

## ORIGINAL ARTICLE

**Acrebol, a novel toxic peptaibol produced by an *Acremonium exuviarum* indoor isolate**M.A. Andersson<sup>1</sup>, R. Mikkola<sup>1</sup>, M. Raulio<sup>1</sup>, L. Kredics<sup>2</sup>, P. Maijala<sup>1</sup> and M.S. Salkinoja-Salonen<sup>1</sup><sup>1</sup> Department of Applied Chemistry and Microbiology, University of Helsinki, POB56, FI 00014 Helsinki University, Finland<sup>2</sup> Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Közép Fásor 52, H-6726 Szeged, Hungary**Keywords**

acrebol, antimycin, mitochondrial toxicity, moldy building, myxothiazol, sperm toxic.

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2008/1170: received 8 July 2008, revised 18 August 2008 and accepted 3 September 2008

doi:10.1111/j.1365-2672.2008.04062.x

**Abstract****Aims:** To identify a toxin and its producer isolated from woody material in a building where the occupants experienced serious ill health symptoms.**Methods and Results:** Hyphal extracts of an indoor fungus, identified as the cycloheximide-tolerant species *Acremonium exuviarum*, inhibited motility of boar spermatozoa ( $EC_{50}$   $5 \pm 2 \mu\text{g}$  of crude solids  $\text{ml}^{-1}$ ) and caused cytolysis of murine neuroblastoma cells (MNA) and feline fetal lung cells (FL). The responsible substances were purified and identified as two structurally similar, heat-stable, novel, toxic peptaibols, 1726 Da and 1740 Da, respectively, with amino acid sequences of Acetyl-Phe-Iva/Val-Gln-Aib-Ile-Thr-Leu-Aib-Pro-Aib-Gln-Pro-Aib-(X-X-X)-SerOH and Acetyl-Phe-Iva/Val-Gln-Aib-Ile-Thr-Leu-Val-Pro-Aib-Gln-Pro-Aib-(X-X-X)-SerOH. Purified acrebol inhibited motility of boar sperm, depleted ATP half-content in 1 day ( $EC_{50}$  of  $0.1 \mu\text{g ml}^{-1}$ ,  $60 \text{ nmol l}^{-1}$ ) depolarised the mitochondria after 2 days, but did not affect the cellular content in NADH. This indicates mitochondrial toxicity. Plate-grown biomass of *A. exuviarum* BMB4 contained 0.1–1% (w/w) of acrebol, depending on the culture medium.**Conclusions:** Acrebol paralysed the energy generation of mammalian cells suggesting that mitochondria were its target of action.**Significance and Impact of the Study:** *Acremonium exuviarum*, as an indoor fungus, is potentially hazardous to health because of the toxic peptaibols that it produces.**Introduction**

Moulds and bacteria are frequent indoor colonizers in moisture-damaged buildings. Bioactive microbial secondary metabolites toxic to mammalian cells are known to occur in water-damaged indoor building material (Andersson *et al.* 1997, 2002; Mikkola *et al.* 2004; Brasel *et al.* 2005). Microbial metabolites are released from contaminated surfaces as respirable fragments (particles below  $2 \mu\text{m}$  in size) into the indoor air of mouldy buildings (reviewed by Gorny and Dutkiewicz 2002; Bloom *et al.* 2007). Exposure to the toxic and bioactive metabolites may contribute to the multivariate symptoms experienced by the occupants of the mouldy buildings (Johanning 1995; Curtis *et al.* 2004; Putus 2005).

Indoor isolates of *Stachybotrys* spp. (Peltola *et al.* 2002; Andersen *et al.* 2003) and *Trichoderma harzianum* (Peltola *et al.* 2001, 2004) have been shown to be toxinogenic. Moulds frequently reported from damaged indoor environments include also species from genera *Acremonium*, *Alternaria*, *Cladosporium*, *Penicillium* and *Wallemia* (Curtis *et al.* 2004; Nielsen *et al.* 2004; Krause *et al.* 2006; De la Luz *et al.* 2007; Pietarinen *et al.* 2008) but their significance to human health is unclear (Lacey 1994). Identifications to species level and toxigenic potential of the indoor contaminants are only rarely reported (Gravesen *et al.* 1994).

In this paper, we screened for toxic metabolites from the microbiota of indoor material from a residence where the occupant(s) experienced building-associated serious ill health symptoms. To detect toxigenicity, we used a new application of the previously described rapid sperm

microassay (Andersson *et al.* 2004) in combination with a detection method for mitochondrial toxins (Hoonstra *et al.* 2003). In this paper, we describe the identification of a new toxigenic indoor fungus, *Acremonium exuviarum* and the structure and cell toxicity of a novel peptaibol named acrebol, produced by this fungus.

## Materials and methods

### Screening for toxic microbes in building materials from a moisture-damaged residence

Wood-based building material (0.5 g) from a water-damaged residence of a Finnish family in St Petersburg, Russia, was rehydrated in polyethylene glycol-peptone water (Andersson *et al.* 1995) for 2 h at +4°C and then plated on tryptic soy agar (TSA; Difco™). The plates were incubated at 16°C to attenuate growth of fast-growing species. The plates contained no fungicide or bactericide. After 3 weeks of incubation, colonies that formed inhibition zones against bacteria or fungi growing on the same plate were picked for toxicity assay. The biomass (2–15 mg) taken from each colony selected from the primary plate was suspended in ethanol (25–50 mg ml<sup>-1</sup>) and heated for 10 min in boiling a water bath. After cooling, aliquots of 5 µl of the extracts were used to expose boar sperms (5 million in 0.2 ml of extended semen) and motility of the sperms was inspected microscopically after 15 min, using a heated stage, as described in Andersson *et al.* (2004).

### Strains, culture media and chemicals

The strain BMB4 was deposited as DSM21752 at the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). *Acremonium exuviarum* CBS 110649 and *Acremonium tubakii* CBS 110360 (type strain) were obtained from the Centraal Bureau voor Schimmelcultures (CBS, Utrecht, The Netherlands); *Aspergillus niger* AVII was from our own collection.

TSA was from Difco™ (Becton Dickinson&Co), malt extract agar (MEA) from Biokar (Beauvais, France) and potato dextrose agar (PDA) from Biokar Diagnostics (Allone, France). Alamethicin (from *Trichoderma viride*), antimycin A, cycloheximide, myxothiazol and valinomycin were from Sigma (St Louis, MO, USA). Purified amylosin was prepared as described in Mikkola *et al.* (2007). Other chemicals were of analytical grade and purchased from local suppliers.

### Preparation of fungal extracts for toxicity assays

Fungal mycelium (500 mg) harvested from agar plates was extracted with methanol as described by Mikkola

*et al.* (2004). Alternatively, the harvested fungal biomass was flooded with 100 ml of ethanol overnight, then centrifuged, and the supernatant was evaporated to dryness at 70°C, weighed and redissolved in ethanol (99%) to concentrations of 10 mg dry solids ml<sup>-1</sup>. The extracts were heated in a boiling water bath for 15 min in capped tubes before use for toxicity assays.

Solubility of the extracted toxic substances in pentane was assessed by evaporating 500 µl of the ethanol extract (10 mg of dissolved solids ml<sup>-1</sup>) to dryness and then covering the dry residue with pentane (1000 µl) for 5 min. Thereafter, the pentane phase was harvested into a new vial, evaporated, the residue weighed and redissolved in ethanol. The pentane-insoluble residue was flooded with water (1000 µl) for 5 min, thereafter the water was removed to a new vial and treated as before. The residual remaining after the washes with pentane and water was redissolved in ethanol. All extracts were adjusted to concentrations corresponding to 3–10 mg of the starting material (ethanol dissolved dry solids) ml<sup>-1</sup>. All extracts were heated at 100°C for 15 min and the toxicity titres were assessed using two-step serial dilutions.

### Characterization of the toxic organism

Light microscopy was performed with phase-contrast microscope from plate cultures. For field emission scanning electron microscope (FESEM), the strain BMB4 was cultivated on TSA-agar-coated glass coverslips for 5 days at room temperature and fixed, dehydrated and examined as previously described (Raulio *et al.* 2008).

