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New Trichobrachins, 11-residue peptaibols from a marine strain of *Trichoderma longibrachiatum*

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ABSTRACT

A marine strain of *Trichoderma longibrachiatum* isolated from blue mussels (*Mytilus edulis*) was investigated for short peptaibol production. Various 11-residue peptaibols, obtained as microheterogenous mixtures after a chromatographic fractionation, were identified by positive mass spectrometry fragmentation (ESI-IT-MS^n, CID-MS^n and GC/EI-MS). Thirty sequences were identified, which is the largest number of analogous sequences so far observed at once. Twenty-one sequences were new, and nine others corresponded to peptaibols already described. These peptaibols belonged to the same peptidic family based on the model Ac-Aib-xxx-xxx-xxx-Aib-Pro-xxx-xxx-Aib-Pro-xxol. They were named trichobrachin A when the residue in position 2 was an Asn, and trichobrachin C when it was a Gln. Major chromatographic sub-fractions, corresponding to purified peptaibols, were assayed for their cytotoxic activity. Trichobrachin A-IX and trichobrachin C exhibited the highest activities. There was an exponential relation between their relative hydrophobicity and their cytotoxicity on KB cells.

1. Introduction

Peptaibols are short linear peptides produced by various filamentous fungi such as *Trichoderma*, *Emericellopsis*, *Stilbella*, *Gliocladium* and *Acremonium*. They are characterized by an acetylated N-terminus, the presence of an amino alcohol such as leucinol (Leuol), isoleucinol (Ileol) or valinol (Valol) at the C-terminus, and a high proportion of a non-proteinogenic amino acid: α-aminoisobutyric acid (Aib).

Peptaibols are produced by a non-ribosomal biosynthesis process involving peptide synthetases as multienzymatic templates. Peptide synthetases possess multiple modules that bind, activate, and condense each specific amino acid to form the peptide product [26]. The number, the organization and the order of modules in the peptide synthetase reflect the size, the complexity and the sequence of the synthesized peptide [21,33]. It seems that the specificity of each module is variable resulting in the synthesis of microheterogenous mixtures [30].

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Peptaibols can be grouped according to the number and the nature of residues: long-sequence peptaibols with 18–20 residues, short-sequence peptaibols with 11–16 residues. The shortest peptaibols (11-residues) have only been observed from species of the genus *Trichoderma* or their teleomorphs in *Hypocrea* [3,8,12,13,27]. A first study on this group of peptides from a marine strain of *T. longibrachiatum* showed some originalities in sequences [28].

Peptaibols exert a broad range of biological activities depending on their chain length and structural characteristics. The peptaibol backbone generally forms a helical structure due to the conformational constraints imposed by the presence of Aib residues. These helical structures allow them to interact with natural or artificial bilayers to form pores or voltage-dependent ion channels increasing membrane permeability [2,6,11,24]. As other Aib containing peptides [40], long peptaibols have been described to exhibit significant antibacterial activity [14,25]. Moreover, they have been reported to display antifungal activity [5,15] antimycoplasmic activities [6,25,30], antiviral activity [39], neuroactivity on blowfly larvae [23] and neuroleptic activity in mice [4,32]. Short sequence peptaibols also form pores and channels on planar lipid bilayer membranes [36]. They present a narrower range of bioactivities including antimycoplasmic [30] and neuroleptic [4] activities but they do not exhibit antibacterial properties [30].

The present work describes the identification of 11-residue peptaibols produced by a marine strain of *T. longibrachiatum*, analyzes the repartition of the different amino-acid residues between the variable positions of the observed sequences and studies the relationship between their hydrophobicity and their cytotoxicity against human cells.

