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20-Residue and 11-residue peptaibols from the fungus *Trichoderma longibrachiatum* are synergistic in forming Na^+/K^+-permeable channels and adverse action towards mammalian cells

Raimo Mikkola[1,*], Maria A. Andersson[1,*], László Kredics[2], Pavel A. Grigoriev[1,3], Nina Sundell[1] and Mirja S. Salkinoja-Salonen[1]

1 Department of Food and Environmental Science, University of Helsinki, Finland
2 Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Hungary
3 Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Russia

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Correspondence
M. S. Salkinoja-Salonen, Microbiology, Department of Food and Environmental Science, University of Helsinki, PO Box 56, Helsinki FI-00014, Finland
Fax: +358 9 19159301
Tel: +358 40 5739049
E-mail: mirja.salkinoja-salonen@helsinki.fi

*These authors contributed equally to this work.

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Certain species of the filamentous fungal genus *Trichoderma* (e.g. *Trichoderma longibrachiatum* and *Trichoderma citrinoviride*) are among the emerging clinical pathogens and also the most common species in the indoor space of mould-damaged buildings. The molecules involved in its pathology are not known. In the present study, we report that 0.5–2.6 wt% of the *T. longibrachiatum* mycelial biomass consisted of thermostable secondary metabolites mitochondriotoxic to mammalian cells. These were identified by LC/MS as one 11-residue and eight 20-residue peptaibols, AcAib-Asn-Leu/Ile-Leu/Ile-Aib-Pro-Leu/Ile-Leu/Ile-Aib-Pro-Leuol/Ileol (1175 Da) and AcAib-Ala-Aib-Ala-Aib-Ala/Aib-Gln-Aib-Val/Iva-Aib-Gly-Leu/Ile-Aib-Pro-Val/Iva-Aib-Val/Iva/Aib-Gln/Glu-Gln-Pheol(1936–1965 Da) (Aib, α-aminoisobutyric acid; Ac, acetyl; Ileol, isoleucinol; Iva, isovaline; Leuol, leucinol; Pheol, phenylalaninol). The toxic effects on boar sperm cells depended on these peptaibols, named trilongins. The trilongins formed voltage dependent, Na^+/K^+ permeable channels in biomembranes. The permeability ratios for Na^+ ions, relative to K^+, of the 11-residue trilongin channel (0.95 : 1) and the 20-residue trilongin channel (0.8 : 1) were higher than those of alamethicin. The combined 11-residue and 20-residue trilongins generated channels that remained in an open state for a longer time than those formed by either one of the peptaibols alone. Corresponding synergy was observed in toxicokinetics. With 11-residue and 20-residue trilongins combined 1 : 2 w/w, an effective median concentration (EC_{50}) of 0.6 $\mu g \cdot mL^{-1}$ was reached within 30 min, and the EC_{50} shifted down to 0.2 $\mu g \cdot mL^{-1}$ upon extended exposure. By contrast, with 11-residue or 20-residue trilonging separately in 30 min of exposure, the EC_{50} values were 15 and 3 $\mu g \cdot mL^{-1}$, respectively, and shifted down to 1.5 and 0.4 $\mu g \cdot mL^{-1}$ upon extended exposure. This is the first report on ion-channel forming peptaibols with synergistic toxicity from *T. longibrachiatum* strains isolated from clinical samples.

Database

- Nucleotide sequence data have been deposited in the GenBank database under accession numbers HQ593512 and HQ593513.

Abbreviations

$\Delta\Psi_m$, mitochondrial transmembrane potential; Ac, acetyl; Aib, α-aminoisobutyric acid; BLM, black lipid membrane; EC_{50}, effective median concentration; FIC, fractional inhibitory concentration; ITS, internal transcribed spacer; MEA, malt extract agar; Pheol, phenylalaninol; TSA, tryptic soy agar.

Introduction

Filamentous fungi from the genus *Trichoderma* (Ascomycota, Hypocreales) are well known as producers of industrial enzymes, especially cellulases [1–3]. Certain members of the genus are included among the promising biocontrol agents as a result of their antagonistic activities against plant pathogenic fungi [4]. In addition, *Trichoderma* strains are also known rarely to cause opportunistic infections in humans, varying from localized to fatal disseminated diseases in particular risk populations, including patients undergoing peritoneal dialysis, transplant recipients and patients with haematological malignancies [5]. Possible sources of infection include water-related sites, air, foods and catheters. Based on the extensive review of Kredics *et al*. [5], nine species from the genus *Trichoderma* (*Trichoderma longibrachiatum*, *Trichoderma citrinoviride*, *Trichoderma pseudokoningii*, *Trichoderma reesei*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma atroviride*, *Trichoderma viride*) have been previously reported from clinical cases. However, several clinical isolates originally identified based on their morphological characters were recently reidentified by sequence-based molecular techniques as *T. longibrachiatum*, which thus proved to be the most frequently occurring, almost exclusive clinical aetiological agent within the genus *Trichoderma* [6,7]. Therefore, it was suggested that the biotechnological and agricultural application of *T. longibrachiatum* should be avoided or at least carefully monitored to minimize possible health risks.

Trichoderma species were reported to be among the dominating microfungi in the indoor environments of water-damaged buildings [8,9]. Their possible association with building-related symptoms of ill health has been suggested [10–12]; however, because a causal relationship could not be established, their actual degree of contribution is yet unknown. The *Trichoderma* species detected in such environments include the clinically relevant species *T. longibrachiatum*, which, along with the closely-related species *T. citrinoviride*, may represent almost half of the *Trichoderma* isolates from building materials [8].

Peptaibiotics represent a constantly growing group of peptide antibiotics with increased interest as a result of their unique bioactivities and conformations [13–17]. They are defined as linear or cyclic polypeptide antibiotics of 4–21 amino acid residues that are characterized by a molecular masses in the range between 500 and 2200 Da, a high α-aminoisobutyric acid (Aib) content, the presence of other non-proteinogenic amino- or lipoamino acids, an acylated N-terminus, and (if linear) a C-terminal residue mostly consisting of a free or acetylated amide-bonded 2-amino alcohol [14]. The subgroup comprises Aib-containing peptides carrying a C-terminal 2-amino alcohol residue, which are referred to as peptaibols [17]. The first report of an Aib-containing antibiotic from the genus *Trichoderma*, compound U-22324 (later renamed as alamethicin), was published in 1967 [18]. Subsequently, it was revealed that the first peptaibol isolated from a *Trichoderma* sp. was actually suzukacillin from '*T. viride*' 63 C-I [19]; however, the presence of Aib in the SZ-hydrolysate was confirmed only 6 years later [20]. The producer strain NRRL 3199 originally identified as *T. viride* was recently reidentified as *Trichoderma arundinaceum*, a member of the *Trichoderma brevicompactum* clade [21], and all other alamethicin-producing *Trichoderma* species (*T. brevicompactum*, *Trichoderma protrudens*, *Trichoderma turrialbense*) also belong to the so-called 'Brevicompactum clade' [14,22]. The occurrence of several peptaibol compounds has been reported also from *Trichoderma* strains belonging to the clinically relevant species *T. longibrachiatum*. These included tricholongins [23], longibrachins [24], trichobrachins [25,26] and trichorovin [25]. However, one of the producer isolates, '*T. longibrachiatum*' CBS 936.69 was recently reclassified as *Trichoderma ghanense* [14] and, until now, only the identities of trichobrachin- and trichorovin-producing *T. longibrachiatum* strains were confirmed by phylogenetic data.

Crude extracts of various *T. longibrachiatum* isolates have been reported to contain thermostable substances that inhibited motility of boar spermatozoa and quenched the mitochondrial transmembrane potential ($\Delta\Psi_m$) of the sperm cells at low exposure concentrations [27]. In the present study, we describe the isolation, structure, toxicity and ion channel-forming activities, as well as synergistic properties, of two different sizes of peptaibols produced by *T. longibrachiatum* isolates originating from agricultural and clinical samples, and also from an indoor environment where serious building-related symptoms of ill health were claimed.

Results

Cell free extracts of *T. longibrachiatum* strains were toxic to porcine sperm cells

Cell extracts of *T. longibrachiatum* isolates (Table 1) originating from clinical ($n = 2$), terrestrial ($n = 3$) and sick building samples ($n = 3$) were assayed for the presence of substances toxic to mammalian cells.

Table 1. Fungal strains examined during the present study, origins and ITS sequence used for identification.Collections: CBS, Centraalbureau voor Schimmelcultures, Utrecht NL; CECT, Spanish type culture collection; CNM, mycological collection of the Spanish National Centre for Microbiology; DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; IMI CABI Bioscience, Egham UK.

