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

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Running title. Adverse effects of *T. longibrachiatum* peptaibols

Abbreviations. Aib, α-aminoisobutyric acid; Ac, acetyl; BLM, black lipid membrane; EC_{50}, effective median concentration; ITS, internal transcribed spacer region; Iva, isovaline; Leuol, leucinol; Ileol, isoleucinol; Pheol, phenylalaninol; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial transmembrane potential

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Summary

Certain species of the filamentous fungal genus *Trichoderma*, e.g. *T. longibrachiatum* and *T. citrinoviride* are among the emerging clinical pathogens and also the most common species in the indoor space of mould-damaged buildings. Molecules involved in its pathology are not known. We report here that 0.5 to 2.6 weight % 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). 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 20-residue trilongins 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 wt / wt, an EC_{50} of 0.6 µg mL^{-1} was reached already within 30 min, and the EC_{50} shifted down to 0.2 µg mL^{-1} upon extended exposure. In contrast, with 11-residue or 20-residue trilonging separately in 30 min exposure the EC_{50} values were 15 and 3 µg mL^{-1}, respectively, and shifted down to 1.5 µg mL^{-1} and 0.4 µg 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.

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 among the promising biocontrol agents due to their antagonistic activities against plant pathogenic fungi [4]. In addition, *Trichoderma* strains are also known to rarely 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 hematologic 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* (*T. longibrachiatum*, *T. citrinoviride*, *T. pseudokoningii*, *T. reesei*, *T. harzianum*, *T. koningii*, *T. atroviride*, *T. viride*) have been previously reported from clinical cases. However, several clinical isolates originally identified based on their morphological characters have been recently reidentified by sequence-based molecular techniques as *T. longibrachiatum*, which thus proved to be the most frequently occurring, almost exclusive clinical etiologic 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 in order to minimize the possible health risks.

Trichoderma species were reported to be among the dominating microfungi in indoor environments of water-damaged buildings [8, 9]. Their possible association with building related ill health symptoms has been suggested [10-12], however, as 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 due to their unique bioactivities and conformations [13-17]. They are defined as linear or cyclic polypeptide antibiotics of 4–21 amino acid residues that are characterised by a molecular weight between 500 and 2200, 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 of Aib-containing peptides carrying a C-terminal 2-amino alcohol residue is 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]. Later it turned out, that the first peptaibol isolated from a Trichoderma sp. was actually suzukacillin from 'Trichoderma viride' 63 C-I [19], however, the presence of Aib in the SZ-hydrolysate was confirmed only six years later [20]. The producer strain NRRL 3199 originally identified as *Trichoderma viride* was recently reidentified as *T. arundinaceum*, a member of the *Trichoderma brevicompactum* clade [21], and all other alamethicin-producing *Trichoderma* species (*T. brevicompactum, T. protrudens, T. 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 *T. 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 membrane potential of the sperm cells at low exposure concentrations [27]. In this report, we describe the isolation, structures, toxic and ion channel-forming activities and synergistic properties of two different sizes of peptaibols produced by *T. longibrachiatum* isolates originating from agricultural and clinical samples as well as from indoor environment where serious building-associated ill health effects were claimed.

Results

Cell free extracts of *Trichoderma longibrachiatum* strains were toxic to porcine sperm cells. Cell extracts of *T. longibrachiatum* isolates (Table 1) originating from clinical (n = 2), terrestrial (n = 4) and sick building samples (n = 3) were assayed for the presence of substances toxic to mammalian cells. 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, mitochondrial inner membrane potential $\Delta\Psi_m$, and the cell membrane permeability barrier to propidium iodide (Table 2). The EC_{50} was 3 to 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 10 fold higher. The eight toxic *T. longibrachiatum* strains were cultivated on TSA, BHI and MEA at 22 °C and at 37 °C for optimising growth and toxin production. The growth for all strains was optimal on MEA at 22 °C and at 37 °C but the production of toxin was higher at room temperature 22 °C. Toxicity of the extracts of Thb and Thd decreased by a factor of 2 to 4 when the extracted biomass was cultivated at 37 °C (Table 2). Toxicity of the extracts increased (by factor of 4) when incubation was extended from 5 d to 15 d. The toxic substances of *T. longibrachiatum* strains were resistant to heat (10 min, 100 °C).

The toxic substances of *Trichoderma 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. 1 A) showed the doubly charged cationised 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 cationised 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 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).

The MS^2 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 to 20 and showed that the C-terminus contained phenylalaninol (Pheol) (Fig. 2 B, C). The MS^2 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 to 13. MS^2 analysis of the doubly charged $[M+2Na]^{2+}$ ion at *m/z* 991 confirmed residues 16 to 20 and 4 to 13 (Fig. 2D). Since the fragment ion 196 Da (sequence between 14-15) 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 to 15 was Pro-Vxx.

The diagnostic fragment ions of the above mass spectrometric analysis of peptaibols are compiled in Table 3. Conclusion of the results recited 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, differences being found only in position 6 (Ala or Aib) and in position 17 (Vxx) or Aib) (Table 5).

