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Isolation, Structure Elucidation and Biological Activities of Trichofumins A, B, C and D, new 11 and 13mer Peptaibols from *Trichoderma* sp. HKI 0276‡

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Abstract: Trichofumins A–D were isolated from cultures of *Trichoderma* sp. HKI 0276 as new 11 and 13mer peptaibols. Similar to 15mer peptaibols they promote morphogenesis of the fungus *Phoma destructiva* and cause hypothermia in mice as a characteristic of neuroleptic activity. Membrane measurements using a synthetic BLM model showed that A, B, C and D increased membrane permeability for cations in a similar manner as was shown for larger peptaibols but with comparably lower efficiency. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: trichofumins; 11 and 13mer peptaibols; structure elucidation; effects on fungal morphogenesis; pigment formation; membrane activity

INTRODUCTION

Peptaibols are helical peptides forming ion-penetrable channels in artificial bilayer membranes [1,2]. They owe this unique property to the presence of hydrophobic amino acids such as α-aminoisobutyric acid (Aib), an acetylated nitrogen terminus and an alcoholic instead of a carboxylic acid group at the *C*-terminus. As an explanation for their biological activities membrane channel formation was established for the 15–20mer peptaibols [1–6]. Peptaibols with >18 amino acids were subjected to various studies of their molecular conformation, ion channel formation and biological activities [3]. However, little information is available on the 'smaller' peptaibols such as 10–14mer representatives (c.f. the trichorozins [4]).

In the course of our continuing search for new peptaibols, *Trichoderma* sp. HKI 0276 was recently disclosed as a producer of the new peptaibols trichofumins A–D (Figure 1). The structures of A–D were elucidated by mass spectrometry (HRESI-MS, ESI-CID-MS/MS and ESI-QqTOF-MS/MS) as will be shown below. Moreover, the biological activities of A–D and their interactions of A–D with an artificial bilayer membrane are reported.

MATERIAL AND METHODS

Compounds A–D were isolated from cultures of *Trichoderma* sp. HKI 0276. The strain was cultivated

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A Acetyl-Val-Gln-Leu-Val-Aib-Pro-Leu-Leu-Aib-Pro-Leuol

B Acetyl-Val-Gln-Leu-Leu-Aib-Pro-Leu-Leu-Aib-Pro-Leuol

C Acetyl-Val-Gln-Val-Aib-Gln-Gln-Leu-Leu-Pro-Leu-Aib-Pro-Leuol

D Acetyl-Val-Gln-Leu-Aib-Gln-Gln-Leu-Leu-Pro-Leu-Aib-Pro-Leuol

Figure 1 Amino acid sequences of trichofumins A, B, C and D.

as a surface culture (60 l) at 26 °C in 500 ml Erlenmeyer flasks containing 100 ml malt medium [5,6] composed as follows (g/l): malt extract 20, glucose 10, yeast extract 1, $(NH_4)_2SO_4$ 5, pH 6.0.

After 20 days of cultivation at 26 °C, the culture broth was harvested and separated by filtration. Thereafter, the culture filtrate and the mycelium were extracted twice with ethyl acetate. The combined extracts were dried and evaporated. The residue (3 g) was subjected to silica gel chromatography (silica gel 60, Merck, 0.063–0.1 mm, column 600 × 40 mm, $CHCl_3$–MeOH, 9 : 1 v/v), and 20 ml portions were collected. Fractions containing trichofumins A–D were detected by ESI-MS. Final purification was done by isocratic preparative HPLC (Spherisorb ODS-2, 5 µm, RP_{18}, Promochem, 250 × 25 mm, acetonitrile H_2O, 83 : 17 v/v; 12 ml/min, 210 nm).

Antimicrobial Activities

The antimicrobial activity of the new peptaibols A–D was determined against several bacteria and fungi by the agar plate diffusion assay [7]. The inducing effect on pigment formation by *Phoma destructiva* was investigated as reported earlier [8,10].