2. Material and methods

2.1. Fungal strain

The fungal strain was isolated from mussels (*Mytilus edulis*) collected in a shellfish-farming area from the estuary of the Loire River (Tharon, France). The isolated strain was identified as *Trichoderma longibrachiatum* Rifai using metabolic profiles on Biolog FF MicroPlates™ and by sequencing the internal transcribed spacer (ITS) regions and a 9-kb portion of the translation elongation factor-1α gene (eEF1a1). The strain was inoculated on tubes in a solid marine-like medium, stored under a paraffin oil layer and conserved in our fungal collection under the reference number MMS 151 (SMAB Marine Fungal Collection, University of Nantes). The isolate also has been deposited in the Canadian Collection of Fungus Cultures (DAOM 234100), and the eEF1a1 sequence for the strain deposited in Genbank (DQ125467).

2.2. Cultures

Dextrose Casein Agar medium (DCA) was prepared with dextrose (40 g), enzymatic digest of casein (10 g) and agar (15 g) in 1 L natural seawater. Petri dishes with DCA (125 mL) were inoculated with *T. longibrachiatum* conidia taken from stock cultures, and the cultures incubated for 10 days at 27 °C prior to harvesting for extraction.

2.3. Extraction and semi-purification

Mycelium and conidia were scraped from the agar surface. The harvested biomass was steeped twice in dichloromethane/methanol (1:2 then 2:1, v/v) for 2 h at room temperature. The combined extract was filtered under vacuum (0.45 μm PTFE membrane filters, Sartorius, Göttingen, Germany), washed with water and evaporated to provide a crude extract which was chromatographed on an open silica gel column (300 mm × 25 mm, 60 Å 35–75 μm, SDS, Peypin, France) with dichloromethane and acetone as eluants. Peptaibols were eluted in acetone, and semi-purified by a second column chromatography (200 mm × 12 mm) on silica gel eluting with dichloromethane/methanol mixtures (90:10; 88:12 and 85:15, v/v).

2.4. HPLC purification

The peptaibol mixture was purified by HPLC on a preparative reverse column (Inertsil ODS-3, 5 μm, 250 mm × 10 mm, Interchim, Montluçon, France). Mobile phase (methanol/water 85:15, v/v) was delivered by a 305-Pump (Gilson, France) at a constant flow rate of 5 mL/min. Volumes of 500 μL of 10 mg/mL sample solution resuspended in methanol/water (85:15, v/v) were injected. Detection was performed at 230 nm with a 115-UV model detector (Gilson, France). Methanol was of Normapur quality (SDS) and distilled prior to use. Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.5. Mass spectrometry analysis

Mass analyses were carried out using an electrospray ionization-ion trap mass spectrometer (ESI-IT/MS) (LCQ, Finnigan, Thermo Separation Products, San Jose, CA, USA). Mass analyses were conducted in positive mode with a capillary temperature of 265 °C.

All spectra were acquired and analyzed by LCQ Xcallibur software (Thermo Separation Products). Total current ion mass spectra (Fulscan mode) were acquired between *m*/z 150 and *m*/z 2000.

For MS analysis, HPLC sub-fractions were infused as methanolic solutions (0.5 μg/mL) directly into the ESI probe with a 250-μL micrometrically automated syringe (Hamilton, Massy, France) at a flow rate of 3 μL/min. Methanol of HPLC quality grade was purchased from Baker (Deventer, Holland).

Microheterogenous mixtures of peptaibols were sequenced according to the method of Mohamed-Benkada et al. [28].

2.6. GC/MS analyses

Peptaibols were hydrolysed by 3N hydrochloric acid for 24 h at 110 °C in hermetically sealed flasks (Fischer bioblock Scientific, Illkirch, France). Amino acids (AA) were converted into AA-isopropyl esters by reaction with 3N hydrochloric isopropanol for 30 min at 110 °C. N-trifluoroacetyl-isopropyl ester derivatives were prepared directly by treatment of AA-isopropyl esters with trifluoroacetic anhydride for 30 min at 110 °C. Standard AA were purchased from Sigma–Aldrich (Pro, Asn, Gln, Val, Leu, Ile), Fluka (Valol, Leuol, Ileol, Aib) and Acros organics (Iva).