Tolerance to cycloheximide (500 µg ml<sup>-1</sup>) was tested on TSA plates. The activity of the applied cycloheximide was confirmed by inhibition of *Asp. niger* AVII (isolated from respirable dust) used as positive control.

### Molecular analyses

Mycelia grown on yeast extract-glucose medium (0.5 g l<sup>-1</sup> yeast extract, 10 g l<sup>-1</sup> glucose, 20 g l<sup>-1</sup> agar l<sup>-1</sup>) at 25°C for 10 days were subjected to DNA isolation by the Gene Elute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Universal fungal primers were used to amplify the internal transcribed spacer (ITS) region of the rRNA gene complex incorporating ITS 1, the 5.8 rRNA gene and ITS 2 (White *et al.* 1990). The amplifications were performed in 50-µl volumes containing 5 µl of 10× polymerase chain reaction (PCR) buffer (ZenonBio, Szeged, Hungary), 200 µmol l<sup>-1</sup> of each dNTP, 2.5 mmol l<sup>-1</sup> of MgCl<sub>2</sub>, 0.5 µmol l<sup>-1</sup> of each primer (ITS1: 5'-TCCGTAGG-TAACCTGCGG-3'; and ITS4: 5'-TCCTCCGCTTATTGATATGC-3'), 2.5 U of *Taq* DNA polymerase (ZenonBio)

and 5  $\mu\text{l}$  of template DNA. PCR reactions were carried out in a T3 thermocycler (Biometra, Göttingen, Germany) with 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min and a final elongation step at 72°C for 1 min. The product was purified using GeneElute™ MINUS ETBrSPIN COLUMNS (Sigma-Aldrich) and then sequenced using the ITS4 primer on an ABI 373 A DNA sequencer (Applied Biosystems Inc., Foster City CA, USA). The sequence (481 bp) was deposited at the NCBI Genbank (accession number: EU706288).

The ITS region sequence comparisons were carried out by BLASTN similarity search (Altschul *et al.* 1990) at the website of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST>). The pairwise alignments were further compared using EMBOSS Matcher program (Huang and Miller 1991) run at the computers of CSC, Espoo, Finland.

#### Cell toxicity assessment of the fungal extracts

Commercially available extended boar semen intended for artificial insemination (AI) and pig breeding was obtained from an artificial insemination station (FABA Sika OY Rauhahlinna, Finland). The semen was extended to 14% in MRA semen extender and contained  $27 \times 10^6$  sperm cells  $\text{ml}^{-1}$  and was used for toxicity testing on the second and third days after preparation. Inhibition of sperm motility, depletion of the electric transmembrane potentials of the mitochondrial membrane ( $\Delta\Psi_m$ ), hyperpolarization of the plasma membrane ( $\Delta\Psi_p$ ) and damage to the integrity barrier of the plasma membrane were measured as described in Hoornstra *et al.* (2003) and Kotiaho *et al.* (2008). Cytotoxicities against somatic cells were tested with feline fetus lung cells (FL) as described previously (Andersson *et al.* 1997) and with murine neuroblastoma cells (MNA) as described by Kulonen *et al.* (1991). ATP and NADH contents of the sperm cells were measured as described by Mikkola *et al.* (2004). The exposures were performed by dispensing 20  $\mu\text{l}$  of the fungal extract or pure substance or dilutions thereof (in ethanol) into 2 ml of the target cell suspension. The toxicity assays were executed as three independent experiments and calibrated with valinomycin.

#### High-performance liquid chromatography (HPLC) purification and quantification of peptaibols

The methanol extract of strain BMB4 was fractionated by reverse phase (RP)-HPLC using a 1100 series LC (Agilent Technology, Wilmington, Del. USA). The column used was Atlantis C18 T3 4.6  $\times$  150 mm, 3  $\mu\text{m}$  (Waters, Milford, MA, USA). The separation of peptaibols was at a

constant flow rate of 1  $\text{ml min}^{-1}$  with a gradient elution that used 0.1% formic acid (A) and methanol (B) as solvents and which deliver 80% B from 0 to 20 min, 80–100% B from 20 to 30 min and 100% B from 30 to 40 min at a flow rate of 1  $\text{ml min}^{-1}$ . For detection, absorbances at wavelengths of 215, 240, 254 and 280 nm were used. The methanol extract of strain BMB4 was fractionated by RP-HPLC using 1100 series LC with DAD detector (Agilent Technology). Fractions were collected once a minute. Concentrations of the peptaibols in the methanol extracts were determined with HPLC (at 215 nm) using alamethicin standards (at 215 nm) as a reference peptaibol.

#### HPLC-mass spectrometry (MS) of peptaibols

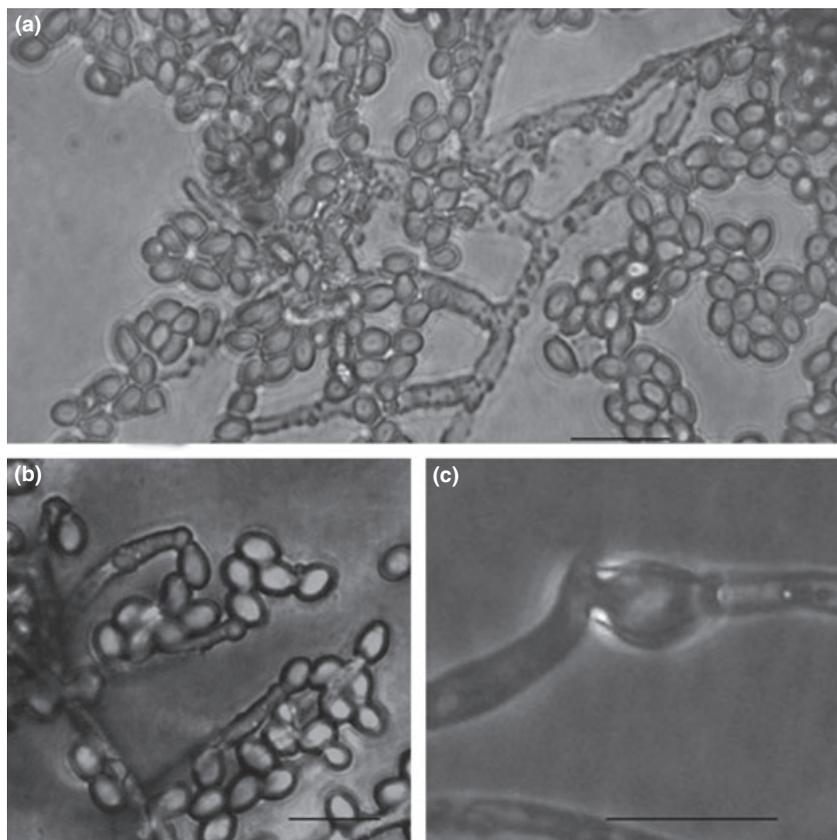
Electrospray ionization ion trap mass spectrometry analysis (HPLC-ESI-IT-MS) was performed using an MSD-Trip-XCT\_plus ion trap mass spectrometer equipped with Agilent ESI source and Agilent 1100 series LC (Agilent Technologies). The HPLC-ESI-IT-MS was performed using positive mode in the mass range of 50–2000  $m/z$ . The elution and run conditions were as described before.

## Results

#### Toxinogenic *Acremonium exuviarum* isolated from a moisture-damaged residence

Decayed wood-based indoor material from a building, where the occupants suffered from indoor air-related, serious ill health symptoms, was screened for toxin-producing microbes. When cultured on TSA, the material yielded  $10^4$ – $10^5$  CFU  $\text{g}^{-1}$  of bacteria and fungi. The plate contained no fungicide or antibiotic to suppress fungal or bacterial growth. Sixty-seven colonies were selected from the primary plates based on their capability to suppress the growth of co-growing bacteria and fungi. Ethanol and methanol extracts of these colonies were prepared and their toxicity was tested using boar spermatozoa as test cells, with motility inhibition within 15 min of exposure as the measured end point. The isolate with the highest toxicity titre ( $\text{EC}_{50} < 10 \mu\text{g}$  of the ethanol-soluble substance  $\text{ml}^{-1}$ ) was selected for characterization.

