Trichopolyn VI: a new peptaibol insecticidal compound discovered using a recombinant *Saccharomyces cerevisiae* screening system

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Introduction

Insecticides are a key element in helping to produce and secure the world’s food supply and help overcome the global hunger problem. It has been predicted that the worldwide market for agricultural chemicals will increase to nearly US$60 billion in 2016. However, the overuse and abuse of existing insecticides has led to many insect pest species evolving widespread resistance. Increasing insecticide resistance is influenced by many factors. Insecticidal tolerance caused by genetic alterations is normally responsible for mechanisms that either reduce the effect of an insecticide on its target site or lead to a decrease in insecticide kinetics, such as absorption, distribution, metabolism and excretion (Herron et al., 2003; Kaufman et al., 2001; Khalighi et al., 2014; Kim et al., 2004; Van Leeuwen et al., 2010; Van Nieuwenhuyse et al., 2009).

Therefore, it is necessary to develop new insecticides, particularly those that have a novel mode of action, to help overcome the global increase in insecticide-resistant agricultural pests.

In this regard, we have devised and implemented a comprehensive screening system to identify and develop new insecticides. We developed a screening program to find ADP/ATP carrier protein (AAC) inhibitors, which could function as mitochondrial respiration inhibitors. Mitochondrial respiration produces ATP, which is transported into intermembrane spaces by the AAC, penetrating the mitochondrial inner membrane. Thus, ATP can be utilized in the cytosol. We prepared *Saccharomyces cerevisiae* yeast organisms by deleting *aac* genes and subsequently transformed some *aac* genes, derived from other pests, to the *S. cerevisiae* ∆aac. The haploid strain of *S. cerevisiae* W303-1B was provided by Dr. Shimizu (Osaka University).
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Recently, this screening system allowed us to isolate a novel mitochondrial respiration inhibitor with lanostane-type triterpenoid, ascosteroside C, produced by Aspergillus flaschentraegeri (Suga et al., in press). We also discovered that a fungus, Trichoderma brevicompactum FKI-6324, isolated from a soil sample, produced a new insecticidal peptide, designated as trichopolyn VI (1), as shown in Fig. 1a. The structure of 1 was elucidated by detailed experiments of ESI-MS/MS and NMR to be a lipophilic amino acid residue with non-proteinic constituents. The structural frame is similar to that of trichopolyn I (Fig. 1b), of the peptaibol family (Daniel and Filho, 2007; Degenkolb and Doehren, 2008; Whitmore and Wallace, 2004).

Since the discovery of alamethicin from the extracts of Trichoderma viride, a number of peptaibols have been isolated from Ascomycetes and imperfect fungi (Payne et al., 1970). The genus Trichoderma, to which imperfect fungi belong, has contributed a particularly large proportion of these peptaibols. In particular, Trichoderma polysporum produces trichopolyns, which contain α-aminoisobutyric acid (Aib) and 2-amino-6-hydroxy-4-methyl-8-oxodecanoyl acid (AHMOD), and have a unique chemical structure characterized by protective fatty acids of 2-methyldecanoic acid (MDA) at the N-terminus and trichodiaminol (Tdol) at the C-terminus. In addition, these compounds are known to have bioactive properties, such as immunosuppressive and antifungal activity, although their mechanism of action remains only partially elucidated (Iida et al., 1999; Toniolo et al., 2001). We screened microbial culture broths to identify active compounds that specifically inhibit the recombinant S. cerevisiae. We consequently isolated a new peptaibol, produced by the fungal strain FKI-6324, from a soil sample collected in Hateruma Island, Okinawa, Japan. In this paper, the taxonomy of the producing fungus, isolation, structure elucidation and biological activity of 1, are described.

Materials and Methods

Microorganism. The ITS sequence of strain FKI-6324 was elucidated and deposited at the DNA Data Bank of Japan with accession number LC032124. The ITS sequence of FKI-6324 was compared to sequences in the GenBank database by BLASTN 2.2.30 analysis (Altschul et al., 1997). The sequence of FKI-6324 was 99.8% similar to that of Trichoderma brevicompactum G.J.S. 04-381 (GenBank accession number DQ000635), and thus the strain FKI-6324 was identified with Trichoderma brevicompactum.