Assay of Hypothermia in Mice (Neuroleptic Effect)

The neuroleptic activity of the new peptaibols was investigated according to Thompson [9,10]. Solutions of peptaibols were administered intraperitoneally in male mice (strain NMR) obtained from the Central Breeding Laboratory for Animals, Beutenberg Campus Jena, in a dosage of 20 mg/kg body weight. The decrease of body temperature (less 2–6 °C) was recorded 30 min after administration. The effect was in the same order of magnitude as was described for chlorpromazine, a known neuroleptic agent [10]. The basal body temperature was measured by a thermistor probe placed in the rectum of mice until a stable temperature was indicated on the thermometer. The colon temperature was recorded 0.5, 1, 3, 5, 7 and 24 h after administration of the trichofumins A–D.

Measurements Employing Lipid Bilayer Membranes

Planar bilayer lipid membranes (BLM) were prepared from soya bean phosphatidylcholine (Sigma, P5638) 20 mg/ml in *n*-heptane [11].

The measuring glass cell (25 ml of total volume) was equipped with a teflon cylinder (1 cm diameter), which contained a hole of 0.5 mm diameter to harbour the BLM. The membranes were formed by painted method on the hole of the teflon cylinder. Formation of the BLM was controlled by the use of a binocular microscope. Both the measuring cell (10 ml outside (*cis*)-volume) and the inner side of Teflon cylinder (*trans*-volume; 1 ml) were filled with a solution of potassium chloride ranging from 100 to 1000 mM depending on the type of experiment.

The membrane current was measured by the voltage-clamp method [12]. The current measuring device consisted of an operational amplifier model Keithly.301 (USA). The amplitude current noise of the amplifier was less than 10^{-13} A in the frequency range of 0.1–20 Hz. Then 1–10 µl of the stock solutions of the peptaibols (0.1–1 mg/ml in methanol) was added into the glass chamber containing the teflon cylinder with a bilayer membrane. The solution was mixed for 5 min by a magnetic stirrer at 500 rotations/min. The progress of the bilayer formation and estimation of its area was monitored on the screen of the PC by the amplitude of the membrane capacitance currents in response to the rectangular shape voltage pulses applied to the membrane. Measurements of the membrane currents corresponding to the applied peptaibol concentration were done 5 min after termination of the process of black membrane formation marked by the constant amplitude of the membrane capacitive current.

RESULTS AND DISCUSSION

The physicochemical properties of the new peptaibols, trichofumins A, B, C and D are shown in Table 1. The structures of A–D (Figure 1) were elucidated by HRESI-MS (Finnigan MAT 95 XL, Finnigan, Bremen, Germany; showing for trichofumin A: m/z 1189.7918 ($([M+H]^+$), calcd. 1189.7929 for $C_{59}H_{105}N_{12}O_{13}$); trichofumin B: m/z 1203.8096 ([M + H], calcd. 1203.8081 for $C_{60}H_{107}N_{12}O_{13}$); trichofumin C: m/z 1444.9026