Strain codes		ITS sequence	
Trichoderma longibrachiatum	Alternate codes and origin	GenBank accession number	Reference
Thb	Moisture-damaged residence, Finland	HQ593512	Present study
Thd	Moisture-damaged residence, Finland	HQ593513	Present study
SzMCThg	Moisture-damaged residence, Finland	EU401573	TU Vienna code C.P.K.1698 [6]
CNM-CM 2171	C.P.K. 1696, foot skin of premature infant with subcutaneous lesions, fatal, Spain	AY920397	[6]
CNM-CM 2277	C.P.K. 2277, sputum of tuberculosis patient, Spain	AY920398	[6]
IMI 291014	C.P.K. 1303; soil, Antarctica	EU401560	[6]
CECT 2412	C.P.K. 2062; CNM-CM 1698; mushroom compost, Wales UK	EU401572	[6]
CECT 20105	C.P.K. 1698; IMI 297702; CNM-CM 1698, biocontrol strain, Egypt	AY585880	[6]
Reference strains			
Trichoderma reesei DSM 768	Synonym *T. reesei* Simmons, *T. viride* QM6a; Cotton canvas, Solomon Islands, Bouganville.		[2,3]
Trichoderma harzianum ES39	Ceiling of a residence, after renovation of Moisture damage, Helsinki, Finland	AY585881	[11]; present study
Acremonium tubakii CBS 110649[a]	Reed sandy soil		[47]

[a] This strain is mistakenly referred to as CBS110360 by Andersson *et al.* [47].

Internal transcribed spacer (ITS) sequences confirmed the identity of the strains as *T. longibrachiatum* (Table 1). Boar sperm cells were used as toxicity indicator cells. The cell free extracts (prepared by heating in methanol at 100 °C) destroyed several cellular functions of boar sperm cells: motility, inner $\Delta\Psi_m$ and cell membrane permeability barrier to propidium iodide (Table 2). The effective median concentration (EC_{50}) was 3–6 μg of the methanol-soluble substance·mL^{-1}. Corresponding extracts from *T. longibrachiatum* DSM 768 or from *Acremonium tubakii* (strain CBS 110649) showed no toxicity up to concentrations ten-fold higher. The eight toxic *T. longibrachiatum* strains were cultivated on tryptic soy agar (TSA), brain heart infusion and malt extract agar (MEA) at 22 °C and at 37 °C to optimize growth and toxin production. The growth for all strains was optimal on MEA at 22 °C and at 37 °C, although the production of toxin was higher at room temperature 22 °C. Toxicity of the extracts of Thb and Thd decreased by a factor two to four when the extracted biomass was cultivated at 37 °C (Table 2). The toxicity of the extracts increased (by factor of four) when incubation was extended from 5 to 15 days. The toxic substances of *T. longibrachiatum* strains were resistant to heat (10 min at 100 °C).

The toxic substances of *T. longibrachiatum* were 20-residue and 11-residue peptaibols

Toxic cell extract of *T. longibrachiatum* strain Thb was fractionated with HPLC. Five peaks in the HPLC elution profile (215 nm) of the Thb extract inhibited the motility of boar spermatozoa (labelled A1–A5 in Fig. 1A). Similarly, fractionated alamethicin (A4665) consisted of four alamethicin F50 peptaibols, with molecular masses of 1962, 1976, 1976 and 1990 Da (labelled B1–B4 in Fig. 1B). HPLC-MS analysis of the toxic fractions A2–A5 of strain Thb extract (Fig. 1A) showed the doubly-charged cationized molecules $[M+2Na]^{2+}$ at *m/z* 991.5 (16.6 min; peak A2), 998.6 (18.8 min; peak A3), 998.5 (22.1 min; peak A4) and 1005.6 (25.8 min; peak A5) and the corresponding triply-charged cationized molecules $[M+3Na]^{3+}$ at *m/z* 668.9, 673.6, 673.6 and 678.1 (Fig. 1C–F). Negatively-charged unprotonated molecules $[M-2H]^{2-}$ at *m/z* 967.6, 974.7, 974.7 and 981.5 were observed in peaks A2–A5, respectively. These experimental values fitted

Table 2. The toxic activities towards porcine spermatozoa by extracts of the fungus *T. longibrachiatum* and reference strains. The cell extracts were prepared from mycelial biomass grown on MEA at 22 °C for 5 days. The toxicity endpoints EC_{50} indicate methanol-soluble substances (μg dry wt·mL^{-1}). Depolarization of mitochondria was recorded by epifluoresence microscopy after staining with the membrane potential sensitive dye JC-1. Exposure time (h).

	Motility inhibition		Depolarization of mitochondria		Relaxed permeability barrier of cell membrane to propidium iodide	
	24 h	72 h	24 h	72 h	24 h	72 h
Strain	EC_{50} (μg dry wt·mL^{-1})					
Cell extracts of *T. longibrachiatum*						
From indoor isolates						
Thb	6 (25)[a]	3 (12)[a]	6	3	6	3
Thd	12 (25)[a]	3 (12)[a]	12	3	12	3
SzMCthg	6	3	6	3	6	3
From clinical isolates						
CNM-CM 2171	12	6	12	6	12	6
CNM-CM 2277	6	3	6	3	6	3
From environmental isolates						
IMI 291014	6	2	12	3	12	3
CECT 2412	6	2	6	2	12	6
CECT 20105	6	3	2	3	12	3
DSM 768	> 100	> 50				
Cell extracts of reference strains						
Trichoderma harzianum ES39	4	2	4	2	4	2
Acremonium tubakii CBS 110649	> 100	50	> 100	100	> 100	100
Reference toxin						
Alamethicin	0.15	0.08	0.15	0.08	0.15	0.08

[a] Toxicity endpoint of extracts (in parentheses) indicate a situation where strains were grown on MEA at 37 °C for 5 days.

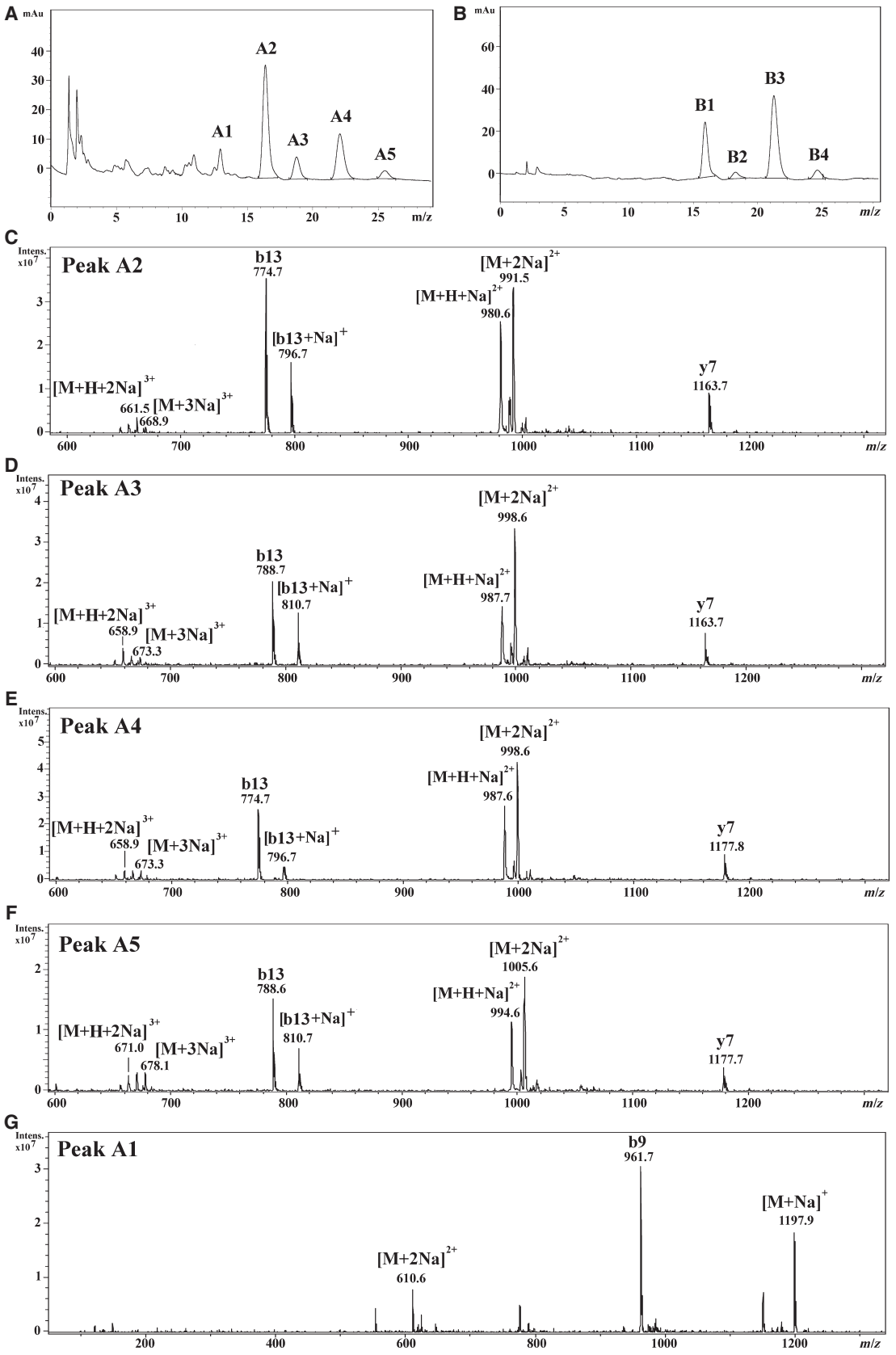
the calculated monoisotopic masses of 1936.1 Da (peak A2), 1950.1 Da (peak A3), 1950.1 Da (peak A4) and 1964.2 Da (peak A5).