Peak A1 (13 min) was also toxic to boar sperm cells. It contained a compound that formed doubly charged cationised molecules $[M+2Na]^{2+}$ at *m/z* 610.6 and a single charged $[M+Na]^{+}$ at *m/z* 1197.9, corresponding to 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 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. The HPLC-MS analysis showed that *T. longibrachiatum* strains contained also other 11-residue peptaibols with sodiated mass ions at *m/z* 1183, 1169, 1155 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. The MS/MS analysis of the precursor ions gave the b ion series are shown in Table 4. Conclusion from the above mass spectrometry data is that the toxic peak A1 of Fig. 1A was a peptaibol with an average molecular weight of 1175.5 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 one Da higher, *m/z* 775 and 789, than corresponding y7 ions, *m/z* 774 and 788, of the trilongins BI-BIV. These were named trilongins CI, CII, CIII and CIV. The 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 the position 18 where Glu was substituted with Gln (Table 5). The trilongins CI-CIV varied also in position 6 (Ala or Aib) and in position 17 (Vxx or Aib) like the trilongins BI-BIV (Table 5). The 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 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 the 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 shows the new sequences (Table 5). The HPLC-MS elution profile of the

peptaibols observed in the methanol extract of the *T. longibrachiatum* strain CECT 20105 is shown in Fig. 3. The sequences and retention times of the 11- and 20-residue peptaibols found are in Table 6. Table 7 compiles the contributions of the different 20-residue trilongins BI-BIV and CI-CIV in the *Trichoderma 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 done using the absorbance at 215 nm and alamethicin as reference.

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 contributed in the different strains to 5-13 wt % of the methanol-soluble matter and the 11-residue peptaibol 0.2 to 0.8 wt %. The toxic peptaibols thus made up 0.5 to 2.6 wt % in the harvested mycelial biomass (320±20 mg per Petri dish of Ø 90 mm) of the investigated *Trichoderma longibrachiatum* isolates. One fully grown culture dish thus contained 1500-8800 µg of the toxic peptaibols.

Toxicity of the purified 20-residue and 11-residue trilongins. Toxicities were measured using boar spermatozoa motility inhibition as the toxicity indicator, separately of the 20-residue trilongins BI-BIV, 11-residue trilongin AI and combination of trilongins (BI-BIV plus AI) in 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 µg mL^{-1} upon extended exposure, whereas the EC_{50} of 11-residue trilongins AI decreased from 15 to 1.5 µg 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 ∑FIC was in all exposure times < 1 and highest ∑FIC (0.2) was observed after 30 min exposure (Table 9).

Panels A–C in Fig. 4 show that the mitochondrial membrane potentials decreased (yellow fluorescence changed to green) upon exposure to 0.4 μg mL^{-1} of the trilongins BI–BIV (Panel B in Fig. 4). This exposure relaxed the plasma membrane permeability barrier towards propidium iodide (red fluorescence) (Fig. 4 E). Interestingly, the dual pattern of staining (calcein-AM with propidium iodide) in Fig. 4 E showed in the proximal part of the sperm tail green fluorescence 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 in Table 9 also show that the *T. longibrachiatum* peptaibols were similarly sperm toxic as the well-known peptaibol alamethicin (EC_{50} 0.15 μg mL^{-1}, exposure time 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 and in Fig. 6 for alamethicin. For each type of the channels, at least four levels of conductance (G) 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 to O4) as 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^+ vs K^+ was 1.35 times higher for trilongin AI and for the trilongins BI-BIV 1.16 ×, and at level O2 the peptaibols figures were 1.36 times and 1.20 times higher respectively, than those of alamethicin F50. The single ion channels remained in an open state 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).

Discussion

We showed here that the fungus *T. longibrachiatum* produced large quantities, 1 to 2 wt % of the mycelial biomass, of thermostable secondary metabolites identified as members of the families of 20-residue (1936 to 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 trilongins 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* [38, 27, 5]. This species is also the most common species colonising mould troubled indoor space [9**]**. The molecules involved in the pathology connected to this species have been unknown so far.

A further novel finding described in this paper was the toxic synergy between the 11-residue and the 20-residue trilongins of *T. longibrachiatum*. Synergy was visible as potentiated toxic action on primary porcine cells as well as extended duration (lifetime) of the ion conducting channels generated in artificial phospholipid membranes (BLM). Synergistic toxicity of different size classes of peptaibols appears not to have been reported before. 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 to 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 µg mL^{-1} and 0.4 µg mL^{-1}, respectively. But when combined 1:2 w/w, the mixture was highly toxic already within 30 min, EC_{50} was 0.6 µg mL^{-1} and shifted down to 0.2 µg mL^{-1} upon extended exposure. In that exposure time also the $\sum$FIC [39] had lowest value (0.2) indicating clearly toxic synergy effect (Table 8). It thus seems that generation of the (pathological) ion conductive channels was speeded up and stabilised by 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 loss of motility and loss of the mitochondrial membrane potential

($\Delta\Psi_m$) at low concentration, EC_{50} of ≤ 0.1 to 0.2 µM. This mammalian cell toxicity threshold appears the lowest reported for *Trichoderma* peptaibols so far. 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 depolarise mitochondria and vacuolise the cytoplasm of hepatocellular cancer cells at exposure to 20µM (~ 40 µg mL^{-1}) [40] and to act as a Ca^{2+} channel agonist in isolated bullfrog cardiac myocytes at 20 µg mL^{-1} (10 µM) [41]. Alamethicin (40 µg mL^{-1}, 20 µM) has been shown to mediate uptake of Ca^{2+} ions by bovine adrenal chromaffin cells [42].

