acids from Fungal Peptaibiotics: Assessing the Potential for Meteoritic Contamination', *Astrobiology* **2011**, *11*, 123–133.
[48] C. R. Röhrich, A. Iversen, W. M. Jaklitsch, H. Voglmayr, A. Vilcinskas, K. F. Nielsen, U. Thrane, H. von Döhren, H. Brückner, T. Degenkolb, 'Screening the Biosphere: the Fungicolous fungus *Trichoderma phellinicola*, a Prolific Source of Hypophellins, New 17-, 18-, 19-, and 20-Residue Peptaibiotics', *Chem. Biodiversity* **2013**, *10*, 787–812.
[49] C. R. Röhrich, W. M. Jaklitsch, H. Voglmayr, A. Iversen, A. Vilcinskas, K. F. Nielsen, U. Thrane, H. von Döhren, H. Brückner, T. Degenkolb, 'Front line defenders of the ecological niche! Screening the structural diversity of peptaibiotics from saprotrophic and fungicolous *Trichoderma/Hypocrea* species', *Fungal Divers.* **2014**, *69*, 117–146.
[50] K. T. Hodge, S. B. Krasnoff, R. A. Humber, '*Tolypocladium inflatum* is the anamorph of *Cordyceps subsessilis*', *Mycologia* **1996**, *88*, 715–719.
[51] C. A. Quandt, K. E. Bushley, J. W. Spatafora, 'The genome of the truffle-parasite *Tolypocladium ophioglossoides* and the evolution of antifungal peptaibiotics', *BMC Genomics* **2015**, *16*, 553.
[52] C. A. Quandt, R. M. Kepler, W. Gams, J. P. M. Araújo, S. Ban, H. C. Evans, D. Hughes, R. Humber, N. Hywel-Jones, Z. Li, J. J. Luangsa-ard, S. A. Rehner, T. Sanjuan, H. Sato, B. Shrestha, G.-H. Sung, Y.-J. Yao, R. Zare, J. W. Spatafora, 'Phylogenetic-based nomenclatural proposals for *Ophiocordycipitaceae* (Hypocreales) with new combinations in *Tolypocladium*', *IMA Fungus* **2014**, *5*, 121–134.
[53] A. E. Papathanassiu, N. J. MacDonald, D. R. Emlet, H. A. Vu, 'Antitumor activity of efrapeptins, alone or in combination with 2-deoxyglucose, in breast cancer *in vitro* and *in vivo*', *Cell Stress Chaperones* **2011**, *16*, 181–193.

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