The toxinogenic isolate BMB4 was a cycloheximide-tolerant filamentous fungus extensively producing ovoid to subglobose conidia of 2–3  $\mu\text{m}$  in diameter, in chains (Figs 1 and 2). The partial sequence (488 bp) of the ITS 1–5.8S rRNA region was determined and found to match in the NCBI Nucleotide database 100% with *A. exuviarum* (Table 1). The strain BMB4 was morphologically similar to the recently described type strain of *A. exuviarum* (CBS 113360). The conidia are formed in chains (Figs 1



**Figure 1** Phase-contrast view of the toxic fungus, *Acremonium exuviarum* BMB4. The panels show hyphae and loose chains of conidia. (a) Collapsing hyphae bearing short, undifferentiated conidiophore (bar, 10  $\mu\text{m}$ ); (b) conidiogenous phialides borne in verticils on short undifferentiated conidiophores (bar, 10  $\mu\text{m}$ ); (c) a putative chlamydospore (bar, 20  $\mu\text{m}$ ). Objective 40 $\times$ . The culture was grown on potato dextrose agar for 5 days.

and 2b,e) and are released from short conidiophores that appear to collapse (Fig. 1a). The FESEM micrographs (Fig. 2) show that the subglobose formed, small-sized conidia, <4  $\mu\text{m}$  in diameter. The small size and the lack of any structures around the conidia indicate facility of airborne spreading. The indoor strain BMB4 was resistant to cycloheximide (500  $\mu\text{g ml}^{-1}$ ) similar to the type strain of this mitosporic ascomycete (Sigler *et al.* 2004).

Pairwise comparison of the ITS region revealed close relationship of *A. exuviarum* especially with species belonging to the genera *Stanjemonium* and *Emericellopsis*. However, with *Acremonium alternatum* (the type species of the genus), the similarity was relatively low, about 80% (Table 1).

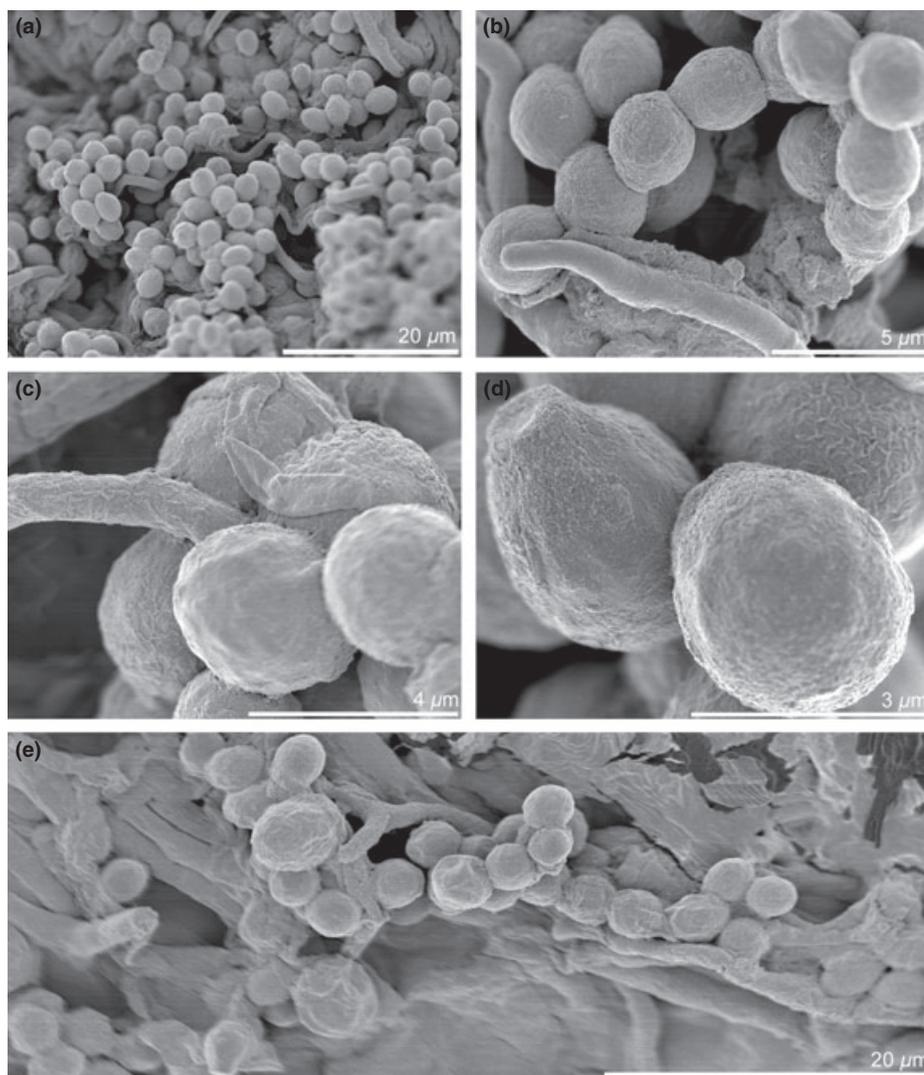
#### Toxicity of fungal extracts prepared from *Acremonium exuviarum*

Toxicity of extracts prepared from *A. exuviarum* BMB4, CBS 113360 and *A. tubakii* CBS 110649 was assessed using boar spermatozoa, FL and MNA cells as targets. The results in Table 2 show that heat-stable methanol-soluble metabolites produced by *A. exuviarum* CBS 113360 and BMB4 inhibited sperm motility after a short exposure (30 min) and a low dose (7  $\mu\text{g d.s. ml}^{-1}$ ).

Depolarization of mitochondria occurred at 10 times higher exposure concentrations, thus representing an effect independent of the inhibition of sperm motility. The permeability barrier of the plasma membrane (exclusion of propidium iodide) was not damaged by these exposures. Similarly prepared extract from *A. tubakii* CBS 110649 had no effect on these toxicity parameters even at 10-fold higher concentrations.

The heat-stable metabolites extracted from *A. exuviarum* BMB4 were cytotoxic to MNA and FL cells at concentrations (2–16  $\mu\text{g d.s. ml}^{-1}$ ) similar to those causing sperm motility inhibition. Interestingly, MNA cells were eight times more sensitive than FL cells: cytolysis occurred after exposure to 2  $\mu\text{g d.s. ml}^{-1}$  of the extract. The toxicity of *A. exuviarum* BMB4 harvested from potato dextrose agar (PDA) or malt extract agar (MEA) plates was 10 times higher than that harvested from TSA plates, and the toxin content of the hyphal biomass increased with culture age from 5 to 14 days (data not shown). The toxin(s) from *A. exuviarum* were more soluble in pentane than in ethanol or methanol but insoluble in water.

The toxic effects on boar sperm cells of *A. exuviarum* BMB4 are visualised in Fig. 3. The spermatozoa exposed to motility-inhibiting concentrations (0.2 to up to



**Figure 2** Field emission electron microscopic images (FESEM) of *Acremonium exuviarum* BMB4 grown on tryptic soy agar for 10 days. The images show hyphae with diameters from 0.5  $\mu\text{m}$  to 3  $\mu\text{m}$  and loose chains of subglobose conidia, 3–4  $\mu\text{m}$   $\times$  2–3  $\mu\text{m}$ . (a,b) Hyphae and chains of conidia, (c,d) collapsing hyphae and subglobose conidia, (e) conidia, conidiophores and hyphae.

