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NOTE

Antitrypanosomal peptaibiotics, trichosporins B-VIIa and B-VIIb, produced by *Trichoderma polysporum* FKI-4452

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In the course of screening for new antitrypanosomal agents, several novel bioactive natural compounds have been discovered and reported by our group.[1–3] Recently, we have isolated two new trichosporin analogs, designated as trichosporins B-VIIa (**1**) and B-VIIb (**2**), together with five known trichosporins (**3**–**7**),[4] from the culture broth of *Trichoderma* sp. FKI-4452. These have proved to exhibit antitrypanosomal activity (Figure 1). In this paper, the fermentation, isolation, structure elucidation and biological activity of these novel trichosporins are described.

Fungal strain FKI-4452 was isolated from a soil sample collected in Yakushima-Island, Kagoshima, Japan by the dilution plating method. The ITS sequence of strain FKI-4452 was determined and deposited at the DNA Data Bank of Japan, with the accession number AB517619, using The *Trich*OKEY program of ITSH (International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy), which suggested that the strain FKI-4452 belongs to *Trichoderma polysporum*.[5] Moreover, FKI-4452 was a 98% match to the nucleotide sequences of *T. polysporum* CBS 820.68 according to the *Tricho*BLAST database of ITSH.[5] Thus, the strain FKI-4452 was identified as *T. polysporum* and designated *Trichoderma polysporum* FKI-4452. There was no report of trichosporin production by *T. polysporum* CBS 820.68.

The strain FKI-4452 was grown and maintained on an LcA agar slant consisting of 0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract and 1.5% agar (adjusted to pH 6.0 before sterilization). A loop of spores of *Trichoderma* sp. FKI-4452 was inoculated into 100 ml of the seed medium, which consisted of 2.0% glucose, 0.2% yeast extract, 0.5% Polypepton (Wako Pure Chemical Industries, Osaka, Japan), 0.05% $MgSO_4{\cdot}7H_2O$, 0.1% KH_2PO_4 and 0.1% agar (adjusted to pH 6.0 before sterilization), in a 500-ml Erlenmeyer flask. The inoculated flask was incubated in a rotary shaker (210 r.p.m.) at 27 °C for 3 days.

For production of **1**, **2**, and the other known trichosporins (**3**–**7**), a 1-ml portion of the seed culture was transferred to each of nine 500-ml Erlenmeyer flasks containing 100 ml of the production medium, consisting of 3.0% soluble starch, 2.0% soybean meal, 1.0% glycerol, 0.3% dry yeast, 0.3% KCl, 0.2% $CaCO_3$, 0.05% $MgSO_4{\cdot}7H_2O$, and 0.05% KH_2PO_4 (adjusted to pH 6.5 before sterilization), fermentation taking place on a rotary shaker (210 r.p.m.) at 27 °C for 6 days.

To the whole culture broth (1.0 l) was added 1.0 l of ethanol, followed by filtration. The filtrate was concentrated under reduced pressure to remove ethanol and then extracted with 1.0 l of ethyl acetate (pH 2). The ethyl acetate layer was concentrated under reduced pressure to afford a crude extract (745 mg). The ethyl acetate extract (426 mg) was applied to an ODS column (Pegasil Prep ODS-7515-12A, 20$\phi\times$120 mm, Senshu Scientific Co., Tokyo, Japan) pre-equilibrated with 20% methanol. The column was eluted with 20, 40 and 60% methanol stepwise (120 ml each) and the active principles were eluted with 80% methanol (120 ml), followed by concentration *in vacuo* to yield a brown material (91.0 mg). The material was purified by HPLC using a Pegasil ODS column (20$\phi\times$250 mm, Senshu Scientific Co.) with 70% CH_3CN at 5 ml min^{-1} detected at UV 210 nm. The retention times of the active fractions 1, 2, 3, and 4 were 16, 18, 20, and 24 min, respectively. The active fractions 1 and 2 were concentrated *in vacuo* to dryness to afford trichosporins B-V (**3**, 9.9 mg) and B-VIb (**4**, 10.0 mg), respectively, as white powders. The active fraction 3 (8.4 mg) was purified by HPLC using an XBridge C8 column (10$\phi\times$250 mm, Waters Co., Milford, MA, USA) with 50%