Multiple Aib residues have been shown essential for generating ion conductive channels in biomembranes by peptaibols [43, 44]. The *T. longibrachiatum* 20-residue trilongins contain 8 or 9 Aib residues, similar to alamethicin, and Ala in position 2 instead of Pro in alamethicin (Table 5). Aib residues were also shown essential for non-endocytic entry of peptaibols to mammalian cells [45].

The 11-residue trilongin AI was toxic also by itself to porcine 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. Similar observation was reported by Ruiz et al. [26] on 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 trilongins AI (Table 5) described in this paper. The other trichobanchins resembling [Ruiz et al. 2007] 11-residue trilongins AO and AII-AIV (Table 5) found in this study were neither toxic to boar sperm cells nor active in BLM experiments.

Cell free extracts prepared from *T. longibrachiatum* mycelial biomass of isolates originating from sick building samples (Table 1) contained 10 weight % of the toxic trilongins. The toxic trilongins might be connected with 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 this study are solely responsible for the toxicity detected in the clinical and indoor isolates strains; this needs further investigations.

Experimental procedures

The fungal strains. Examined strains 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, residence associated ill health symptoms (Table 1). *Trichoderma* sp. was cultured from insulation material of the bathroom on tryptic soy agar (TSA) plates as the principal fungal coloniser. Cell free extracts were prepared in methanol of 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 malt extract agar (MEA) at 22° C. The isolates were identified based on the sequences of the internal transcribed spacer (ITS) region. DNA-isolation, amplification of the ITS-region, amplicon purification and sequencing were done as described earlier [47]. The sequence of the ITS-region was analysed with the aid of the program TrichOKey 2.0 [49]. The ITS-sequences were deposited in the GenBank database (Table 1).

Preparation of cell extracts, purification and mass spectrometry of the toxins. The strains were grown on MEA plates for the indicated times harvested into methanol. Methanol extracts of the mycelial biomass were processed and analysed as described by Andersson *et al*. [47]. The HPLC and HPLC-ESI-IT-MS analysis was done as described [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 absorbance at wavelength of 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 to 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 (min-max) 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^{o}C) as described previously [47].

Functional stainings. The number of cells with plasma membrane relaxed permeation of propidium iodide and depleted mitochondrial transmembrane potential (loss of $\Delta\Psi_m$) were recorded by microscopic assessment of cells stained with calcein-AM, propidiumiodide and the membrane potential sensitive dye JC-1. Details of these protocols were described earlier [47].

Bilayer lipid membrane (BLM) analysis. The BLM (black lipid membrane) 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 executed as previously described [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 (0.3 mm i.d.) in the teflon wall separating the aqueous solutions of 2 M KCl or of 2 M NaCl, in 20 mM Tris-Cl, pH 7.0 at 15 ^{o}C.

Synergy effects of peptaibols. Synergy effects of peptaibols were estimated using the fractional minimal inhibitory concentrations (FIC) method. The sum of FIC (∑FIC) values below 1, =1 and above 1 indicate synergy, additivity and antagonism, respectively [39]. The ∑FIC for long (A) and short (B) peptaibols were calculated using the equation

$$\sum \mathrm{FIC} = \frac{FIC(A+B)}{FIC(A)} + \frac{FIC(A+B)}{FIC(B)}$$

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). The other chemicals were of analytical quality, obtained from local suppliers.

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Table 1. Fungal strains examined during the study, origins and ITS sequence used for identification.

Strain codes	Alternate codes and origin	ITS sequence	Reference
Trichoderma longibrachiatum		GenBank Accession #	
Thb	moisture-damaged residence, Finland	HQ593512	this study
Thd	moisture-damaged residence, Finland	HQ593513	this 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	AY585880	[6]

	strain, Egypt		
Reference strains			
Trichoderma reesei DSM 768	synonym *T. reesei* Simmons, *T. viride* QM6a; cotton canvas, Solomon Islands, Bouganville.		[2], anamorph of *Hypocrea jecorina,* [3]
Trichoderma harzianum ES39	Ceiling of a residence, after renovation of moisture damage, Helsinki, Finland	AY585881	[11]; this study
Acremonium tubakii CBS 110649[1]	Reed sandy soil		[47]

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. [1]This strain is mistakenly referred as CBS110360 by Andersson et al. [47].

Table 2. The toxic activities towards porcine spermatozoa by extracts of the fungus *Trichoderma longibrachiatum* and reference strains. The cell extracts were prepared from mycelial biomass grown on MEA at 22ºC for 5 d. The toxicity endpoints indicate methanol-soluble substances, µg dry wt per mL. Depolarisation of mitochondria was recorded by epifluoresence microscopy after staining with the membrane potential sensitive dye JC-1.

	Motility inhibition		Depolarisation of mitochondria		Relaxed permeability barrier of cell membrane to propidium iodide	
Exposure time, hours	24	72	24	72	24	72
	Toxicity endpoint to sperm cells EC_{50} (µg dry wt mL^{-1})					
Cell extracts of *T. longibrachiatum*						
From indoor isolates						
Thb	6 (25)[1]	3 (12)[1]	6	3	6	3
Thd	12 (25)[1]	3 (12)[1]	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

[1] Toxicity endpoint of extracts (in parentheses) indicate situation where strains were grown on MEA at 37 ºC for 5 d.

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^2 and MS^3 analysis.

Diagnostic ions	Trilongin							
	BI	BII	BIII	BIV	CI	CII	CIII	CIV
	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 obtained from MS^2 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
	m/z										
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

Table 5. Amino acid sequences of the trilongins A0-AIV, BI-BIV, CI-CIV produced by *T. longibrachiatum* strains and alamethicin (alm).