**Table 1** Pairwise nucleotide similarity comparison of the internal transcribed spacer region among selected fungi

	<i>Acremonium exuviarum</i> CBS 113360	<i>Stanjemonium ochroroseum</i> CBS 656.79	<i>Emericellopsis donezkii</i> CBS 489.71	<i>Acremonium tubakii</i> CBS 111360	<i>Acremonium alternatum</i> isolate 2261	<i>Bionectria ochroleuca</i> CBS 193.94
The strain BMB4 (this study)	100	97.3	97.1	96.2	82.5	79.5
<i>Stanjemonium ochroroseum</i>		100	96.1	96.0	82.7	80.1
<i>Emericellopsis donezkii</i>			100	97.4	81.6	79.6
<i>Acremonium tubakii</i>				100	82.5	77.5
<i>Acremonium alternatum</i>					100	79.8

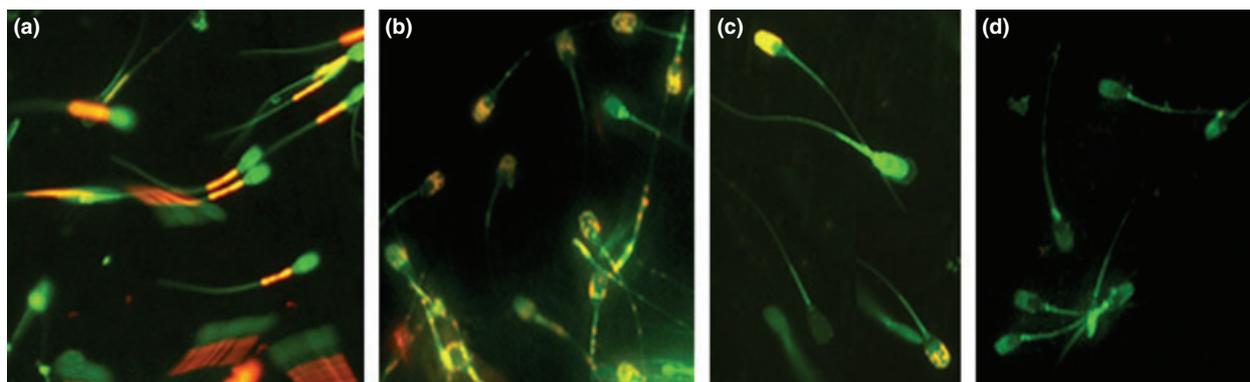
The pairwise alignments (481 bp) were compared using emboss Matcher program (Huang and Miller 1991) run at the computers of CSC, Espoo, Finland. GenBank nucleotide accession numbers: the strain BMB4 (EU706288), *A. exuviarum* CBS 113360 (AY882946), *A. alternatum* isolate 2261 (AM924160), *A. tubakii* CBS111360 (AY632654), *S. ochroroseum* CBS 656.79 (AY632672), *E. donezkii* CBS 489.71 (AY632658), *B. ochroleuca* CBS 193.94 (AF210686).

**Table 2** Toxicity of metabolites from *Acremonium exuviarum* and *Acremonium tubakii* towards boar spermatozoa, feline fetus lung cells (FL) and murine neuroblastoma cells (MNA)

Target cell:	Boar spermatozoa, loss of							FL	MNA		
	Motility			$\Delta\Psi_m$			Plasma membrane integrity	Cytotoxicity			
Exposure time:	30 min	1 day	3 days	30 min	1 day	3 days	3 days	1 day	4 days	1 day	4 days
Fungal strain	EC <sub>50</sub> $\mu\text{g ml}^{-1}$ (dissolved substances from the crude extract of hyphal biomass)										
<i>A. exuviarum</i> BMB4	7	6	3	66	24	6	>50	>300	16	150	2
<i>A. exuviarum</i> CBS 113360	10	10	5	70	40	10	ND	ND	ND	ND	ND
<i>A. tubakii</i> CBS 110649	>250	>100	50	>250	>100	100	ND	ND	ND	ND	ND

The toxicity was assayed by exposing the mammalian target cells to heat-treated (100°C) methanol extracts prepared from plate cultures (5 days at 20–22°C) of the *Acremonium* strains grown on tryptic soy agar plates. The EC<sub>50</sub> values were calculated from the toxicity titres of the fungal extracts, obtained by three parallel assays with a dilution step of 2. Loss of the mitochondrial membrane potential ( $\Delta\Psi_m$ ) was observed as the shift of orange fluorescence emission to green of JC-1-stained cells. Loss of the plasma membrane integrity was recorded at permeability to propidium iodide in viability stained cells. Cytotoxicity was visible as cell lysis.

ND, not determined.



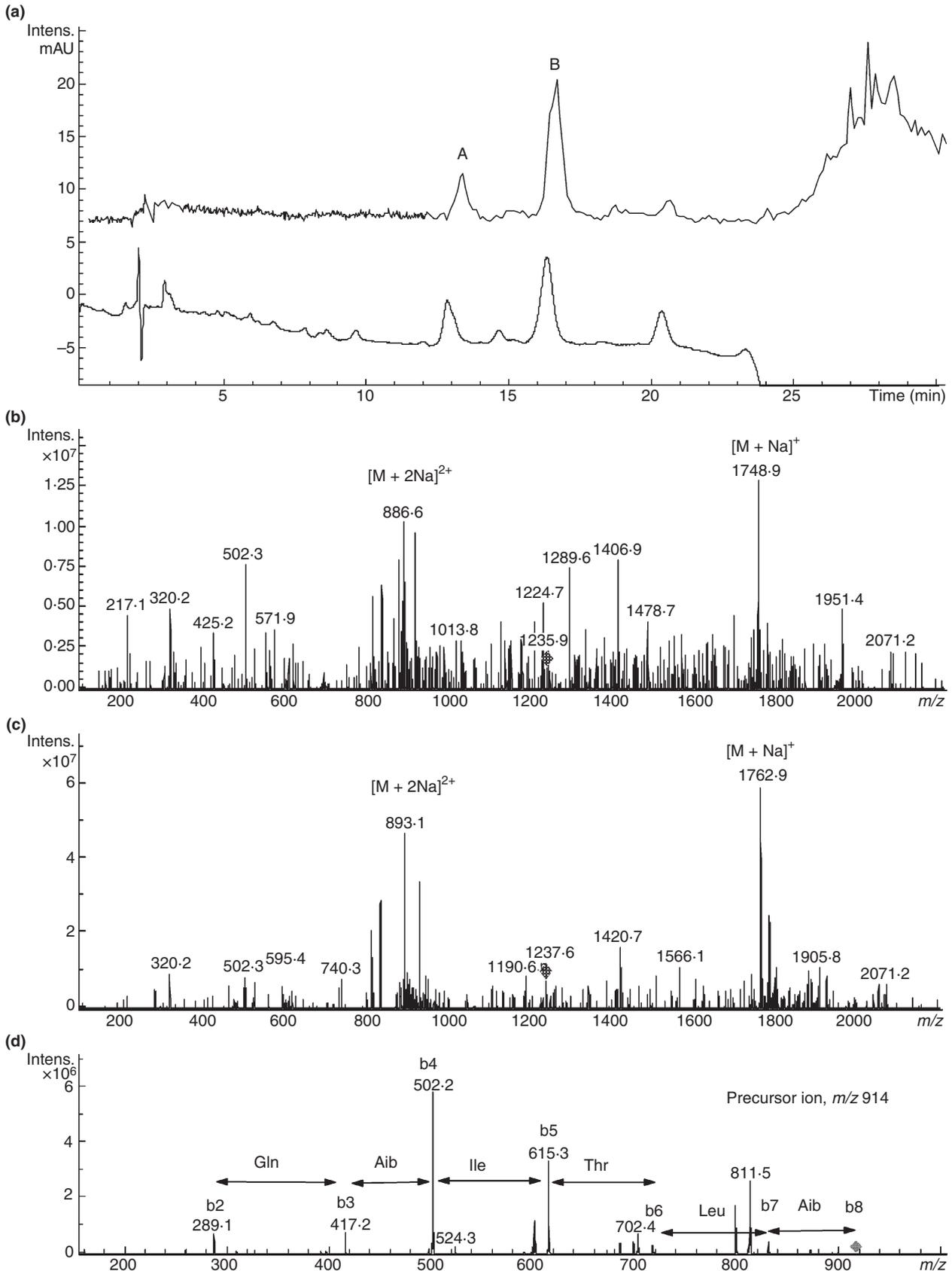
**Figure 3** Effects of increasing concentrations of cell extracts from PDA grown *Acremonium exuviarum* BMB4 on the electric transmembrane potentials of boar sperm cells after 1 day of exposure. After exposure, the sperms were stained with the membrane potential stain JC-1. The panels show epifluorescence images of the sperms exposed to: (a) 0  $\mu\text{g}$ ; (b) 1.4  $\mu\text{g ml}^{-1}$ ; (c) 2.8  $\mu\text{g ml}^{-1}$ ; (d) 9.0  $\mu\text{g ml}^{-1}$  of ethanol-soluble, heat-treated substances of the fungal extract. Bright orange fluorescence emission of JC-1 indicates high  $\Delta\psi$  (–140 mV) and bright green indicates lower  $\Delta\psi$  (–100 mV) of the interior side of the membrane. The vehicle-exposed sperm cells (a) and those exposed to  $\leq 0.8 \mu\text{g ml}^{-1}$  (not shown) of the extract fluoresced orange in the mid piece (location of the mitochondria,  $\Delta\psi_m$ ) and green in the head (plasma membrane,  $\Delta\psi_p$ ). (b) This shows cells with decreased  $\Delta\psi_m$  (green) in the mid piece. The head is hyperpolarized, indicated by yellow patches amidst the green fluorescence. (c) This shows depolarized (green) mitochondria, some cells with hyperpolarized head (yellow patches). (d) This shows sperm cells (pale green) that have lost both  $\Delta\psi_m$  and  $\Delta\psi_p$ . Scale: the sperm head is c. 5  $\mu\text{m} \times 7 \mu\text{m}$ .