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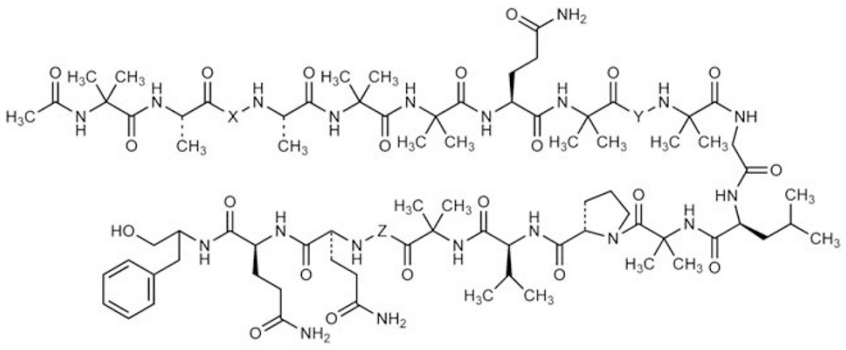

	X	Y	Z
Trichosporin B-VIIa (**1**)	Aib	L-Ile	L-Iva
Trichosporin B-VIIb (**2**)	Aib	L-Leu	Aib
Trichosporin B-V (**3**)	L-Ala	L-Ile	Aib
Trichosporin B-VIb (**4**)	L-Ala	L-Ile	L-Iva
Trichosporin B-VIa (**5**)	Aib	L-Ile	Aib
Trichosporin B-IIIa (**6**)	L-Ala	L-Leu	Aib
Trichosporin B-IVb (**7**)	L-Ala	L-Leu	L-Iva

Figure 1 Structures of trichosporins B-VIIa (**1**) and B-VIIb (**2**) and other known trichosporins isolated (**3**–**7**).

CH_3CN at 2.5 ml min^{-1} and was detected at UV 210 nm. The retention times of the active fractions 3-1 and 3-2 were 30 and 35 min, respectively. They were concentrated *in vacuo* to dryness to afford trichosporins B-VIa (**5**, 3.7 mg) and B-IIIa (**6**, 3.0 mg), respectively, as white powders. The active fraction 4 (9.8 mg) was purified by HPLC using an XBridge C8 column (under the same conditions as described above). The retention times of the active fractions 4-1, 4-2 and 4-3 were 38, 41 and 44 min, respectively. They were concentrated *in vacuo* to dryness to afford **1** (2.2 mg), trichosporin B-IVb (**7**, 2.0 mg) and **2** (0.8 mg), respectively, as white powders.

Compounds **1** and **2** showed strong IR absorptions at 3340 (NH) and 1630 (*C*=O) cm^{-1}, which are characteristic of peptide bonds. The molecular weights of **1** and **2** were elucidated by ESI-MS to be 1978 and 1964, respectively. Analysis of their ^1H NMR spectra revealed the presence of an acetyl methyl group at δ_H 2.1, a phenyl group of phenylalaninol (Pheol) residue at δ_H 7.1–7.4, α-proton methine signals of amino acid residues at δ_H 4.0–5.5, and some singlet methyl groups of α-aminoisobutylic acid (Aib) residues at δ_H 1.5–1.6. Thus, **1** and **2** were considered to belong to the peptaibiotic family.[6]

The amino-acid composition and their absolute configurations in **1** were elucidated as Gly, L-Ala, Aib, L-Pro, L-Val, L-isovaline (L-Iva), L-Leu, L-Ile and L-Gln/Glu by HPLC analysis of its hydrolysate using a chiral column (Sumichiral OA-5000 (4.6ϕ×150 mm), 2 mM $CuSO_4$, 1.0 ml min^{-1}, UV 254 nm, 40 °C).[7] The absolute configuration of phenylalaninol was not elucidated. The sequence of **1** was elucidated by FAB-MS fragmentation analysis. In FAB-MS analysis, the complementary fragment ions at *m/z* 1191 and 788, which were considered to be formed from the entire molecule by cleavage of the labile peptide bond between Aib and Pro,[8] were observed. The *m/z* 1191 ion generated fragment ions at *m/z* 1106, 993, 936, 851, 738, 525, 440, 355, 284, 199 and 128, which were interpreted as the successive losses of Aib, Leu, Gly, Aib, Ile, Gln+Aib, Aib, Aib, Ala, Aib, Ala, and Ac-Aib, by comparison with other known trichosporins isolated. Conversely, the *m/z* 788 ion generated the fragment ions at *m/z* 637, 509, 381, 282 and 197, which were interpreted as the successive losses of Pheol, Gln, Gln, Iva, Aib and Pro+Val. Based on the results mentioned above, the complete structure of **1** was elucidated to be Ac–Aib–Ala–Aib–Ala–Aib–Aib–Gln–Aib–Ile–Aib–Gly–Leu–Aib–Pro–Val–Aib–Iva–Gln–Gln–Pheol (Figure 1), representing a new analog of trichosporin B series. Therefore, we designated **1** as trichosporin B-VIIa.