	Sequence																				Identical or positionally isomeric compound	
Peptaibol	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**		**Ref**
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]

trilongin AIII c	AcAib	Asn	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol
trilongin AIII d	AcAib	Asn	Vxx	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxxol
trilongin A II a	AcAib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Vxxol
trilongin AII b	AcAib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Lxxol
trilongin AII c	AcAib	Asn	Lxx	Lxx	Aib	Pro	Vxx	Lxx	Aib	Pro	Lxxol
trilongin AII d	AcAib	Asn	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol

tv29-11-II f	[29]
trichobrachin A-III	[26]
trichorovin TV-Ia	[26]
trichobrachin III-2b	[29]
tv29-11-II b	[29]
hypojecorin A 3	[30]
trichobrachin A-IVc	[26]
trichobrachin A-VIII a	[26]
trichorovins TV-Vb/VIb	[26]
trichorozin I	[26]
trichobrachins III- 6a/ 8b/9c	[30]
hypojecorin A 6	[30]
trichobrachin A-VIII d	[26]
trichobrachin A-VIII e	[26]
harzianin HB 1	[26]
trichobrachin A-VIII b	[26]
trichorovin TV-VIIa	[26]
tv29-11-II a	[29]
trichobrachins III-7b/8a/9a	

											hypojecorin A 12	[29]
												[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] trichorovins TV-XI/ XII-a/b [26] trichorozin III [26] trichobrachins II-Fa/ Ga/Gb/Ha [26] hypojecorins A 15/16 [30] [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	this study
trilongin CIV	AcAib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Glu	Gln	Pheol	new	this 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]

Ac: acetyl, Aib: aminoisobutyric acid, Vxx:Val/Iva, Lxx: Leu/Ile, Lxxol: Leuol/Ileol, Vxxol: Valol/Ivaol, Pheol: phenylalaninol

Table 6. The sequences and retention times of the 11- residue and 20- residue peptaibols of *T. longibrachiatum* strain CECT 20105.

Peptaibol	$[M+2Na]^{2+}$	Sequence	Fraction[1]	t_R (min)
11-residue peptaibol	*m/z*			
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

[1]The HPLC peaks in Fig. 1, Ac: acetyl, U: aminoisobutyric acid, Vx : Val/Iva, Lx: Leu/Ile, Lxol: Leuol/Ileol, Vxol: Valol/Ivaol, Fol: phenylalaninol

Table7. Molecular masses, characteristic ions and percentages of the 20-residue peptaibols in the methanol extractable metabolomes of different *T. longibrachiatum* strains. Origins of the strains are shown in Table 1. The figures 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*		per cent of the 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

Table 8. Concentrations 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.

Strain	11-residue trilongin AI	20-residue trilongins BI-BIV	trilongins CI-CIV
		mg mL^{-1}	
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).

Peptaibol	EC_{50} µg mL^{-1}		
Exposure time	30 min	1 d	2 d
trilongin AI	15	1.5	1.5
trilongins BI-BIV	3	0.6	0.4
trilongin AI + trilongins BI-BIV[1]	0.6	0.2	0.2
Synergy effect			
∑FIC	0.2	0.5	0.6

[1] contain trilongins BI-BIV and AI in mass ratio of 2:1, respectively.

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

		Conductance levels (G)							
		O1		O2		O3		O4	
	Conductance	pS		pS		pS		pS	
Peptaibols	Medium/ratio		Na/K		Na/K		Na/K		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