0.8  $\mu\text{g d.s. ml}^{-1}$ ) of the extract from *A. exuviarum* BMB4 showed no loss of the electric transmembrane potentials of the plasma membrane ( $\Delta\Psi_p$ ) or of the mitochondria ( $\Delta\Psi_m$ ). Thus, the sperm motility was inhibited at concentrations lower than required for depolarizing the mitochondria.

#### The toxic compound from *Acremonium exuviarum* BMB4 is a novel type of peptaibol

HPLC analysis of the toxic extracts of *A. exuviarum* BMB4 (Fig. 4a) contained only two peaks (eluting at 13 min and 16 min) toxic in the boar sperm motility

**Figure 4** High-performance liquid chromatography-mass spectrometry (MS) analysis of heat-stable substances extracted from *Acremonium exuviarum* BMB4. (a) The total ion (upper curve) and ultraviolet (UV; 215 nm, lower curve) chromatograms of methanol extract of the strain BMB4 (the toxic fractions are marked as A and B). (b) The single- and double-charged sodium-cationized molecular ions at  $m/z$  1749 and 886 of compound A. (c) The single- and double-charged sodium-cationized molecular ions at  $m/z$  1763 and 893 of compound B. (d, e) The fragmentation pattern (b series ions) of compound A obtained by MS/MS analysis selecting the precursor ions at  $m/z$  914 and 1407. (f, g) The fragmentation pattern (b series ions) of compound B obtained by MS/MS analysis selecting the precursor ions at  $m/z$  928 and 1238. (h) The MS4 analysis using the precursor ion at  $m/z$  1225. (i, j) The MS/MS analysis of the compounds A and B using sodium-cationized precursor ions at  $m/z$  1749 and 1763, respectively.



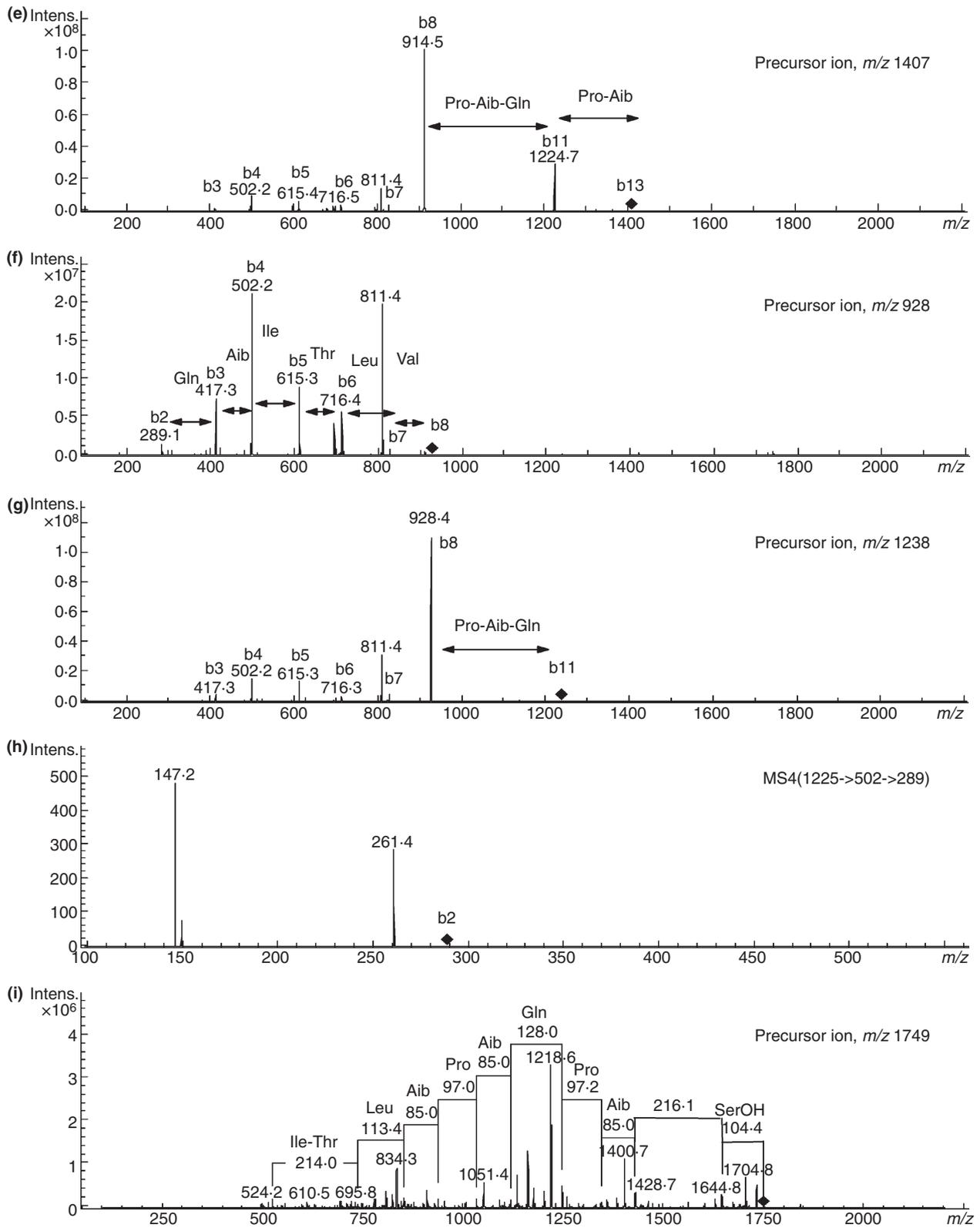


Figure 4 (Continued)

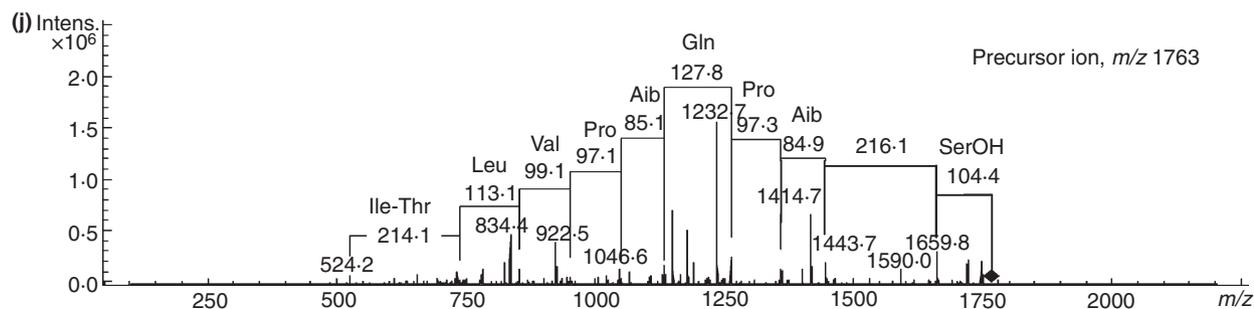


Figure 4 (Continued)

assay. Mass spectrometric analysis of peak A (13 min) gave a single charged sodium adduct  $[M+Na]^+$  at  $m/z$  1748.9 (highest abundance), a double charged  $[M+2Na]^{2+}$  adduct at  $m/z$  886.1 and a double charged  $[M+2H]^{2+}$  adduct at  $m/z$  864.5 (Fig. 4b). These mass ions matched the molecular weight 1726, indicated as compound A. Peak B (16 min) contained single charged sodium adduct  $[M+Na]^+$  at  $m/z$  1762.9 (highest abundance), double charged  $[M+2Na]^{2+}$  adduct at  $m/z$  893.1 and double charged  $[M+2H]^{2+}$  adduct at  $m/z$  871.4 (Fig. 4c). These mass ions matched the molecular weight of 1740, indicated as compound B.