MS/MS analysis of y7 ions at *m/z* 788 and *m/z* 774 and the MS^3 analysis of the mass ion *m/z* 624 (y6) produced y-series fragments revealing residues 16–20 and showed that the C-terminus contained phenylalaninol (Pheol) (Fig. 2B,C). MS/MS analysis of b13 ion at *m/z* 1163 of peak A2 (Fig. 2A) and the MS^3 analysis of the mass ion at *m/z* 440 (b5) produced b-series fragments showing that the N-terminus contained an acetyl group (Ac) and the revealed residues 1–13. MS/MS analysis of the doubly-charged $[M+2Na]^{2+}$ ion at *m/z* 991 confirmed residues 16–20 and 4–13 (Fig. 2D). Because the fragment ion 196 Da (sequence between 14 and 15) matched with the cleavage of Pro-Vxx and, knowing that the bond between the complementary ion pairs Aib and Pro is weak [28], it was concluded that amino acid sequence 14–15 was Pro-Vxx.

The diagnostic fragment ions of the above MS analysis of peptaibols are reported in Table 3. The conclusion based on the results presented above is that the compounds eluting as peaks A2–A5 in Fig. 1A were 20-residue peptaibols with an acetylated α-aminoisobutyric acid at the N-terminus and Pheol at the C-terminus. We named these peptaibols trilongins BI, BII, BIII and BIV, respectively. Their sequences were closely similar to one another, with differences only being only at position 6 (Ala or Aib) and at position 17 (Vxx) or Aib).

Peak A1 (13 min) was also toxic to boar sperm cells. It contained a compound that formed doubly-charged cationized molecules $[M+2Na]^{2+}$ at *m/z* 610.6 and a single-charged $[M+Na]^{+}$ at *m/z* 1197.9, corresponding to

Fig. 1. HPLC-UV and HPLC-MS analysis of peptaibols produced by *Trichoderma longibrachiatum* Thb. (A) HPLC-UV (215 nm) chromatograms of methanol extract from strain Thb and methanol solution of alamethicin (B). (C) Doubly-charged sodiated molecular ions at *m/z* 991, b13 ion at *m/z* 774 and y7 ion at *m/z* 1163 of peak A2 from (A). (D–F) The corresponding ions of peaks A2–A5 from (A). (G) Doubly-charged sodiated molecular ion at *m/z* 1197 and b9 ion at *m/z* 961 of peak A1 from (A).


A
mAu
A1
A2
A3
A4
A5
m/z
B
mAu
B1
B2
B3
B4
m/z
C
Peak A2
b13
774.7
[M+2Na]2+
991.5
[M+H+Na]2+
980.6
[b13+Na]+
796.7
y7
1163.7
[M+H+2Na]3+
661.5
[M+3Na]3+
668.9
D
Peak A3
[M+2Na]2+
998.6
b13
788.7
[b13+Na]+
810.7
[M+H+Na]2+
987.7
y7
1163.7
[M+H+2Na]3+
658.9
[M+3Na]3+
673.3
E
Peak A4
[M+2Na]2+
998.6
b13
774.7
[M+H+Na]2+
987.6
[M+H+2Na]3+
658.9
[M+3Na]3+
673.3
[b13+Na]+
796.7
y7
1177.8
F
Peak A5
[M+2Na]2+
1005.6
b13
788.6
[M+H+Na]2+
994.6
[b13+Na]+
810.7
[M+H+2Na]3+
671.0
[M+3Na]3+
678.1
y7
1177.7
G
Peak A1
b9
961.7
[M+Na]+
1197.9
[M+2Na]2+
610.6
m/z

the molecular mass of 1174.9 Da shown in Fig. 1G. MS/MS analysis using m/z 610.6 as the precursor ion revealed the sequence Lxx-Lxx-Aib-(Pro-Lxx)-Lxx-Aib-Pro-Lxxol (Fig. 2E). The remaining mass ion at m/z 264 matched the sodium adduct of the residue AcAib-Asn. In MS/MS analysis of the mass ion m/z 962 (Fig. 1G), corresponding to the acylium ion b9, the sequence of Lxx-Aib-Pro-Lxx-Lxx-Aib was found (Fig. 2F). The deduced amino acid sequence showed that this compound was a peptaibol containing 11 residues with an acetylated N-terminus and Lxxol as the C-terminus. HPLC-MS analysis showed that *T. longibrachiatum* strains also contained another 11-residue peptaibols with sodiated mass ions at m/z 1155, 1169, 1183 and 1211. The HPLC fractions containing these peptaibols showed no toxicity in the boar sperm assay. The sequences of these 11-residue peptaibols were determined by LC-MS/MS analysis using the double-charged $[M+2Na]^{2+}$ ions as precursor ions. MS/MS analysis of the precursor ions that gave the b ion series is shown in Table 4. The conclusion based on the above MS data is that the toxic peak A1 of Fig. 1A was a peptaibol with a mean molecular weight of 1175.5 Da and an amino acid sequence of AcAib-Asn-Lxx-Lxx-Aib-Pro-Lxx-Lxx-Aib-Pro-Lxxol. It was named trilongin AI (Table 5). The sequences and the identical or positionally isomeric compounds of the 11-residue peptaibols (named trilongins A0–AIV) are shown in Table 5.

Diversity of peptaibols among the toxigenic *T. longibrachiatum* strains

The three toxigenic indoor *T. longibrachiatum* isolates, Thb, Thd and SzMCThg (Table 1), produced the same 11-residue and 20-residue trilongins A0–AIV and BI–BIV. When the clinical and environmental isolates of *T. longibrachiatum* (IMI 291014, CECT 20105, CNM–CM 2277, CECT 2412 and CNM–CM; Table 1) were analyzed with LC/MS, four additional 20-residue peptaibols were found. These new peptaibols contained y7 ions 1 Da higher, m/z 775 and 789 than the corresponding y7 ions (m/z 774 and 788) of trilongins BI–BIV. These were named trilongins CI, CII, CIII and CIV. MS/MS analysis of y7 ions of the 20-residue peptaibols CI–CIV (Table 3) revealed amino acid sequences resembling those of the y7 ions of trilongins BI–BIV, except from position 18 where Glu was substituted with Gln (Table 5). Trilongins CI–CIV also varied also at cposition 6 (Ala or Aib) and at position 17 (Vxx or Aib), similar to trilongins BI–BIV (Table 5). MS/MS analysis of b13 ions of trilongins CI–CII at m/z 1163 and CIII–CIV at m/z 1177 showed that the fragmentions were identical to the corresponding fragmentations of trilongins BI–BII (at m/z 1163) and BIII–BIV (at m/z 1177) (Table 3). The deduced amino acid sequences of trilongins BI–BIV and CI–CIV (Table 5) are based on the MS/MS analyses using y7 ions, b13 ions and doubly-charged $[M+2Na]^{2+}$ sodiated molecules as the precursor ions. Trilongins CIII and CIV show the new sequences (Table 5). The HPLC-MS elution profile of the peptaibols observed in the methanol extract of *T. longibrachiatum* strain CECT 20105 is shown in Fig. 3. The sequences and retention times of the 11- and 20-residue peptaibols found are provided in Table 6. Table 7 compiles the contributions of the different 20-residue trilongins, BI–BIV and CI–CIV, in the *T. longibrachiatum* strains.

Quantification of peptaibols

The fragmentation patterns of alamethicin F50 were similar to those of trilongins BI–BIV and CI–CIV and contained y7 ion at m/z 774. Therefore, y7 ion of alamethicin at m/z 774 and the corresponding y7 ions, m/z 774, 775, 788 and 789 of the 20-residue trilongins BI–BIV and CI–CIV were used for the quantifications. The quantification of trilongin AI was performed by monitoring A_{215} and alamethicin as a reference.