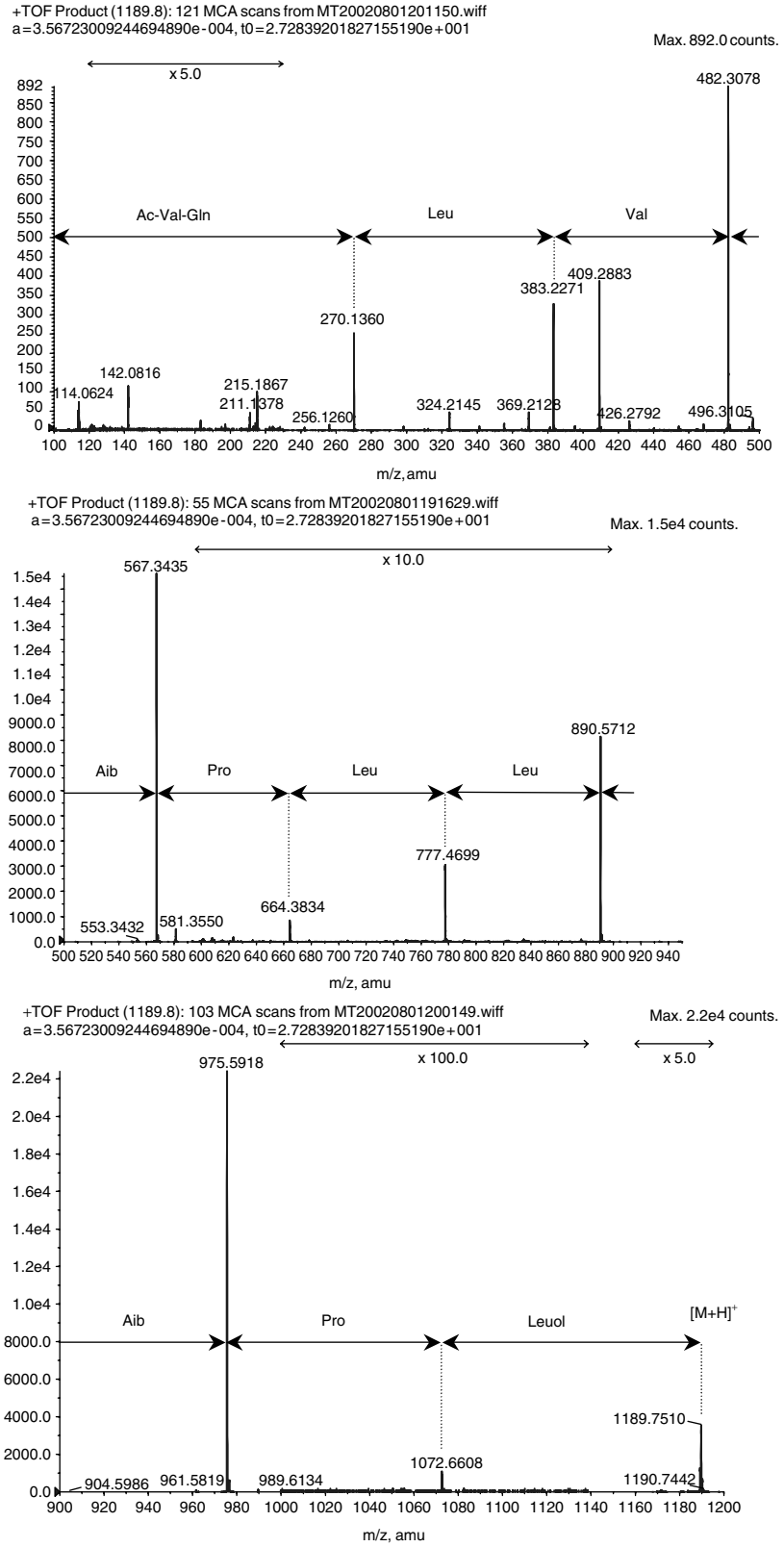

Figure 2 Diagnostic fragment formation by trichofumin A during ESI-QqTOF-MS/MS.

Table 1 Physico-chemical properties of trichofumins A–D

Trichofumin	A	B	C	D
Appearance	White solid	White solid	White solid	White solid
Melting point	157–160 °C	162–164 °C	167–169 °C	178–180 °C
HRESI-MS (m/z)	1189.7918	1203.8096	1444.9026	1458.9163
$[M+H]^+$	calcd. 1189.7929	calcd. 1203.8081	calcd. 1444.9017	calcd. 1458.9174
Molecular formula	$C_{59}H_{105}N_{12}O_{13}$	$C_{60}H_{107}N_{12}O_{13}$	$C_{69}H_{120}N_{16}O_{17}$	$C_{70}H_{122}N_{16}O_{17}$
$[\alpha]_D^{25}$ (MeOH, 3 mg/ml)[a]	—	−1.6°	−3.7°	−4.2°
R_t on HPLC (min)[b]	15.1	15.6	14.7	14.5

[a] Propol polarimeter (Dr Kernchen, Seelze, Germany).
[b] Nucleosil 100-5, RP18, 250 × 4.6 mm, 1 ml/min, 210 nm gradient acetonitrile/H_2O trifluoroacetic acid.