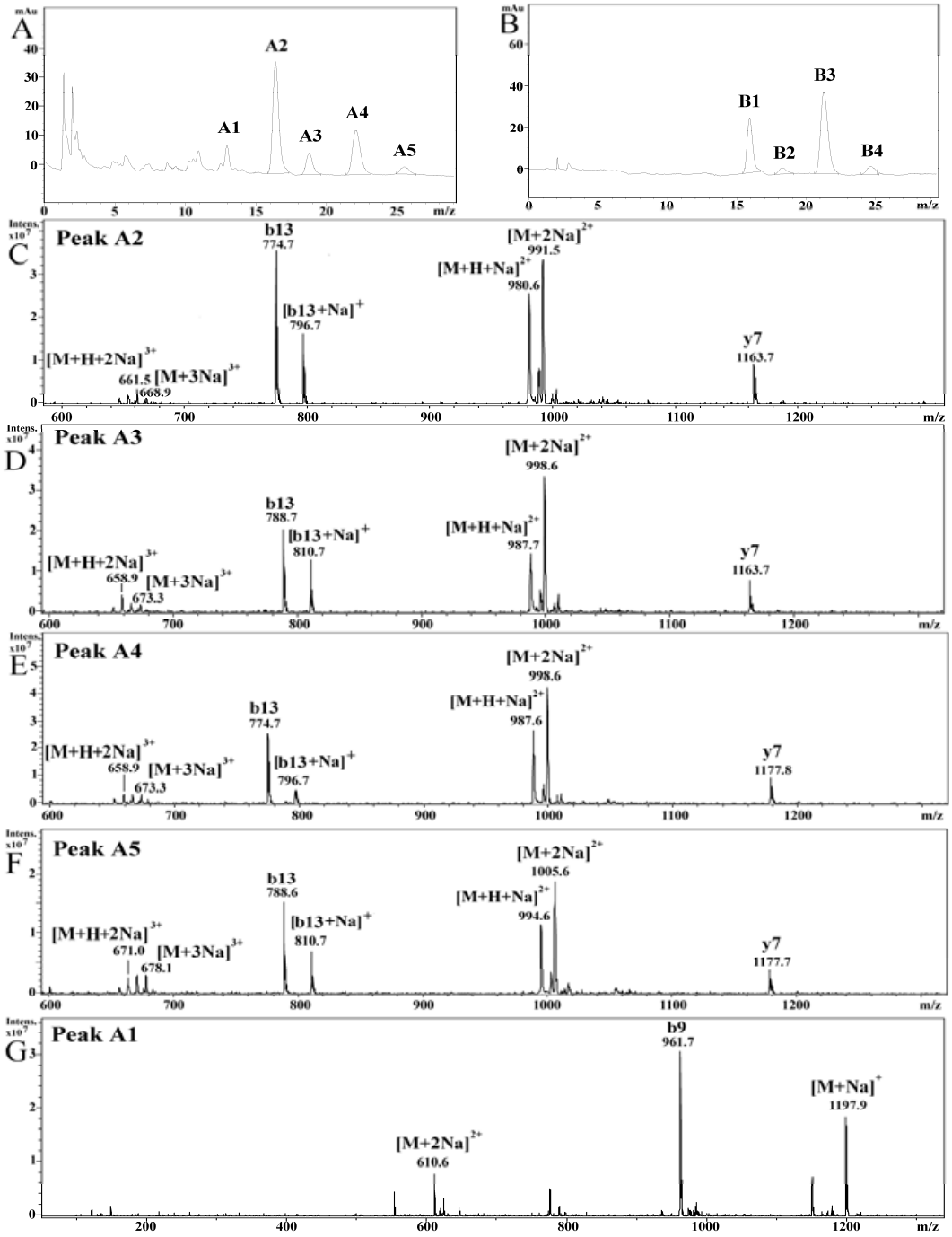
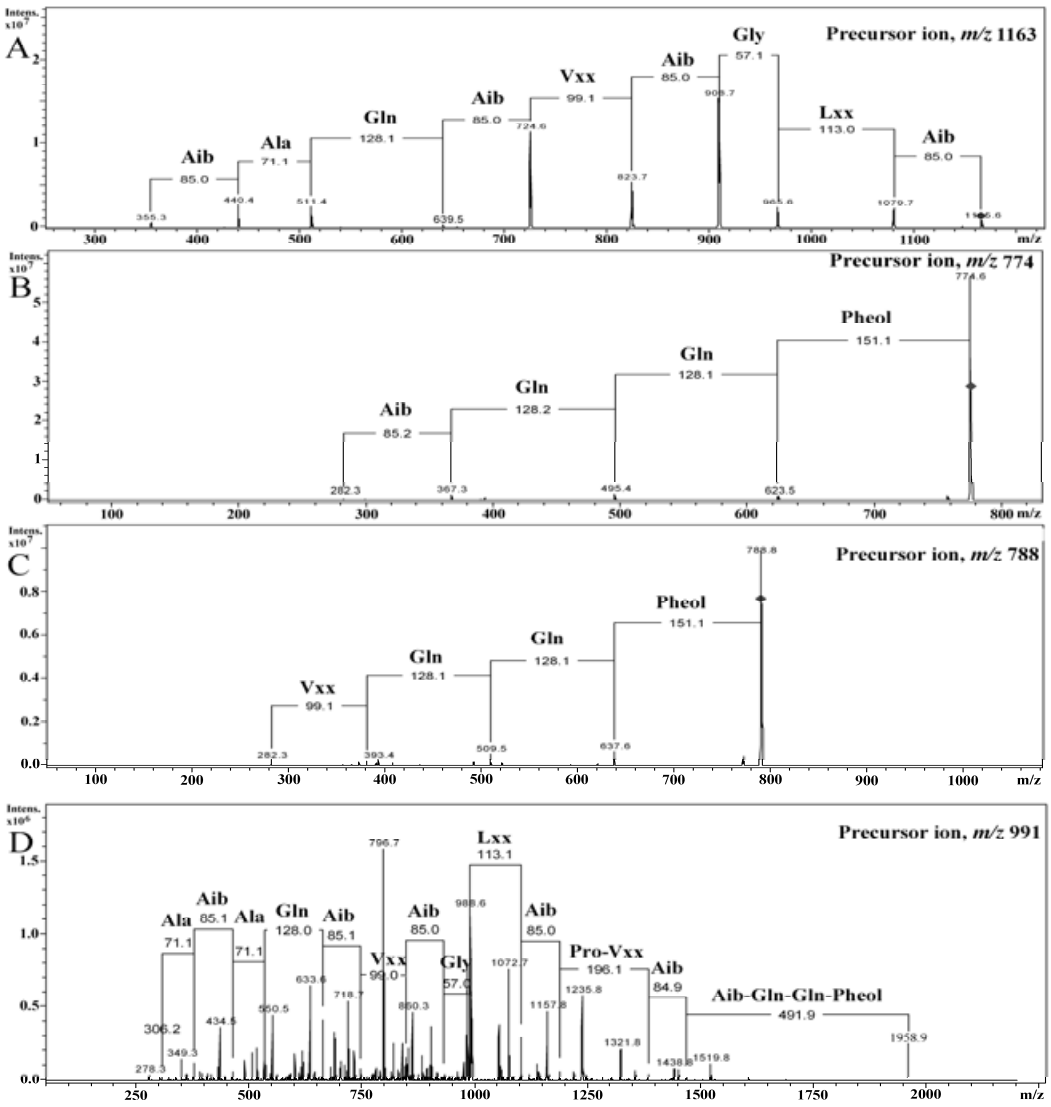