The concentrations of the eight 20-residue trilongins BI–BIV and CI–CIV and the 11-residue trilongin AI in the methanol extracts of *T. longibrachiatum* strains are shown in Table 8. Of the total harvested biomass, 10–20% (w/w) was methanol-soluble. The 20-residue peptaibols in the different strains contributed to 5–13 wt% of the methanol-soluble matter and the 11-residue peptaibol contributed to 0.2–0.8 wt%. The toxic peptaibols thus made up 0.5–2.6 wt% of the harvested mycelial biomass (320 ± 20 mg per Petri dish of diameter 90 mm) of the investigated *T. longibrachiatum* isolates. One fully grown culture dish thus contained 1500–8800 μg of the toxic peptaibols.

Fig. 2. MS/MS fragmentation patterns and amino acid sequences of peptaibols found in methanol extract of *T. longibrachiatum* Thb. (A) The amino acid sequence of y7 ion at *m/z* 1163 (Fig. 1C, peak A2). The sequences of b13 ions at m/z 774 (Fig. 1C, peak A2) and 788 (Fig. 1D, peak A3) are shown in (B) and (C), respectively. (D) The sequence of doubly-charged sodiated molecular ions at *m/z* 992 (Fig. 1C, peak A2). The sequences of doubly-charged sodiated molecules at *m/z* 611 and b9 ion at *m/z* 961 (Fig. 1G, peak A1) are shown in (E) and (F), respectively.

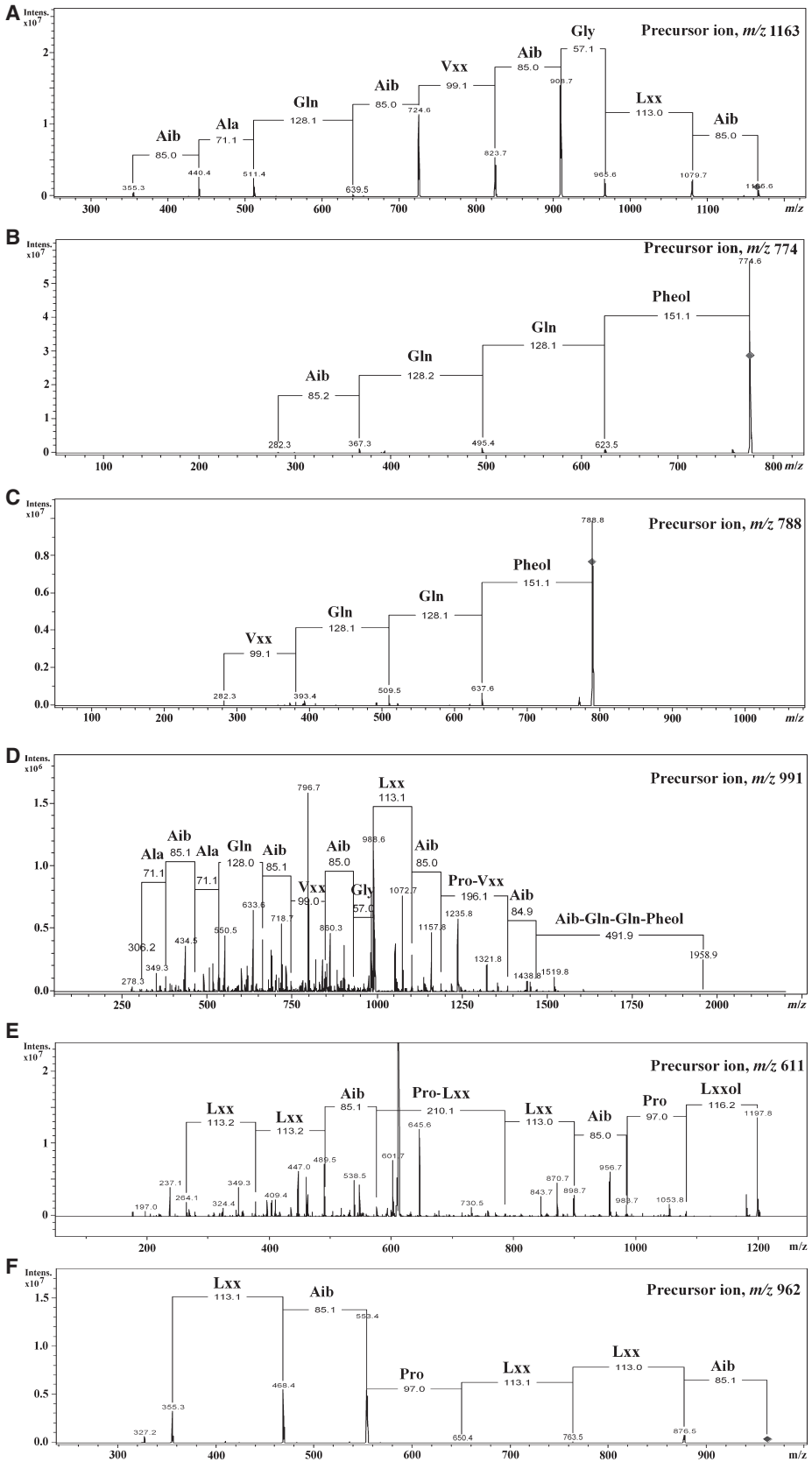

A
Intens. x10^7
Precursor ion, m/z 1163
Aib 85.0
Ala 71.1
Gln 128.1
Aib 85.0
Vxx 99.1
Aib 85.0
Gly 57.1
Lxx 113.0
Aib 85.0
355.3
440.4
511.4
639.5
724.6
823.7
908.7
965.6
1079.7
1166.6
m/z
B
Intens. x10^7
Precursor ion, m/z 774
Aib 85.2
Gln 128.2
Gln 128.1
Pheol 151.1
282.3
367.3
495.4
623.5
774.6
m/z
C
Intens. x10^7
Precursor ion, m/z 788
Vxx 99.1
Gln 128.1
Gln 128.1
Pheol 151.1
282.3
393.4
509.5
637.6
788.8
m/z
D
Intens. x10^6
Precursor ion, m/z 991
Ala 71.1
Aib 85.1
Ala 71.1
Gln 128.0
Aib 85.1
Vxx 99.0
Aib 85.0
Gly 57.0
Lxx 113.1
Aib 85.0
Pro-Vxx 196.1
Aib 84.9
Aib-Gln-Gln-Pheol 491.9
278.3
306.2
349.3
434.5
550.5
633.6
718.7
796.7
850.3
988.6
1072.7
1157.8
1235.8
1321.8
1438.8
1519.8
1958.9
m/z
E
Intens. x10^7
Precursor ion, m/z 611
Lxx 113.2
Lxx 113.2
Aib 85.1
Pro-Lxx 210.1
Lxx 113.0
Aib 85.0
Pro 97.0
Lxxol 116.2
197.0
237.1
264.1
324.4
349.3
409.4
447.0
489.5
538.5
601.7
645.6
730.5
843.7
870.7
898.7
956.7
983.7
1053.8
1197.8
m/z
F
Intens. x10^7
Precursor ion, m/z 962
Lxx 113.1
Aib 85.1
Pro 97.0
Lxx 113.1
Lxx 113.0
Aib 85.1
327.2
355.3
468.4
553.4
650.4
763.5
876.5
m/z

Table 3. The $[M+Na]^+$ and $[M+2Na]^{2+}$ ions of trilongins BI–BIV and CI–CIV and the diagnostic fragment mass ions of b13 and y7 series ions observed by MS/MS and MS^3 analysis.