The amino acid sequences of the compounds A and B were determined by tandem mass spectrometric analysis (MS/MS), which showed that both compounds contained aminoisobutyric acid (Aib). The *N*-termini were acetyl modified phenylalanines (AcPhe) and the *C*-termini were SerOH, suggesting that they are peptaibols. Amino acid sequences of the two peptaibols were partially resolved. The MS/MS analysis showed that b series ions arose from fragmentation of the precursors  $m/z$  at 914 (Fig. 4d) and 1407.6 (Fig. 4e) of peptaibol A. Using the precursor ion  $m/z$  914 (Fig. 4d) the b2–b8 series mass ions were detected corresponding to the amino acid sequence Gln-Aib-Thr-Leu-Aib. The mass ion  $m/z$  at 289.1 (b2) matched an acetylated *N*-terminal sequence AcePhe-Iva/Val. The MS4 analysis also showed that the

*N*-terminus contained Phe (Fig. 4h). Figure 4e shows b series mass ions b8–b11 corresponding to the amino acid sequences Pro-Aib-Gln and b11–b13 mass ions corresponding to Pro-Aib obtained using precursor ion  $m/z$  at 1407.

Figure 4f and g shows a similar b series of mass ions from the precursor ion  $m/z$  at 928 and 1238 of the peptaibol B as before (Fig. 4d,e) shown for peptaibol B. However, the peptaibol B contained Val (b7–b8) instead of Aib (b7–b8, Fig. 4d). Figure 4b and c revealed that the fragmentation of peptaibols already occurred in the ion source (ESI) as some of the b series ions are visible at  $m/z$  502, 914, 1224 and 1407 (peptaibol A, Fig. 3b) and at  $m/z$  502, 928, 1238 and 1421 (peptaibol B, Fig. 4c).

The MS/MS spectra of the sodium-cationized precursor ions at  $m/z$  1749 (peptaibol A) and 1763 (peptaibol B; Fig. 4i,j) confirmed the b series mass ions (b4–b13) of the amino acid sequence Ile-Thr-Aib-Leu-Pro-Aib-Gln-Pro-Aib (peptaibol A) and Ile-Thr-Val-Leu-Pro-Aib-Gln-Pro-Aib (peptaibol B). Specifically, the b series ions (b8–b13) of the amino acid sequences Pro-Aib-Gln-Pro-Aib of peptaibols A and B were found. The *C*-termini of the two peptaibols were SerOH (Fig. 4i,j). Peptaibols A and B had identical toxic effects on exposed spermatozoa when tested separately (data not shown). The peptaibols A and B were therefore combined and named acrebol. Table 3 summarizes the deduced amino acid sequence of

**Table 3** Deduced partial amino acid sequences of the acrebols A and B produced by *Acremonium exuviarum* BMB4. The sequences of cephaibols from *Acremonium tubakii* are shown for comparison\*

Peptaibol	Residue no.																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
	Sequence																	
Acrebol A	Ace	Phe	Iva/Val	Gln	Aib	Ile	Thr	Leu	Aib	Pro	Aib	Gln	Pro	Aib	X	X	X	SerOH
Acrebol B	Ace	Phe	Iva/Val	Gln	Aib	Ile	Thr	Leu	Val	Pro	Aib	Gln	Pro	Aib	X	X	X	SerOH
Cephaibol Q	Ace	Phe	Iva	Gln	Aib	Ile	Thr	Aib	Leu	Aib	Pro	Gln	Aib	Hyp	Aib	Pro	Phe	SerOH
Cephaibol P	Ace	Phe	Iva	Gln	Aib	Ile	Thr	Aib	Leu	Aib	Hyp	Gln	Aib	Hyp	Aib	Pro	Phe	SerOH

\*Schiell et al. (2001).

X, uninterpreted residue(s); Ace, acetyl; Aib, aminoisobutyric acid; Hyp, hydroxyproline.

acrebols A and B and shows differences between those and the closest similar known peptaibols (cephaibols Q and P).

### Cell toxicity of the purified acربول

The combined acrebols were investigated for toxicity using boar sperm cells similar to the crude extracts of metabolites (Table 2, Fig. 3). Acربول (100 ng ml<sup>-1</sup>) inhibited sperm motility after 1 and 2 days of exposure

(Table 4). The sperm cells displayed no change in any membrane potential ( $\Delta\Psi_m$  or  $\Delta\Psi_p$ ; Fig. 5a,b) after 1 day of exposure to 100–300 ng ml<sup>-1</sup> of acربول. After exposure to 400 ng of purified acربول, the majority of the sperm cells were immotile but exhibited preserved  $\Delta\Psi_m$  and  $\Delta\Psi_p$ . A minority of the cells exhibited hyperpolarized  $\Delta\Psi_p$  in the head. Exposure to 800 ng ml<sup>-1</sup> of acربول depolarized the mitochondria in 1 day and most of the sperm heads exhibited hyperpolarized plasma membranes. After exposure to 1200 ng of acربول, the transmembrane

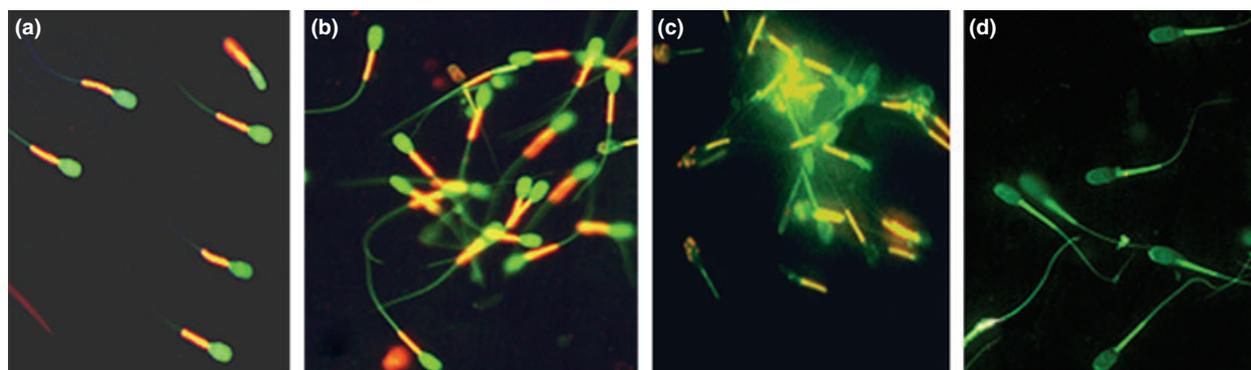
**Table 4** Toxic endpoints of acربول, the purified toxin from *Acremonium exuviarum* BMB4, and selected microbially produced reference substances on boar spermatozoa as target cells

Exposure time	Toxic end point†, EC <sub>50</sub> , ng dry substance ml <sup>-1</sup> of extended boar semen					
	Motility inhibition	Depolarization of mitochondria		Depletion of cellular		Plasma membrane integrity
		1 and 2 days	1 day	3 days	ATP	
Acربول	100	800	100	100	ND† (>1000)	ND (>4000)
Reference substances with known functions						
Alamethicin – Cation channel former*	150	150	150	150	150	150
Amylosin – Cation channel former‡	30	30	30	30	30	ND (>600)
Valinomycin – Potassium ionophore*	2	2	2	ND (>20)	ND (>1000)	ND (>1000)
Oligomycin – Inhibitor of mitochondrial ATP synthase*	500	ND (>50 000)	ND (>5000)	ND (>5000)	ND (>5000)	ND (>50 000)
Myxothiazol – Inhibitor of cytochrome b-c1 segment*	1	>100	1	1	ND (>200)	ND (>100)
Antimycin A – Inhibitor of electron transfer at complex III*	5	>100	5	5	ND (>100)	ND (>100)

\*Sigma catalogue (2004–2005).