Figure 1. HPLC-UV and HPLC-MS analysis of peptaibols produced by *Trichoderma longibrachiatum* Thb. Panel A, HPLC-UV (215 nm) chromatograms of methanol extract from strain Thb and methanol solution of alamethicin (Panel B). Panel C, doubly charged sodiated molecular ions at *m/z* 991, b13 ion at *m/z* 774 and y7 ion at *m/z* 1163 of peak A2 from panel A. Panels D to F show the corresponding

ions of peaks A2-A5 from panel A. Panel G shows a doubly charged sodiated molecular ion at *m/z* 1197 and b9 ion at *m/z* 961 of peak A1 from panel A.



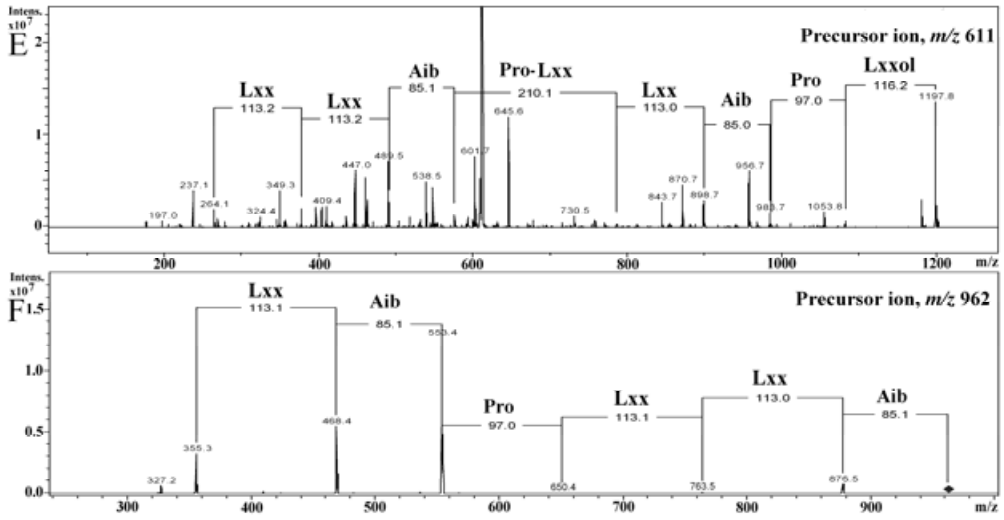


Figure 2. MS/MS fragmentation patterns and amino acid sequences of peptaibols found in methanol extract of *Trichoderma longibrachiatum* Thb. Panel A, the amino acid sequence of y7 ion at *m/z* 1163 (Fig. 1 C, peak A2). The sequences of b13 ions at m/z 774 (Fig. 1C, peak A2) and 788 (Fig. 1D, peak A3) are shown in Panels B and C, respectively. Panel 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 Panels E and F, respectively.

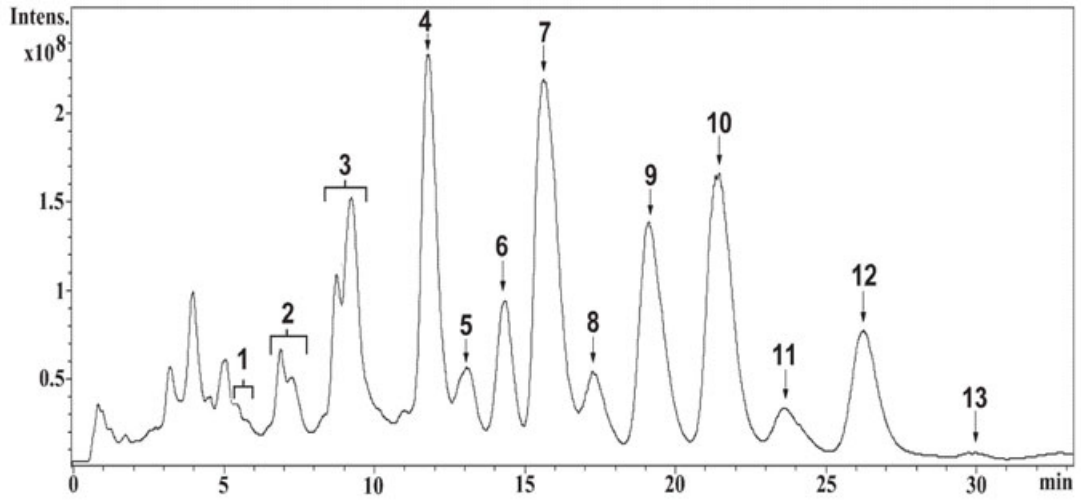


Figure 3. Total ion chromatogram of the HPLC-MS analysis of the *T. longibrachiatum* strain CECT 20105 peptaibols. The peak numbers refer to the 11- residue peptaibols (1-5) and 20-residue peptaibols (6-13).