	Trilongin							
	BI	BII	BIII	BIV	CI	CII	CIII	CIV
Diagnostic ions	*m/z*							
[M+Na]+	1958	1972	1972	1986	1959	1973	1973	1987
$[M+2Na]^{2+}$	991	998	998	1005	992	999	999	1006
b13	1163	1163	1177	1177	1163	1163	1177	1177
b12	1078	1078	1092	1092	1078	1078	1092	1092
b11	965	965	979	979	965	965	979	979
b10	908	908	922	922	908	908	922	922
b9	823	823	837	837	823	823	837	837
b8	724	724	738	738	724	724	738	738
b7	639	639	653	653	639	639	653	653
b6	511	511	525	525	511	511	525	525
b5	440	440	440	440	440	440	440	440
b4	355	355	355	355	355	355	355	355
b3	284	284	284	284	284	284	284	284
b2	199	199	199	199	199	199	199	199
b1	128	128	128	128	128	128	128	128
y7	774	788	774	788	775	789	775	789
y6	623	637	623	637	624	638	624	638
y5	495	509	495	509	496	510	496	510
y4	367	381	367	381	367	381	367	381
y3	282	282	282	282	282	282	282	282
y2	197	197	197	197	197	197	197	197

Table 4. The $[M+Na]^+$,$[M+2Na]^{2+}$ mass ions and the b series mass ions (*m/z*) obtained from MS/MS analysis of $[M+2Na]^{2+}$ mass ions of 11-residue peptaibols of *T. longibrachiatum* strains.

			b Series mass ions								
11-residue peptaibol	$[M+Na]^+$	$[M+2Na]^{2+}$	b10	b9	b8	b7	b6	b5	b4	b3	b2
Trilongin AIV a	1155	589	1039	941	856	743	644	547	462	363	264
Trilongin AIV b	1155	589	–	956	870	757	644	547	462	363	264
Trilongin AIV c	1155	589	1039	941	856	757	644	547	462	363	264
Trilongin AIII a	1169	596	1067	969	884	771	–	561	476	377	264
Trilongin AIII b	1169	596	1053	956	870	757	–	561	476	377	264
Trilongin AIII c	1169	596	1067	969	884	771	–	561	476	363	264
Trilongin AIII d	1169	596	1053	956	870	757	–	561	476	363	264
Trilongin AII a	1183	603	1067	969	884	771	–	561	476	363	264
Trilongin AII b	1183	603	1081	983	898	785	–	575	490	377	264
Trilongin AII c	1183	603	1067	969	884	785	672	575	490	377	264
Trilongin AII d	1183	603	1067	969	884	771	672	575	490	377	264
Trilongin AII e	1183	603	1067	969	884	771	–	561	476	377	264
Trilongin AI	1197	610	1080	983	898	785	672	575	489	377	264
Trilongin A0	1211	617	1095	998	913	800	687	589	504	391	278

Toxicity of the purified 20-residue and 11-residue trilongins

Toxicities were measured using boar spermatozoa motility inhibition as the toxicity indicator, separate from the 20-residue trilongins BI–BIV, 11-residue trilongin AI and a combination of trilongins (BI–BIV plus AI) in a mass ratio of 2 : 1. As shown in Table 7, the EC_{50} of 20-residue trilongins BI–BIV decreased from 3 to 0.4 $\mu g \cdot mL^{-1}$ upon extended exposure, whereas the EC_{50} of 11-residue trilongin AI decreased from 15 to 1.5 μg

Table 5. Amino acid sequences of trilongins A0–AIV, BI–BIV and CI–CIV produced by *T. longibrachiatum* strains and alamethicin (Alm).Ileol, isoleucinol; Iva, isovaline; Vxx, Val/Iva; Lxx: Leu/Ile; Lxxol, Leuol/Ileol; Vxxol, Valol/Ivaol.

	Sequence																					
Peptaibol	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Identical or positionally isomeric compound	Reference
Trilongin AIV a	AcAib	Asn	Vxx	Vxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxxol										Trichobrachin A-VII j	[26]
																					TV29-11-I d	[29]
Trilongin AIV b	AcAib	Asn	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol										Trichobrachin A-VII c	[26]
																					Trichobrachin III-9e	[29]
																					TV29-11-I b	[29]
																					Hypojecorin A 1	[30]
Trilongin AIV c	AcAib	Asn	Vxx	Vxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Lxxol										Trichobrachin A-VII i	[26]
Trilongin AIII a	AcAib	Asn	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol										Trichobrachin A-IV	[26]
																					Trichorovin TV-IIb	[26]
																					Trichobrachin III-3b	[29]
																					TV29-11-II a	[29]
																					Hypojecorin A 5	[30]
Trilongin AIII b	AcAib	Asn	Lxx	Vxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxxol										Trichobrachin A-IVd	[26]
																					TV29-11-II f	[29]
Trilongin AIII c	AcAib	Asn	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol										Trichobrachin A-III	[26]
																					Trichorovin TV-Ia	[26]
																					Trichobrachin III-2b	[29]
																					TV29-11-II b	[29]
																					Hypojecorin A 3	[30]
Trilongin AIII d	AcAib	Asn	Vxx	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxxol										Trichobrachin A-IVc	[26]
Trilongin A II a	AcAib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol										Trichobrachin A-VIII a	[26]
																					Trichorovins TV-Vb/VIb	[26]
																					Trichorozin I	[26]
																					Trichobrachins III- 8b/9c	[30]
																					Hypojecorin A 6	[30]
Trilongin AII b	AcAib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Lxxol										Trichobrachin A-VIII d	[26]
Trilongin AII c	AcAib	Asn	Lxx	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxxol										Trichobrachin A-VIII e	[26]
																					Harzianin HB 1	[26]
Trilongin AII d	AcAib	Asn	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol										Trichobrachin A-VIII b	[26]
																					Trichorovin TV-VIIa	[26]
																					TV29-11-II a	[29]
																					Trichobrachins III-7b/8a/9a	[29]
																					Hypojecorin A 12	[30]
Trilongin AII e	AcAib	Asn	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol										Trichobrachin A-VIII c	[26]
																					Trichorovin TV-Va	[26]
Trilongin AI	AcAib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol										Trichobrachin A-IX	[26]
																					Harzianin HK-VI	[26]

Table 5. (Continued).

Peptaibol	Sequence 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Identical or positionally isomeric compound	Reference
																					Trichorovins TV-XI/XII-a/b	[26]
																					Trichorozin III	[26]
																					Trichobrachins II-Fa/Ga/Gb/Ha	[30]
																					Hypojecorins A 15/16	[30]
Trilongin A0	AcAib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol										Trichobrachin C-I/C-II	[26]
																					Trichorovin TV-XIII	[26]
																					Trichorozin IV	[26]
																					Hypomurocins A-V/Va	[30]
																					Trichobrachins III-16a/17/18	[29]
																					TV29-11-V b	[29]
																					Trichobrachins III- I/J	[30]
																					Hypojecorins A 17/18	[30]
Trilongin BI	AcAib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol	Gliodeliquescin A	[31]
																					Trichoaureocin 3	[32]
																					Trichobrachins II-5/6	[33]
																					Longibrachin A I	[24]
																					Trichokonin VI	[34]
Trilongin BII	AcAib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol	Trichoaureocin 4	[32]
																					Suzukacillin 10a	[35]
																					Trichobrachins II-7/8/9	[33]
																					Longibrachin A II	[24]
																					Trichokonin VII	[34]
Trilongin BIII	AcAib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol	Trichoaureocin 5	[32]
																					Trichosporin B-IVc	[36]
																					Trichobrachin II-10	[33]
																					Longibrachin A III	[24]
																					Trichokonin VIII	[34]
Trilongin BIV	AcAib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol	Trichoaureocin 6	[32]
																					Longibrachin A IV	[24]
Trilongin CI	AcAib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Glu	Gln	Pheol	Longibrachin B II	[24]
Trilongin CII	AcAib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Glu	Gln	Pheol	Longibrachin B III	[24]
Trilongin CIII	AcAib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Glu	Gln	Pheol	New	Present study

Table 5. (Continued).

Peptaibol	Sequence																			Identical or positionally isomeric compound	Reference
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Trilongin ClV	AcAib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Glu	Gln	Pheol	New	Present study
Alm F50/5	AcAib	Pro	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol		[37]
Alm F50/6a	AcAib	Pro	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Val	Gln	Gln	Pheol		[37]
Alm F50/6b,7,8a	AcAib	Pro	Aib	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol		[37]
Alm F50/8b	AcAib	Pro	Aib	Aib	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol		[37]

mL^{-1}. The EC_{50} of trilongins (BI–BIV plus AI) decreased from 0.6 to 0.2 μg·mL^{-1} upon extended exposure and the mixture of trilongins was a stronger motility inhibitor than the trilongins alone (Table 9) or any of the crude extracts (Table 2). The calculated synergy effect based on the sum of fractional inhibitory concentrations (∑FIC) was < 1 for all exposure times and the lowest ∑FIC (0.2) was observed after 30 min of exposure (Table 9).

Figure 4A–C shows that $\Delta\Psi_m$ decreased (yellow fluorescence changed to green) upon exposure to trilongins BI–BIV at a concentration of 0.4 μg·mL^{-1} (Fig. 4B). This exposure relaxed the plasma membrane permeability barrier towards propidium iodide (red fluorescence) (Fig. 4E). Interestingly, the dual pattern of staining (calcein-AM with propidium iodide) in Fig. 4E showed green fluorescence in the proximal part of the sperm tail, which is absent in the distal part of the tail, indicating that the mitochondrial inner membrane retained the calcein-AM cleavage products (green fluorescence). The results provided in Table 9 also show that the *T. longibrachiatum* peptaibols were as similarly sperm toxic as the well-known peptaibol alamethicin (EC_{50} 0.15 μg·mL^{-1}, exposure time of 1 day; Table 2).