†Measured and calculated as for Table 2; ND, not detectable (the highest tested concentration).

‡Mikkola *et al.* (2007).



**Figure 5** Effects of purified acربول on the electric transmembrane potentials,  $\Delta\Psi_m$  and  $\Delta\Psi_p$  of boar sperm cells after 1 day of exposure. The staining with JC-1 and microscopy were carried out as for Fig. 3. The lowest concentration of pure acrebols A and B that immobilized the sperms was 100 ng ml<sup>-1</sup>. (a) Sperms exposed to the vehicle (ethanol) only; (b) sperms exposed to 300 ng ml<sup>-1</sup> of acربول. The cells were immotile but the  $\Delta\Psi_m$  and  $\Delta\Psi_p$  of the sperm cells were similar to those of the vehicle-exposed cells in (a); (c) 400 ng ml<sup>-1</sup> of immotile sperm cells with mostly preserved  $\Delta\Psi_m$ . c. 20% of the sperm cells had depolarized mitochondria and spotwise hyperpolarized head; (d) 1000 ng ml<sup>-1</sup> of acربول. All cells were immotile and both  $\Delta\Psi_m$  (mid piece) and  $\Delta\Psi_p$  (head) were lost. Scale: the sperm head is c. 5  $\mu\text{m}$   $\times$  7  $\mu\text{m}$ .

**Table 5** Amino acid sequences of the peptaibols from *Acremonium exuviarum* BMB4, from *Acremonium* spp. and its teleomorph *Emericellopsis* spp. and reference peptaibols

Peptaibol and its producer	Sequence†
Acrebol A*; <i>A. exuviarum</i> BMB4	AcF J/V Q U I T L U P U Q P U — S OH**
Acrebol B*; <i>A. exuviarum</i> BMB4	AcF J/V Q U I T L V P U Q P U — S OH
Cephaibol Q‡; <i>Acremonium tubakii</i> FH	AcF J Q U I T U L U O Q U O U P F S OH
Cephaibol P‡; <i>A. tubakii</i> FH	AcF J Q U I T U L U P Q U O U P F S OH
XR586‡; <i>Acremonium persicinum</i>	AcW J Q U I T U L U P Q U O J P F G OH
Emerimicin IIA‡; <i>Emericellopsis microspora</i>	AcW I Q U I T U L U O Q U O U P F OH
Zervamicin ZII2‡; <i>Emericellopsis salmosynnemata</i>	AcW I Q U I T U V U O Q U O U P F OH
Zervamicin ZII3‡; <i>Emericellopsis salmosynnemata</i>	AcW V Q U I T U L U O Q U O U P F OH
Zervamicin ZIIA‡; <i>Emericellopsis salmosynnemata</i>	AcW I Q U I T U L U O Q U O U P F OH
Reference peptaibols	
Alamethicin; <i>Trichoderma brevicompactum</i> complex§	AcU P U A U A Q U V U G L U P U U E Q F
Peptaibol ES39 IV and V; <i>Trichoderma harzianum</i> Rifai¶	AcU S A U L/I Q V/J L/I U A L/I U P L/I U U Q L OH

\*From Table 3.

†Schiell *et al.* (2001) and Vertesy *et al.* (2003).

‡<http://www.cryst.bbk.ac.uk/peptaibol/welcome.html>.

§O'Reilly and Wallace (2003) and Degenkolb *et al.* (2006).

¶Peltola *et al.* (2004).

\*\*— is uninterpreted residue(s); Ac is acetyl; U is aminoisobutyric acid; O is hydroxyproline; J is isovaline.

electric potentials were lost as indicated by only weak green emission (Fig. 5d). These patterns of toxic effects were similar to those obtained with the crude extracts (Table 2), indicating that acrebol was the causative agent. Based on the toxicity titres measured for the crude extracts and for purified acrebol we calculated that *A. exuviarum* BMB4 mycelia contained 1 and 10 mg of acrebol g<sup>-1</sup> (dry wt.) of hyphal biomass grown on TSA and PDA, respectively.

The toxic endpoints of purified acrebol on boar spermatozoa are compared in Table 4 with those of the other microbial toxins known to affect the energy metabolism of mammalian cells. After 1 day of exposure to 100 ng ml<sup>-1</sup> of acrebol, the sperm cells lost more than half of their ATP. Exposure to 400–800 ng ml<sup>-1</sup> causes mitochondria to depolarize and the ATP content is dropped to ≤0.2 pmol l<sup>-1</sup>, i.e. close to the background value for cell-free semen extender. The cellular NADH and the permeability barrier of the plasma membrane (assessed by penetration of propidium iodide) were not affected by these exposures, indicating that the loss of sperm motility and depolarization of mitochondria were

not caused by disruption of the plasma membrane. After 2 days of acrebol exposure the NADH was still preserved, but the mitochondria became depolarized in the immobilized sperm cells. This indicated inhibition of mitochondrial functions.

Table 4 shows that the toxic effects provoked by acrebol were different from those of amyloisin and the peptaibol alamethicin. These substances depleted the sperm cells from ATP and NADH at the same exposure concentrations that inhibited motility. Alamethicin (but not amyloisin) destroyed the plasma membrane permeability barrier. The potassium ionophore valinomycin had no effect on the sperm cell ATP although it simultaneously depolarized mitochondria and immobilized the cells. Acrebol depolarized the mitochondria of sperm cells only long after motility was lost, so the motility loss was not caused by the depolarization. Oligomycin, an inhibitor of the mitochondrial ATP synthase neither depolarized the mitochondria nor affected the cellular ATP at concentrations 10 times above those that inhibited motility. These results show that the effects of acrebol on sperm cells differed from these inhibitors. Exposure to antimycin A and

myxothiazol, blockers of the respiratory chain in the mitochondria, inhibited motility and decreased cellular ATP. The subsequent depolarization of mitochondria occurred after a lag period, and the cellular NADH was preserved. These toxic responses are very similar to those provoked by acrebol and confirmed the assumption that acrebol inhibited sperm motility by interfering with mitochondrial functions.

## Discussion

*Acremonium exuviarum* from water-damaged indoor building material was identified as a producer of a hydrophobic, heat-stable substance toxic to mammalian cells. Extracts prepared from the hyphal biomass of this fungus inhibited motility of boar sperm and caused cytolysis of FL and MNA cells at exposure concentrations of 2–7  $\mu\text{g}$  of crude extract (d. wt.)  $\text{ml}^{-1}$ . This extreme toxicity matches with an unknown toxin (other than satratoxin) from water-damaged indoor environment, emitted by *Stachybotrys chartarum* HMR4 (Peltola *et al.* 1999, 2002).

As far as we know, this paper is only the second report on *A. exuviarum*, a species for the first time isolated from cast-off skin of a lizard in a Californian zoo (Sigler *et al.* 2004). The strain isolated from moist building material in St. Petersburg, Russia shares with the original Californian isolate an identical ITS sequence and morphology and similar toxinogenicity. Its ecology is not known. *Acremonium* species are cosmopolitan filamentous fungi, common in soil and plant debris, also known for their keratinolytic activity (Marcondes *et al.* 2007) and as effective cellulase producers (Zuccaro *et al.* 2004; Ikeda *et al.* 2007). *Acremonium* and *Stanjemonium* are closely related anamorphs of the pharmaceutically important teleomorph clade *Emericellopsis* (Sigler *et al.* 2004; Ovchinnikova *et al.* 2007).