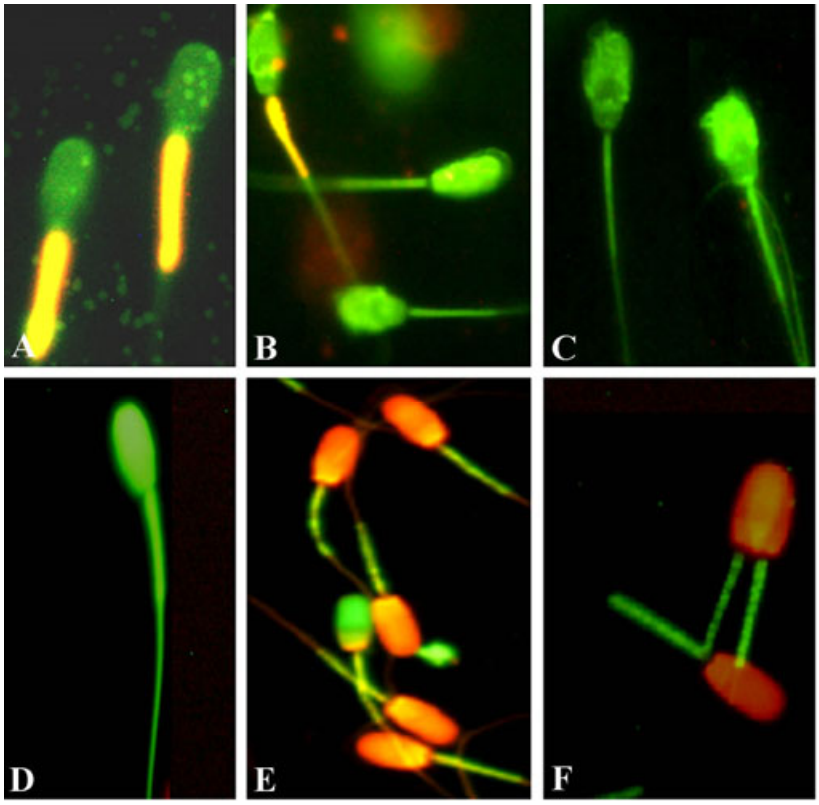


Figure 4. Toxic responses of boar sperm cells to 20-residue trilongins BI-BIV purified from *T. longibrachiatum* Thb. The cells were stained with the membrane potential responsive dye JC-1 (A,B,C, top row) or with the live-dead stain calcein AM-propidium iodide (D,E,F, bottom row). A, exposed to vehicle only (motile); B, exposed to 0.4 μg mL^{-1} (nonmotile) or C, to 0.8 μg 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 panel A, is lost in panels B, C, due to exposure to trilongins BI-BIV. Panel D, exposed to vehicle only, panel E, exposed to 0.4 μg mL^{-1} of trilongins BI-BIV, and panel F, to 0.8 μg mL^{-1} of the trilongins BI-BIV. Exposure to the trilongins resulted into relaxed permeability of the cell membrane towards propidium iodide, visible as nuclei showing red fluorescence (Panels E, F). In panels E and F the proximal part of the tail showed green fluorescence, indicating 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 μm × 8 μm × 2 μm, length of the tail: 55 to 67 μm.

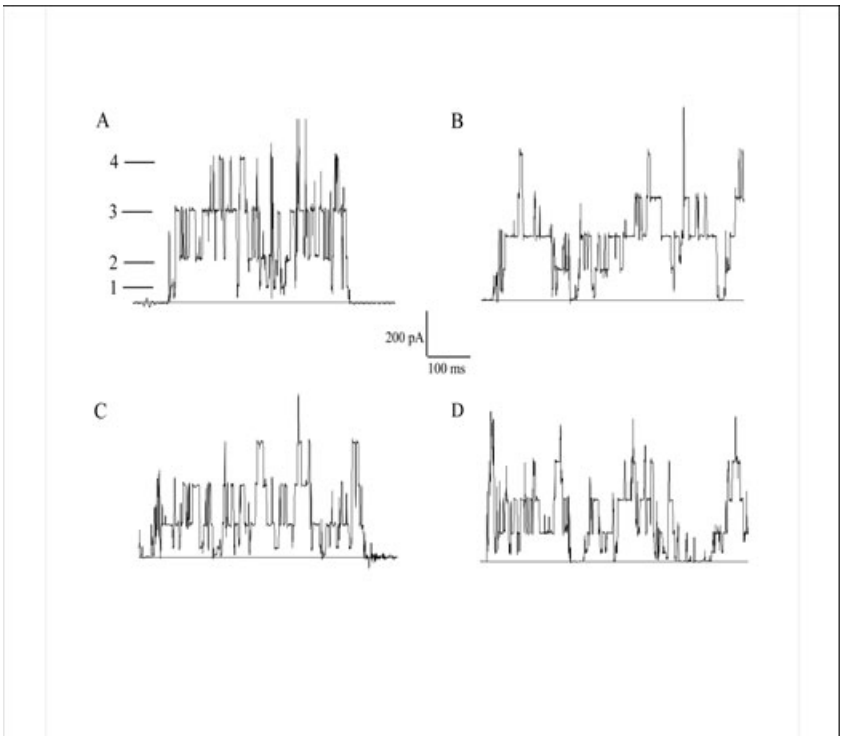


Figure 5. Currents of single ion channels of the 20-residue trilongins BI-BIV and of the 11-residue trilongin AI. A. trilongins BI-BIV in 2 M KCl, V = 260 mV; B. trilongins BI-BIV in 2 M NaCl, V = 260 mV; C. trilongin AI in 2 M KCl, V = 230 mV; D. trilongin AI in 2 M NaCl, V = 240 mV. The peptaibols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