Peptaibols from *T. longibrachiatum* form K^+/Na^+ permeable channels in lipid membranes

Single-channel recordings of voltage-dependent channels formed in 2 M KCl and in 2 M NaCl by trilongins BI–BIV and trilongin AI are shown in Fig. 5, as well as in Fig. 6 for alamethicin. For each type of channel, at least four levels of conductance through the single channels were resolved. The single channel conductances provoked by the peptaibols of *T. longibrachiatum* Thb and by alamethicin in NaCl and in KCl are listed in Table 10. The ratios of Na^+ relative to K^+ were higher for the trilongins at each of the four conductance levels (O1–O4) compared to the reference substance alamethicin F50 (Table 10). When tested individually, the 11-residue trilongin AI displayed channels with higher relative conductance ratios (Na^+ : K^+) than the channels formed by the 20-residue trilongins BI–BIV. Compared to alamethicin F50 at level O1, the benefit of Na^+ versus K^+ was 1.35-fold higher for trilongin AI and 1.16-fold higher for trilongins BI–BIV and, at level O2, the peptaibols values were 1.36- and 1.20-fold higher, respectively, than those of alamethicin F50. The single ion channels remained in an open state for a longer time in the case of the combination of the long peptaibols (trilongins BI–BIV) and the short peptaibol (trilongin AI) (Fig. 7A) than for the long peptaibols alone (Fig. 7B).

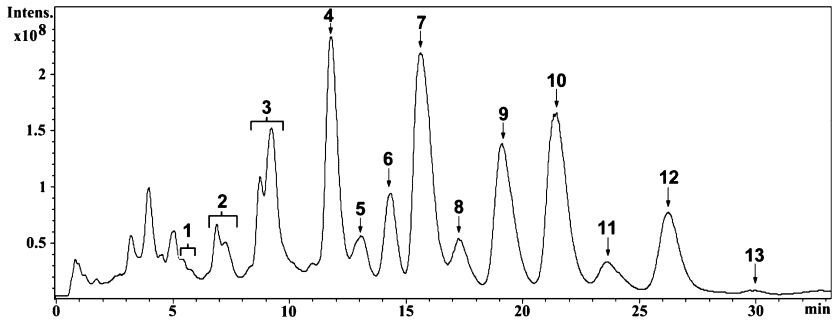


Fig. 3. Total ion chromatogram of the HPLC-MS analysis of the *T. longibrachiatum* strain CECT 20105 peptaibols. The peak numbers refer to the 11-residue peptaibols (1–5) and 20-residue peptaibols (6–13).

Table 6. The sequences and retention times of the 11- residue and 20- residue peptaibols of *T. longibrachiatum* strain CECT 20105.Ac, acetyl; Ileol, isoleucinol; Iva, isovaline; U, aminoisobutyric acid; Vx, Val/Iva; Lx, Leu/Ile; Lxol, Leuol/Ileol; Vxol, Valol/Ivaol; Fol, phenylalaninol.

Peptaibol	$[M+2Na]^{2+}$ (*m/z*)	Sequence	Fraction[a]	t_R (min)
11-residue peptaibol				
Trilongin A IV a	1155	AcU N Vx Vx U P Vx Lx U P Lxol	1	5.3–6.0
Trilongin AIV b	1155	AcU N Vx Vx U P Lx Lx U P Vxol		
Trilongin AIV c	1155	AcU N Vx Vx U P Lx Vx U P Lxol		
Trilongin AIII a	1169	AcU N Lx Vx U P Lx Lx U P Vxol	2	6.5–7.8
Trilongin AIII b	1169	AcU N Lx Vx UP Vx Lx U P Lxol		
Trilongin AIII c	1169	AcU N Vx Lx U P Lx Lx U P Vxol		
Trilongin AIII d	1169	AcU N Vx Lx UP Vx Lx U P Lxol		
Trilongin AII a	1183	AcU N Vx Lx U P Lx Lx U P Lxol	3	8.4–9.3
Trilongin AII b	1183	AcU N Lx Lx U P Lx Lx U P Vxol		
Trilongin AII c	1183	AcU N Lx Lx U P Lx Vx U P Lxol		
Trilongin AII d	1183	AcU N Lx Lx U P Vx Lx U P Lxol		
Trilongin AII e	1183	AcU N Lx Vx U P Lx Lx U P Lxol		
Trilongin AI	1197	AcU N Lx Lx U P Lx Lx U P Lxol	4	11.8
Trilongin A0	1211	AcU Q Lx Lx U P Lx Lx U P Lxol	5	13.1
20-residue peptaibol				
Trilongin BI	1958	AcU A U A U A Q U Vx U G Lx U P Vx U U Q Q Fol	6	14.4
Trilongin CI	1959	AcU A U A U A Q U Vx U G Lx U P Vx U U E Q Fol	7	15.6
Trilongin BII	1972	AcU A U A U A Q U Vx U G Lx U P Vx U Vx Q Q Fol	8	17.2
Trilongin CII	1973	AcU A U A U A Q U Vx U G Lx U P Vx U Vx E Q Fol	9	19.1
Trilongin CIII	1973	AcU A U A U U Q U Vx U G Lx U P Vx U U E Q Fol	10	21.4
Trilongin BIII	1972	AcU A U A U U Q U Vx U G Lx U P Vx U U Q Q Fol	11	23.6
Trilongin CIV	1987	AcU A U A U U Q U Vx U G Lx U P Vx U Vx E Q Fol	12	26.2
Trilongin BIV	1986	AcU A U A U U Q U Vx U G Lx U P Vx U Vx Q Q Fol	13	29.8

[a] HPLC peaks in Fig. 1.

Discussion

In the present study, we show that the fungus *T. longibrachiatum* produced large quantities (1–2 wt% of the mycelial biomass) of thermostable secondary metabolites identified as members of the families of 20-residue (1936–1965 Da, five to eight isoforms per strain) and 11-residue (1175 Da) peptaibols. These peptaibols were mitochondriotoxic toward porcine sperm cells at submicromolar exposure concentrations. The metabolites named trilongins BI–BIV and trilongin AI formed voltage-dependent, Na^+/K^+ conductive channels in biomembranes. *T. longibrachiatum* is an emerging human pathogen and the main pathogen in the fungal genus *Trichoderma* [5,27,38]. This species is also the most common species colonizing mould troubled indoor space [9]. The molecules involved in the pathology associated with this species have remained unknown to date.

A novel finding described in the present study was the toxic synergy between the 11-residue and the 20-residue trilongins of *T. longibrachiatum*. Synergy

Table 7. Molecular masses, characteristic ions and percentages of the 20-residue peptaibols in the methanol extractable metabolomes of different *T. longibrachiatum* strains. The origins of the strains are shown in Table 1. Values were calculated based on the detected y7 ions.

		Characteristic ions		*Trichoderma longibrachiatum* strains					
		y7	b13	Thb	CNM-CM 2171	CNM-CM 2277	IMI 291014	CECT 2412	CECT 20105
Peptaibol	MW	*m/z*		Percentage of total amount of peptaibols					
Trilongin BI	1936	774	1163	49	23	20	55	40	5
Trilongin CI	1937	775	1163	6	14	31	13	12	40
Trilongin BII	1950	788	1163	16	1	8	1	15	3
Trilongin CII	1951	789	1163	–	2	8	–	1	13
Trilongin BIII	1950	774	1177	21	36	7	25	20	2
Trilongin CIII	1951	775	1177	2	21	17	5	5	27
Trilongin BIV	1964	788	1177	5	3	4	–	7	2
Trilongin CIV	1965	789	1177	–	–	4	–	–	8

was visible as a potentiated toxic action on porcine sperm cells, as well as an extended duration (lifetime) of the ion conducting channels generated in artificial phospholipid membranes [black lipid membrane (BLM)]. The synergistic toxicity of different size classes of peptaibols does not appear to have been reported previously. The toxicokinetics of the combined 11-residue trilongin AI and 20-residue trilongins BI–BIV differed from those of the one-sized peptaibol: when tested singly on boar sperm cells, it took 1–3 days of exposure for the 11-residue trilongin AI and for the 20-residue trilongins BI–BIV to reach EC_{50} values of 1.5 and 0.4 $\mu g \cdot mL^{-1}$, respectively. When combined 1 : 2 w/w, the mixture was highly toxic within 30 min; EC_{50} was 0.6 $\mu g \cdot mL^{-1}$ and shifted down to 0.2 $\mu g \cdot mL^{-1}$ upon extended exposure. In that exposure time, the ∑FIC [39] had lowest value (0.2), indicating a clearly toxic synergy effect (Table 9). It appears that the generation of the (pathological) ion conductive channels was speeded up and stabilized by the simultaneous presence of the two different sizes of trilongins compared to channels formed by trilongins of identical size.