Fermentation of the fungal strain. One loop of the strain FKI-6324 grown on an LcA slant (0.1% glycerol, 0.08% KH₂PO₄, 0.02% K₂HPO₄, 0.02% MgSO₄·7H₂O, 0.02% KCl, 0.2% NaNO₃, and 1.5% agar, pH 6.0) was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of a seed culture medium (2% glucose, 0.2% yeast extract, 0.05% MgSO₄·7H₂O, 0.5% Polypepton (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.1% KH₂PO₄, and 0.1% agar, pH 6.0) and incubated on a rotary shaker at 27°C for 3 days. Ten milliliters of the seed culture was inoculated into each of 4 Ulpack 47 (culture bags) (HOKKEN Co. Ltd., Tachigi, Japan) containing a production medium (350 g of water-sodden rice). Static fermentation was continued at 27°C for 14 days.
Isolation. The solvents for extractions and column chromatography were reagent grade and used without further purification. Those used for HPLC were analytical grade. Silica gel column chromatography was carried out using Merck Silica gel 60 (0.063–0.200 µm). An ODS column was conducted on YMC-gel ODS-A (150 µm). HPLC separations were performed on a Pegasil ODS SP100 column, 5 µm particle size (20 × 250 mm, Senshu Scientific Co. Ltd., Tokyo, Japan). Preparative TLC was carried out using a Merck silica gel 60 F254 (20 × 20 cm).

Measurement of UV spectrum, Optical rotation and IR spectrum. The UV spectrum was measured using a Hitachi UV/Vis spectrometer U-2810 (Hitachi High-Technologies Corporation, Tokyo, Japan). Optical rotation was recorded by means of a JASCO P-2200 polarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The IR spectrum was measured using a Horiba fourier transform infrared spectrometer FT-710 (Horiba Ltd., Kyoto, Japan).

ESI-MS/MS and NMR analysis. The sample was injected into the electrospray ion source of a QSTAR Elite ESI quadruple time-of-flight (Q-TOF) MS instrument (AB Sciex, Framingham, MA, USA) coupled to an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on an Inertsil ODS-4 (3.0 × 250 mm, GL Sciences Inc., Tokyo, Japan) at 40°C. As for gradient elution, solvent A was water with 2 mM ammonium acetate and solvent B was methanol with 2 mM ammonium acetate. The gradient elution was pro-
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**Table 1.** Selective growth inhibition activity of trichopolyn VI (1) against various recombinant *Saccharomyces cerevisiae*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>µg/disk</th>
<th>Δaac S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Empty vector strain</td>
</tr>
<tr>
<td>Trichopolyn VI</td>
<td>10</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>—</td>
</tr>
<tr>
<td>Leucinostatins</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>—</td>
</tr>
<tr>
<td>Strobilurin B</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Carboxin</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
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Results of paper disk assays for the empty vector strain (endogenous AAC-disrupted and single-copy type yeast shuttle vector pRS314YA2P-expressing strain), *Tribolium castaneum* strain (endogenous AAC-disrupted and *Tribolium castaneum* AAC-expressing), *Acyrthosiphon pisum* strain (endogenous AAC-disrupted and *Acyrthosiphon pisum* AAC-expressing). Test compounds were dissolved in MeOH at appropriate concentrations. Inhibition zone diameter (millimeter). — No inhibition.

The culture was extracted with 1.7 L of 50% ethanol. After filtration, the filtrate was concentrated in vacuo to remove the ethanol. The resultant aqueous solution was extracted with an equal volume of ethyl acetate and cone voltage = 20, 35 and 50 V. Liquid chromatography/mass spectrometry (LC/MS) analysis was performed by a high-resolution ESIMS (HRESIMS; R = 10,000, the tolerance for mass accuracy was 10 ppm). NMR spectra (1D and 2D) were recorded in CDCl3 using an Agilent Technologies NMR system spectrometer (400 MHz for 1H and 100 MHz for 13C). Chemical shifts were referenced relative to the residual solvent signals (δH = 7.26 ppm/δC = 77.0 ppm).

**Yeast growth inhibition assay.** We evaluated the growth inhibitory effect of trichopolyn VI, leucinostatins, strobilurin B and carboxin using the paper disk method on agar plates inoculated with recombinant *S. cerevisiae* of the WB-12 pRS314YA2P/y2NtcAAC strain (endogenous AAC-disrupted and *Tribolium castaneum* AAC-expressing strain, accession number XP_973257) or the WB-12 pRS314YA2P/y2NapAAC strain (endogenous AAC-disrupted and *Acyrthosiphon pisum* AAC-expressing strain) in a glucose-containing medium. Sterile filter disks impregnated with 10 µL of each compound solution were placed on the agar plate, and the plates were incubated at 30°C for 48 h. WB-12 pRS314YA2P strain (endogenous AAC-disrupted and single-copy type yeast shuttle vector pRS314YA2P-expressing strain) in a glucose-containing medium was also tested as a reference.