The indoor isolate, *A. exuviarum* BMB4, produced two toxic, closely similar peptaibols, acrebols A and B. Peptaibols are defined as linear amphiphatic peptides with 5–20 amino acids, rich in amino isobutyric acid (Aib), containing nonproteinogenic amino acids, with an acetylated *N*-terminus, the *C*-terminus reduced to an amino alcohol (Degenkolb *et al.* 2003) and produced non-ribosomally (Marahiel *et al.* 1997; Raap *et al.* 2005; Wei *et al.* 2005; Grünwald and Marahiel 2006). The amino acid sequence of acrebols A (1726 Da) and B (1740 Da) was resolved, excepting for 2–3 residues near the *C*-terminus. The sequences showed that they represent a novel structure. The mass of the unexplained residues was 216 Da. It is therefore expected that the acrebols A and B contain 16–17 amino acid residues. Acrebol sequence is closely similar to that of the peptaibols, cephaibol Q (1856 Da) and

cephaibol P (1872 Da) produced by *A. tubakii* strain FH (Schiell *et al.* 2001; Bunkoczi *et al.* 2003; Vertesy *et al.* 2003), which contains 17 amino acid residues. The *N*-terminal phenylalanine, *C*-terminal seryl alcohol and sequence of residues 2–6 of acrebol are similar to the cephaibols P and Q, but residues at positions 7, 8, 9, 10, 12 and 13 differ (Table 3).

More than 300 peptaibols have been described to date and were divided into nine subfamilies (SF1–9) according to their primary structure (Chugh and Wallace 2001; Whitmore *et al.* 2003; Szekeres *et al.* 2005). The cephaibols and five peptaibols produced by the closely related species *Acremonium persicinum*, *Emericellopsis microspora* and *Emericellopsis salmosynnemata* (Table 5), belong to the subfamily SF3 by the following criteria: they have an aromatic amino acid at the *N*-terminus (most members also at *C*-terminus); imino acids (proline, hydroxyproline) at positions 10, 13, 15; a highly conserved threonine at position 6 and the two glutamines, at positions 3 and 11. Acrebols A and B match with these criteria for SF3 except for the imino acids, which in acrebol were found at positions 9, 11.

The high content of  $\alpha,\alpha'$ -substituted amino acids,  $\alpha$ -aminoisobutyric acid and isovaline (indicated by letters U and J in Table 5) in acrebols A and B indicates membrane-modifying activity. These residues restrict, by steric hindrance, the range of angles their polypeptide backbones can adopt, predisposing them to helical secondary structures (Prasad and Balaram 1984; Chugh and Wallace 2001; O'Reilly and Wallace 2003; Raap *et al.* 2005). Acrebols contained  $\alpha$ -aminoisobutyric acid and isovaline at positions 2, 4, 8, 10 and 13, except for position 8 of acrebol B where it was valine (Table 5). This 2-4-8-10-13 J/U motif may be important for the high toxicity of the acrebols towards mammalian cells. Similar motif is present in cephaibols P, Q (*A. tubakii*), peptaibol XR586 (*A. persicinum*), emerimicin ZII-2 (*E. microspora*) as well as in zervamicins ZII-3 and ZIIA (*E. salmosynnemata*) except that in the latter the isovaline at position 2 was replaced by isoleucine or valine (Table 5).

The known biological activities of peptaibols are based on the formation of ion-conducting channels or nonspecific permeability changes in cellular membranes leading to leakage of cytoplasmic materials and damaging the osmotic balance of the cell (O'Reilly and Wallace 2003; Szekeres *et al.* 2005; Leitgeb *et al.* 2007). In intact eukaryotic cells, the channel-forming peptaibols invade the plasma membrane and also possess affinity to the mitochondrial inner membrane because the insertion and pore formation is driven by  $\Delta\Psi_m$  (Wallace and Starkow 2000).

In this study, the toxic acrebols were detected using a bioassay based on inhibition of motility in exposed boar spermatozoa (Andersson *et al.* 1997, 2004). Motility in

boar spermatozoa may be inhibited by toxins targeting three cellular functions: (i) damaging the plasma membrane integrity barrier; (ii) forming ion-conducting channels through the plasma membrane or (iii) by interference with mitochondrial functions (Hoornstra *et al.* 2003). Toxins targeting the plasma membrane are characterized by simultaneous depolarization of the plasma membrane and mitochondria and depletion of cellular ATP and NADH. Mitochondrial toxicity in boar spermatozoa is indicated by loss of motility, instant or delayed mitochondrial depolarization, decrease in cellular ATP and preservation of cellular NADH contents (Suominen *et al.* 2001; Hoornstra *et al.* 2003; Mikkola *et al.* 2004).

Acrebol exposure rapidly inhibited sperm motility, decreased cellular amount of ATP, caused mitochondrial depolarization after a lag period but did not decrease cellular NADH. This indicates that acrebol inhibited sperm motility by not interfering with the permeability barrier or voltage-dependent cation conductance as do the peptaibols of *Trichoderma harzianum* ES39 (Peltola *et al.* 2001, 2004). The retention of NADH and the preserved mitochondrial membrane potential in sperm cells immobilized by acrebol show that glycolysis and ion balance in the cytosol were unaffected whereas the synthesis of ATP by oxidative phosphorylation, needed for maintaining the progressive motility, were inhibited. The toxic action of acrebol resembled that of the known mitochondriotoxins myxothiazol and of antimycin A, which also induced loss of ATP and depolarized the mitochondria without affecting the cell content of NADH or damaging the cell membrane integrity. However, the antibiotics myxothiazol (a thiazol) and antimycin A (a macrolide) are structurally unrelated to peptaibols.

Immunosuppressive and neuroleptic effects have been reported for cephaibols Q and P (Schiell *et al.* 2006), as well as antiparasitic (Vertesy *et al.* 2003) and antihelminthic (Schiell *et al.* 2001) activities. Neuroleptic activity and induction of behavioural effects in rats was reported for zervamicins (Ovchinnikova *et al.* 2007). Thus these peptaibols, closely similar to the acrebols, possess toxic activity towards mammals. Cell toxicology of peptaibols has been rarely studied. We tested the type strain *A. tubakii* CBS 110649, but found no indication of toxic metabolites. The original producer of cephaibols Q and P, *A. tubakii* FH DSM 12774 (Schiell *et al.* 2006) was not available through culture collections.

*Acremonium* is a heterogeneous genus and includes species with low taxonomic relatedness (Rehner and Samuels 1995; Glenn *et al.* 1996). This is also supported, in this study, by the ITS comparison of several *Acremonium* species, which represent low similarity values. *Acremonium exuviarum* shows better ITS affinity to genera

*Emericellopsis* and *Stanjemonium*. These genera also show morphological similarities to *A. exuviarum*, such as conidia-forming phialides (Gams *et al.* 1998). Phialide structures appear to be common especially among the anamorphic genera of the order *Hypocreales*, where genera *Acremonium*, *Emericellopsis* and *Stanjemonium* are connected. *Hypocreales*, which includes families *Hypocreaceae*, *Clavicipitaceae* and *Bionectriaceae*, is the only fungal order where peptaibiotics have been detected (Degenkolb *et al.* 2007). The order includes *Trichoderma*, *Gliocladium* and other genera well known for producing wood-degrading enzymes, peptaibols and other peptaibiotics as well as pharmaceuticals. No peptaibiotics are known from fungal phyla other than *Ascomycota* (Degenkolb *et al.* 2007).

The high mammalian cell toxicity of acrebols makes the presence of the producer species, *A. exuviarum*, a threat for human health and makes its presence worth looking for, especially when problems with poor indoor environment are of concern.

## Acknowledgements

This research was supported by the Academy of Finland for the CoE 'Photobiomics' (grant 118637) and 'Microbial Resources' 2002–2007 (grant 53305). L. Kredics is the grantee of the Janos Bolyai Research Scholarship (Hungarian Academy of Sciences). Prof. Marjatta Raudaskoski and Dr Alexey Kruglov are acknowledged for their expert advice. The authors are grateful to Dr Pirjo Veijalainen and Dr Christine Ek-Kommonen (Evira, Veterinary Virology) for supplying the animal cell cultures. They also thank the Viikki Science Library and the Faculty Instrument Centre for the expert services and Leena Steininger, Hannele Tukiainen and Tuula Suortti for many kinds of help.

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