Exposure of porcine spermatozoa to purified trilongins (*T. longibrachiatum*) or to alamethicin (*T. arundinaceum*) resulted in a loss of motility and the loss of $\Delta\Psi_m$ at low concentration (EC_{50} of $\leq$ 0.1–0.2 μM). This mammalian cell toxicity threshold appears the to be lowest reported for *Trichoderma* peptaibols so far. The amino acid sequence of trichokonin VI is similar to the 20-residue trilongin BI (Table 5). Trichokonin VI produced by *T. pseudokoningii* MF2 was recently reported to depolarize mitochondria and vacuolize the cytoplasm of hepatocellular cancer cells on exposure to 20 μM (~ 40 $\mu g \cdot mL^{-1}$) [40] and to act as a Ca^{2+} channel agonist in isolated bullfrog cardiac myocytes at 20 $\mu g \cdot mL^{-1}$ (10 μM) [41]. Alamethicin (40 $\mu g \cdot mL^{-1}$; 20 μM) has been shown to mediate the uptake of Ca^{2+} ions by bovine adrenal chromaffin cells [42].

Multiple Aib residues were shown to be essential for generating ion conductive channels in biomembranes by peptaibols [43,44]. The *T. longibrachiatum* 20-residue

Table 8. Concentrations ($mg \cdot mL^{-1}$) of 11 and 20-residue peptaibols in the crude methanolic extracts of different *T. longibrachiatum* strains (10 mg dry weight·mL^{-1}). Amino acid sequences of the peptaibols are shown in Table 5.

	11-residue	20-residue	
Strain	Trilongin AI	Trilongins BI–BIV	Trilongins CI–CIV
Thb	0.08	0.74	0.06
CNM-CM2171	0.02	0.82	0.48
CNM-CM2277	0.02	0.28	0.42
IMI 291014	0.06	0.82	0.18
CECT 2412	0.02	0.74	0.16
CECT 20105	0.05	0.06	0.44

Table 9. Toxicity endpoints for motility inhibition of boar spermatozoa exposed trilongins BI–BIV, AI, a mixture of these two and the calculated synergy effects (∑FIC).

	EC_{50} ($\mu g \cdot mL^{-1}$)		
Peptaibol	30 min	1 day	2 day
Trilongin AI	15	1.5	1.5
Trilongins BI–BIV	3	0.6	0.4
Trilongin AI + trilongins BI–BIV[a]	0.6	0.2	0.2
Synergy effect			
∑FIC	0.2	0.5	0.6

[a] Contains trilongins BI–BIV and AI in a mass ratio of 2 : 1, respectively.

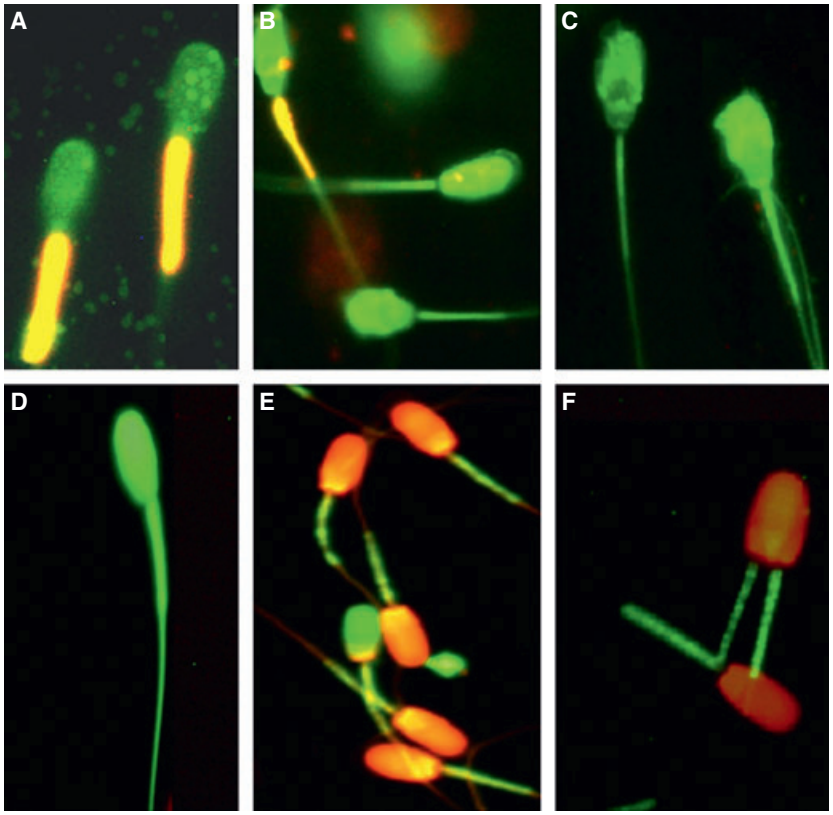


Fig. 4. Toxic responses of boar sperm cells to 20-residue trilongins BI–BIV purified from *T. longibrachiatum* Thb. The cells were stained with the membrane potential responsive dye JC-1 (A, B, C, top row) or with the live-dead stain calcein AM-propidium iodide (D, E, F, bottom row). (A) Exposed to vehicle only (motile); (B) exposed to 0.4 $\mu g \cdot mL^{-1}$ (nonmotile) or (C) to 0.8 $\mu g \cdot mL^{-1}$ (nonmotile) of the pooled trilongins BI–BIV. The membrane potential ($\Delta\psi_m$) of the mitochondrial sheath, located in the proximal part of the sperm tail, high in (A), is lost in (B) and (C) as a result of exposure to trilongins BI–BIV. (D) Exposed to vehicle only; (E) exposed to 0.4 $\mu g \cdot mL^{-1}$ of trilongins BI–BIV; and (F) exposed to 0.8 $\mu g \cdot mL^{-1}$ of trilongins BI–BIV. Exposure to the trilongins resulted in a relaxed permeability of the cell membrane towards propidium iodide, visible as nuclei showing red fluorescence (E, F). (E, F) The proximal part of the tail showed green fluorescence, indicating the retention of the fluorescent cleavage products by cellular esterases. These were absent in the distal part of the tail. Magnification, × 400. The size of the sperm head is 4 × 8 × 2 μm; the length of the tail is 55–67 μm.

trilongins contain eight or nine Aib residues, similar to alamethicin, and Ala in position 2 instead of Pro in alamethicin (Table 5). Aib residues were also shown essential for the non-endocytic entry of peptaibols to mammalian cells [45].

The 11-residue trilongin AI by itself was toxic also to porcine sperm cells with or without contribution of the 20-residue peptaibols, even though 11 amino acids are most likely too short to span across the phospholipid membrane of mammalian cells. Wada *et al.* [46] suggested a head-to-tail model for channel formation in BLMs by the 11-residue trichorovin XIIa. A similar observation was reported by Ruiz *et al.* [26] for trichobrachin A-IX (a toxic 11-residue peptaibol, also known as trichorovin TV-XIIa) from a marine isolate of *T. longibrachiatum* MMS 151, with an amino acid sequence identical to that of trilongin AI (Table 5) described in the present study. The other trichobanchins resembling [26] 11-residue trilongins A0 and AII–AIV (Table 5) found in the present study were neither toxic to boar sperm cells, nor active in the BLM experiments.

Cell free extracts prepared from a *T. longibrachiatum* mycelial biomass of isolates originating from sick building samples (Table 1) contained 10 w% of the toxic trilongins. The toxic trilongins might be related to the higher human pathogenicity of *T. longibrachiatum* among the species of the genus *Trichoderma*. However, we do not claim that the bioactive peptaibols described in the present study are solely responsible for the toxicity detected in the clinical and indoor isolates strains, which remains a subject requiring further investigation.