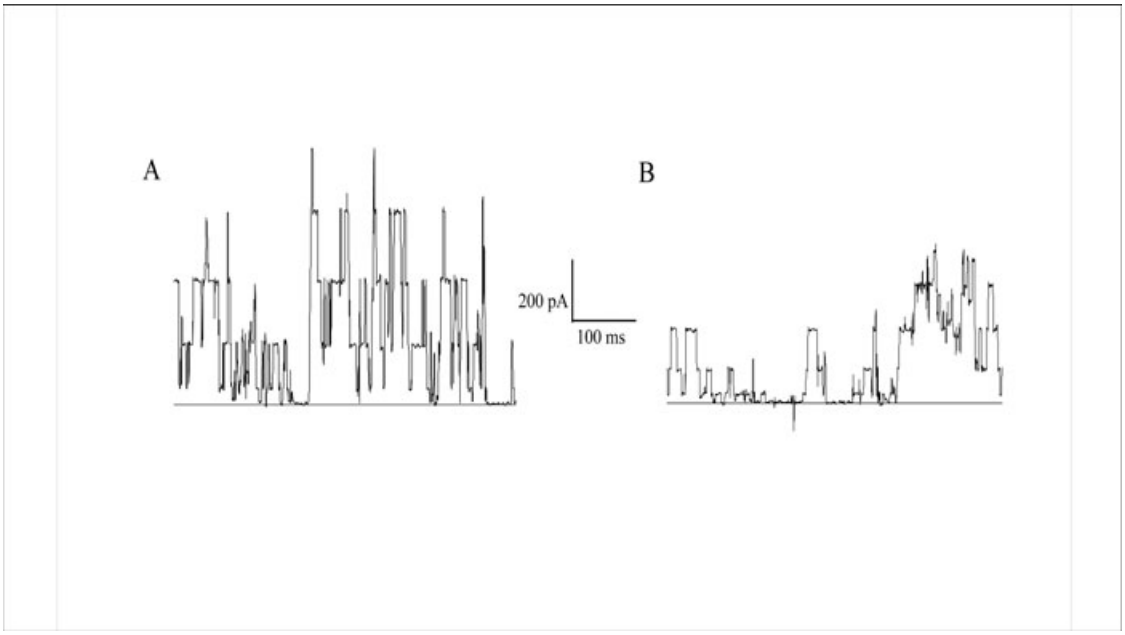


Figure 6. Currents of the single ion channels of alamethicin (2 nM) in 2 M KCl, V = 230 mV (A) and in 2 M NaCl, V = 220 mV (B).

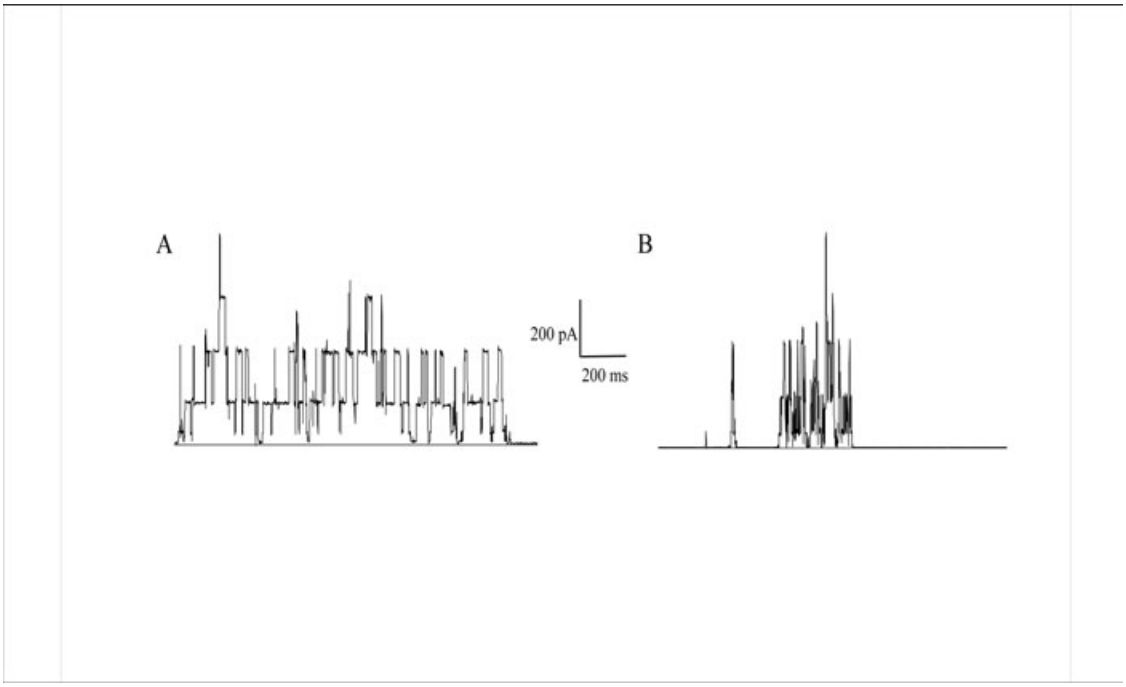


Figure 7. Currents of the single ion channels in 2 M KCl, V = 260 mV. The 20-residue trilongins BI-BIV amended with (panel A) or not amended (panel B) with the 11-residue trilongin AI. The tested peptaibol solutions were the same as those used for Fig. 5.