($[M+H]^+$, calcd. 1444.9017 for $C_{69}H_{120}N_{16}O_{17}$); and trichofumin D: m/z 1458.9163 ($[M+H]^+$, calcd. 1458.9174 for $C_{70}H_{122}N_{16}O_{17}$). The sequence of amino acids in A–D was deduced from ESI-CID-MS/MS (Triple Quadrupole Mass Spectrometer Quattro (VG Biotech, Altrincham, England) and ESI-Qq-TOF-MS/MS (API QStar Pulsar, Hybrid Quadrupole-TOF mass spectrometer, Applied Biosystems, MDS Sciex, USA) due to the diagnostic B-type fragments arising from cleavage of the amide bonds. An example for structure elucidation of A–D is depicted in Figure 2.

Hydrolysis of A–D, derivatization of the amino acids by Marfey's reagent [18] and HPLC-analysis

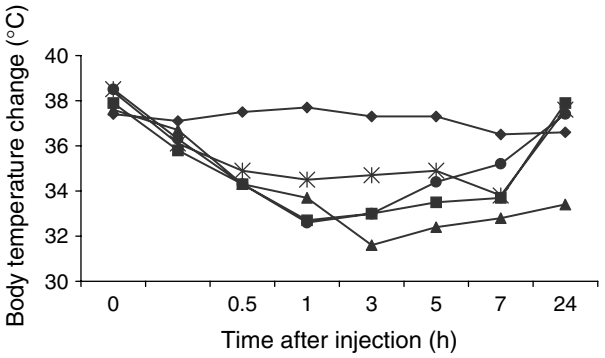

Figure 4 Induction of hypothermia in mice by A–D (♦ control; ■ trichofumin A; ▲ trichofumin B; ● trichofumin C; ∗ trichofumin D).

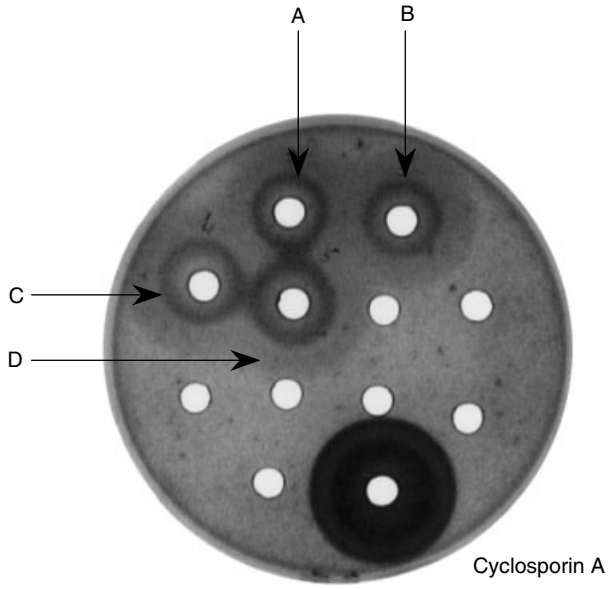

Figure 3 Effect of trichofumins A–D on morphogenesis of *Phoma destructiva* (70 h incubation, 1 mg/ml (MeOH), 23 °C). Cyclosporin A was used as standard.

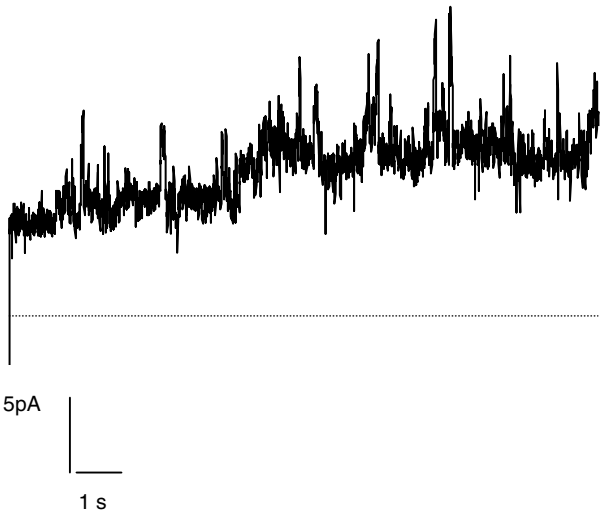

Figure 5 Trace of the membrane current in time in presence of trichofumin A (0.3 μM). Conditions: 500 mM KCl, membrane voltage 170 mV. Note characteristic pulse shape of the current corresponding to the opening and closing of the discrete ion conducting pores.

of the derivatives showed the presence of L-valine, L-leucine, L-glutamine, L-proline, L-leucinol and α-aminoisobutyric acid (Aib).