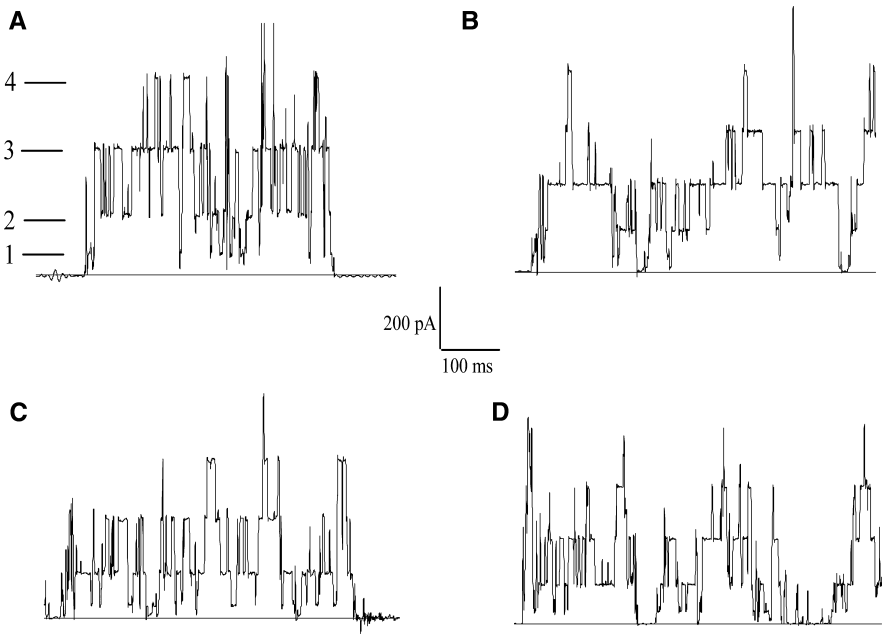


Fig. 5. Currents of single ion channels of the 20-residue trilongins BI–BIV and of the 11-residue trilongin AI. (A) Trilongins BI–BIV in 2 M KCl, $V = 260$ mV; (B) trilongins BI–BIV in 2 M NaCl, $V = 260$ mV; (C) trilongin AI in 2 M KCl, $V = 230$ mV; (D) trilongin AI in 2 M NaCl, $V = 240$ mV. The peptaibols were added to 2 nM.

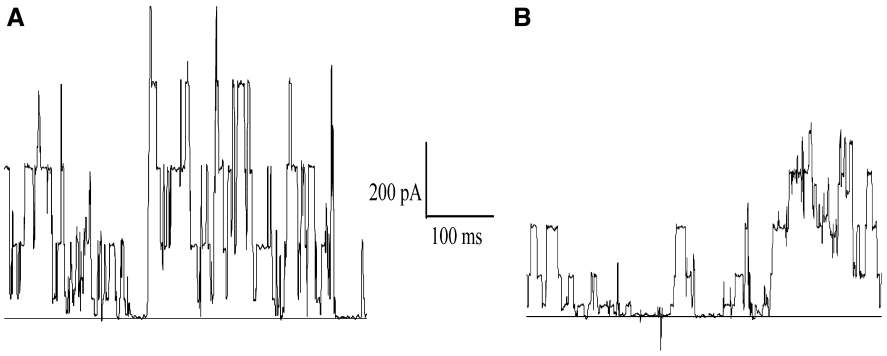


Fig. 6. Currents of the single ion channels of alamethicin (2 nM) in 2 M KCl, $V = 230$ mV (A), and in 2 M NaCl, $V = 220$ mV (B).

Table 10. The four conductance (pS) levels (O1–O4) generated by trilongins BI–BIV, AI and alamethicin in the BLM experiment. Media: 2 M NaCl or 2 M KCl in 10 mM Tris buffer (pH 7.0).

		Conductance levels							
Peptaibols	Medium/ratio	O1 (pS)	Na/K	O2 (pS)	Na/K	O3 (pS)	Na/K	O4 (pS)	Na/K
Trilongin AI	NaCl	180		500		1040		1730	
	KCl	190		700		1550		2440	
			0.95		0.71		0.67		0.71
Trilongins BI–BIV	NaCl	170		480		1000		1640	
	KCl	210		740		1600		2500	
			0.81		0.64		0.63		0.66
Alamethicin	NaCl	140		420		1000		1600	
	KCl	200		800		1700		2600	
			0.70		0.52		0.59		0.61

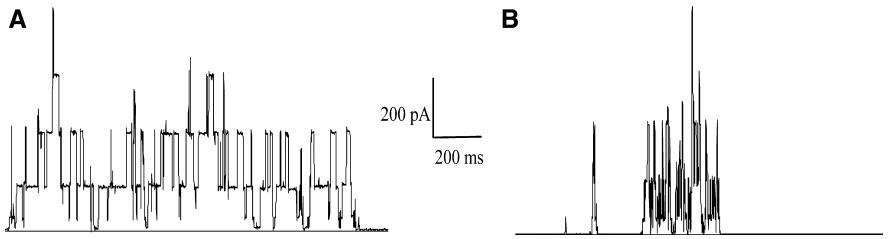


Fig. 7. Currents of the single ion channels in 2 M KCl, $V = 260$ mV. The 20-residue trilongins BI–BIV amended with (A) or not amended (B) with the 11-residue trilongin AI. The tested peptaibol solutions were the same as those used in Fig. 5.

Experimental procedures

The fungal strains

The strains examined are described in Table 1 [2,3,6,11,47]. The indoor isolates of *T. longibrachiatum*, Thb, Thd, Thg originated from Oulu, northern Finland, a moisture-damaged residence of a family of two adults and three children suffering from serious, bulding-associated symptoms of ill health (Table 1). *Trichoderma* sp. was cultured from insulation material of the bathroom on TSA plates as the principal fungal colonizer. Cell-free extracts were prepared in methanol for 15 separate colonies and tested for toxicity by the rapid boar spermatozoan assay [48]. The toxic colonies were further cultivated to obtain pure cultures on MEA at 22 °C. The isolates were identified based on the sequences of the ITS region. DNA isolation, amplification of the ITS region, amplicon purification and sequencing were performed as described previously [47]. The sequence of the ITS region was analyzed using TRICHOKEY, version 2.0 [49]. The ITS sequences were deposited in the GenBank database (Table 1).

Preparation of cell extracts, purification and MS of the toxins

The strains were grown on MEA plates for the indicated times and harvested into methanol. Methanol extracts of the mycelial biomass were processed and analyzed as described by Andersson *et al.* [47]. HPLC and HPLC-ESI-IT-MS analyses were peformed as described previously [47], except that the eluents used for the HPLC separations were 0.1% formic acid (A) and methanol (B), using isocratic elution with 80% of B for 25 min at a flow rate of 1 mL·min^{-1}. For detection, the monitoring of A_{215} was used. Alamethicin was used as a reference compound.

Toxicity assays with porcine sperms as indicator cells

Sperm cells were exposed by dispensing 1–20 μL of the methanolic fungal extract or the pure substance(s) or vehicle only (methanol) into 2.0 mL of extended boar semen (Figen Ltd, Tuomikylä, Finland), which was used as delivered (27×10^6 sperm cells·mL^{-1}).

Toxicity assays were performed in triplicate with the serial (step = 2) dilutions of the test substance, each as three or more parallels with two biological replicates. The results are given as the median unless the range (minimum – maximum) is indicated. The vehicle only (ethanol; 96 vol%) control was prepared for each dilution step. Sperm motility was read by microscopy (on a heated stage, 37 °C) as described previously [47].

Functional staining

The number of cells with plasma membrane relaxed permeation of propidium iodide and depleted $\Delta\Psi_m$ were recorded by microscopic assessment of cells stained with calcein-AM, propidiumiodide and the membrane potential sensitive dye JC-1. The details of these protocols have been described previously [47].

BLM analysis

The BLM technique was used to measure ion conductivity changes of phospholipid membrane in response to the presence of HPLC-purified peptaibols from the *Trichoderma* strains. The experiments were performed as described previously [50]. For the single channel conductances, soybean phosphatidylcholine dissolved in heptane (20 mg·mL^{-1}) was used to form a lipid bilayer membrane covering the circular hole (inner diameter 0.3 mm) in the teflon wall separating the aqueous solutions of 2 M KCl or 2 M NaCl in 20 mM Tris-Cl (pH 7.0) at 15 °C.

Synergy effects of peptaibols

Synergy effects of peptaibols were estimated using the FIC method. ∑FIC values < 1, = 1 and > 1 indicate synergy, additivity and antagonism, respectively [39]. The ∑FIC for long (A) and short (B) peptaibols was calculated using the equation:

$$\Sigma FIC = \frac{FIC\ (A+B)}{FIC\ (A)} + \frac{FIC\ (A+B)}{FIC\ (B)} \quad (1)$$

where the FIC(A) and FIC(B) are EC_{50} values of separate long (A) and short (B) peptaibols, respectively, and the FIC (A + B) is the EC_{50} value of the mixture of peptaibols A and B in the motility biotest with boar sperm cells.

Reagents and media

Alamethicin and soybean phosphatidylcholine were obtained from Sigma-Aldrich (St Louis, MO, USA). JC-1, calcein-AM and propidium iodide were obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical quality and were obtained from local suppliers.

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