The amino-acid composition and their absolute configurations in **2** were elucidated as Gly, L-Ala, Aib, L-Pro, L-Val, L-Leu and L-Gln/Glu by HPLC analysis of its hydrolysate using a chiral column. The sequence of **2** was elucidated by FAB-MS fragmentation analysis as for **1**. The complementary fragment ions at *m/z* 1191 and 774, which were considered to be formed from the entire molecule by cleavage between Aib and Pro, were observed. The *m/z* 1191 ion generated the same fragment ions as **1**, which suggested the successive losses of Aib, Leu, Gly, Aib, Leu, Gln+Aib, Aib, Aib, Ala, Aib, Ala and Ac-Aib. On the other hand, the *m/z* 774 ion generated the fragment ions at *m/z* 623, 495, 367, 282 and 197, which were interpreted as the successive losses of Pheol, Gln, Gln, Aib, Aib and Pro+Val. Based on the results mentioned above, the complete structure of **2** was shown to be Ac–Aib–Ala–Aib–Ala–Aib–Aib–Gln–Aib–Leu–Aib–Gly–Leu–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol (Figure 1), which was also a new analog and was named trichosporin B-VIIb. The other five known trichosporins were also identified, as shown in Figure 1, by the same procedure described above.

The antitrypanosomal activity of the compounds was measured using a method described previously.[1] As shown in Table 1, all trichosporins showed antitrypanosomal activities against *Trypanosoma brucei brucei* strain GUTat 3.1. Among them, **5** showed the most potent activity, with an IC_{50} value of 0.16 μg ml^{-1} and the highest selectivity (cytotoxicity against MRC-5 cells/antitrypanosomal activity) of 60 times. Compounds **1** and **2** showed moderate inhibitory activities, with IC_{50} values of 0.92 and 6.1 μg ml^{-1}, respectively. There is no report of the antitrypanosomal activity of trichosporins. Trichosporins belong to the peptaibiotic family. Our group has previously reported that some peptaibiotics (leucinostatins and alamethicin I) exhibit antitrypanosomal activities.[3] Therefore, trichosporins might have the same inhibitory mechanism, which is related to their membrane-interacting properties. Trichosporin B series has been

Table 1 *In vitro* antitrypanosomal activity and cytotoxicity of trichosporins B-VIIa (1) and B-VIIb (2) and other known trichosporins isolated (3-7)

	IC_{50} ($\mu g\,ml^{-1}$)		
Compound	*Anti-trypanosomal activity (T. b. brucei GUTat 3.1)*	*Cytotoxicity (MRC-5)*	*Selectivity index (SI)*
Trichosporin B-VIIa (**1**)	0.92	13.3	14.4
Trichosporin B-VIIb (**2**)	6.10	21.3	3.5
Trichosporin B-V (**3**)	0.54	14.1	26.0
Trichosporin B-VIb (**4**)	0.37	11.1	30.1
Trichosporin B-VIa (**5**)	0.16	9.6	60.1
Trichosporin B-IIIa (**6**)	0.38	5.3	13.9
Trichosporin B-IVb (**7**)	1.01	10.6	10.5
Suramin*	1.58	>100	>63

*Antitrypanosomal drug used clinically.

reported to uncouple the oxidative phosphorylation in rat liver mitochondria[9] and form ion channels in lipid bilayer membranes.[10]

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