Compounds A–D thus represent new members of the 'smaller' peptaibols containing 11–14 amino acids, c.f. trichorozins from *Trichoderma harzianum*, trichogin-A-IV from *Trichoderma longibrachiatum* and trichorovins from *Trichoderma viride* [4]. Trichofumins A–D displayed moderate antibacterial activity against Gram-positive bacteria such as *Bacillus subtilis* ATCC 6633 in a concentration of >50 μg/ml during the agar well diffusion assay [17]. No activity was found against fungi and yeasts in the same concentration.

Administration of A–D to surface cultures of the phytopathogenic fungus *Phoma destructiva* caused an accelerated morphogenesis of hyphae as was indicated by earlier onset of brownish pigmentation. The effect of A–D on this fungus depending on the incubation time is shown in Figure 3. The pigment formation was comparable to the standard inducer cyclosporin A after 70 h.

Up to now little information has been available on the biological properties of the smaller peptaibols. Compounds A–D caused hypothermia in mice after intraperitoneal application in the same range of concentration and order of magnitude as the 15mer peptaibols ampullosporin A, B and D [8]. This effect can be taken as a measure of neuroleptic activity [9,10]. The vital functions (shock, climbing, refusal) were altered strongly after application of A, B, C or D. The decrease of body temperature after

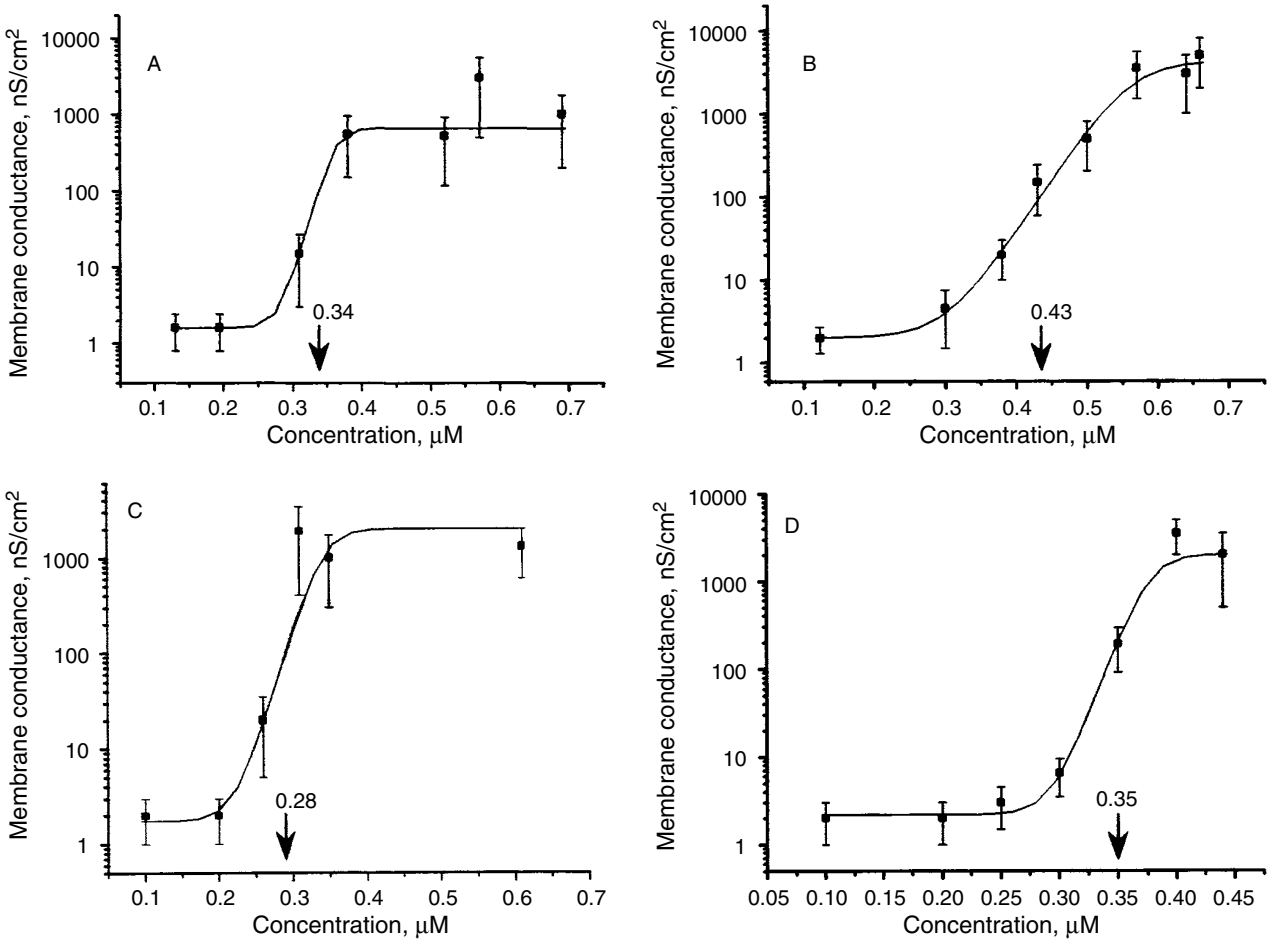

Figure 6 Membrane conductance as a function of the peptaibol concentration: (a) for trichofumin A, (b) for trichofumin B, (c) for trichofumin C and (d) for trichofumin D. The functions are saturated at about the same level of the membrane conductance 1000–2000 nS/cm^2, and the midpoints between two levels of the sigmoids corresponding to the relative effectiveness of the peptaibols are: 0.34 μM for trichofumin A, 0.43 μM for trichofumin B, 0.28 μM for trichofumin C and 0.35 μM for trichofumin D.

intraperitoneal dosage of A–D (20 mg/kg) is depicted in Figure 4.

Compounds A–D exerted these effects on mice and the fungus *Phoma destructiva* although the sequences of amino acids are different from those of the ampullosporins A, B and D. There are visible variations in the activities of A, B, C and D indicating that changes of the constituting amino acids caused alterations of biological activity.

Trichofumins A–D were shown to interact with artificial bilayer lipid membranes suggesting the formation of unstable pores as was reported for other peptaibols [13]. Figure 5 shows traces of membrane current measured in the presence of compound A suggesting that pores with a short life-time were formed. Almost identical pictures were observed with compounds B, C and D. The increase of electric conductivity of the bilayer membrane was dependent on the concentrations of peptaibols A, B, C or D which were given to the *cis*-side of the membrane (Figure 6).

Thereby, the 13mer peptaibols C and D displayed membrane effects comparable to the 11mer peptaibols A and B. The results are compatible with the view that even 11–13mer peptaibols are capable of disturbing the integrity of synthetic bilayer membranes by forming pores or channel-like aggregates. However, their efficiency and channel stability is lower than observed with the 15–20mer representatives of the peptaibol family [4,13]. No membrane activity was observed with peptaibolin [14] as a 5mer representative of the peptaibol structure. It can be suggested that the capacity of channel formation decreases in parallel with the decreasing number of the constituting amino acids. Probably a peptide chain of less than 10 amino acids would not enable membrane activity.

Ampullosporins A, B and D, on the one side, and trichofumins A–D on the other thus show comparable biological activities and effects on conductivity of artificial bilayer membranes. Hence, limited permeabilization of cellular membranes could be responsible, at least partly, for the observed effects of 11–15mer peptaibols on fungal morphogenesis and induction of hypothermia in mice. It is well known that nutrient limitation in microbes promotes their cytodifferentiation [15]. It can be suggested that effects comparable to nutrient limitation can be caused by limited permeabilization of the cytoplasmic membrane. Otherwise, disturbance of ion fluxes of synaptic cells in the presence of peptaibols could explain the observed induction of hypothermia in mice and neuroleptic effects in rats [16].

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