**Results and Discussion**

The culture was extracted with 1.7 L of 50% ethanol. After filtration, the filtrate was concentrated in vacuo to remove the ethanol. The resultant aqueous solution (4.0 L) was extracted with 1.7 L of 50% ethanol. The flow rate was 0.5 mL/min, the injection volume was 5 µL, and UV spectrum detection was carried out by a photodiode array detector. Fragmentation was recorded for 30 min in the m/z region from 100 to 2,000 Da with the following instrument parameters: ion spray voltage = 5,500 V, source gas = 50 L/min, curtain gas = 30 L/min, declustering potential = 50 V, focusing potential = 250 V, temperature = 450°C, detector voltage = 2,300 V, and cone voltage = 20, 35 and 50 V. Liquid chromatography/mass spectrometry (LC/MS) analysis was performed by a high-resolution ESIMS (HRESIMS; R = 10,000, the tolerance for mass accuracy was 10 ppm). NMR spectra (1D and 2D) were recorded in CDCl3 using an Agilent Technologies NMR system spectrometer (400 MHz for 1H and 100 MHz for 13C). Chemical shifts were referenced relative to the residual solvent signals (δH = 7.26 ppm/δC = 77.0 ppm).

**Compound 1** was obtained as a colorless amorphous solid. It was soluble in CH3OH, acetonitrile, CHCl3, and...
DMSO but not in H₂O. The Rₚ value in the solvent system of CHCl₃-CH₃OH (4:1) was 0.55 on a silica gel TLC (Merck 60 F254); [α]D20-27°-45.5° (c 0.1, CH₃OH); IR: KBr νmax 3432, 1670, 1538 cm⁻¹; UV (CH₃OH) λmax 384 (5100), 203 (4600), 226 (sh, 21700). The molecular formula of I was elucidated to be C₆₅H₉₈N₆₀₂ based on HR-ESI-MS ([M+H]+, m/z 1188.8329: calculated for C₆₅H₉₈N₆0₂, 1188.8323). The IR spectrum displayed carbyl and amine absorption at 1670 and 3432 cm⁻¹, respectively, suggesting the presence of peptide bonds.

The amino acid sequence of I was elucidated by the observation of a series of acyl fragment ions using ESI-MS/MS similarly to in the case of many peptaibols (Iwatsuki et al., 2010; Krause et al., 2006). The characteristic mass differences of Δm = 71.0 and 85.1 Da indicated the presence of Ala and Ile residues which were observed in the series of acyl fragment ions resulting from ESI-MS/MS analysis of trichopolyns (Fig. 2a) (Krause et al., 2006). Additionally, the hallmarks of acyl fragment ions were also identified as MDA+Pro (m/z = 266.2 Da) and Tdol (Δm = 132.1 Da) in I, respectively (Fig. 2a). The Δm of 195.1 did not coincide with that of other trichopolyns observed as AHMOD (Δm = 213) (Iida et al., 1999). Thus, I was suggested to possess a dehydration product (AMOD: 2-amino-4-methyl-8-oxodeca-6-enoic acid) in the side chain, based on the observation of the mass difference of Δm = 18 Da, when compared with AHMOD of trichopolyn I (Fig. 1) (Iida et al., 1999; Krause et al., 2006). As the ESI-MS/MS could not distinguish the mass differences of Δm = 113.1 as Leu or Ile in I, the presence of Ile was confirmed by NMR analysis. The CH₃CH(CH₃)₂CH₂CH₃ residue of Ile was elucidated from coupling between each adjacent proton by gCOSY and ¹H NMR spectrum, along with the data in the literature (Figs. 2b and 2c) (Pruksakorn et al., 2010). The existence of AMOD was also elucidated by ¹H NMR and gCOSY as shown in Figs. 2b and 2c, along with the data in the literature (Pruksakorn et al., 2010). The geometry of the olefin was elucidated as trans on the basis of the coupling constant (J = 15.6 Hz). The presence of three triplet methyl in ¹H NMR confirmed the existence of MDA, AMOD and Ile. Eventually, based on the ESI-MS/MS and NMR spectral data, the structure of I was elucidated as a dehydrated form of trichopolyn I and named trichopolyn VI. Compound I has the unique characteristic of the presence of an AMOD unit, which is rarely observed in peptaibol family compounds (Pruksakorn et al., 2010).

The biological activities of the compounds were evaluated using Δaac S. cerevisiae in a glucose-containing medium and insect aac-transformed S. cerevisiae in a glycerol-containing medium. The former yeast cannot export ATP from mitochondria to the cytosol and thus can only grow in a glucose-containing medium. The latter can grow in a glycerol-containing medium due to the presence of the transformed aac. The transformation allows the AAC in the mitochondrial inner membrane to transport ATP from the matrix to intermembrane spaces. Compound I inhibited the growth of insect aac-transformed S. cerevisiae but showed only weak growth inhibition against empty vector transformed S. cerevisiae (Table 1). We also evaluated some mitochondrial respiration inhibitors and uncouplers and found that the leucinostatins, strobilurin B and carboxin inhibited insect aac-transformed S. cerevisiae, specifically. In previous reports, the modes of action of strobilurin B and carboxin have been revealed to be via uncoupling of mitochondria, as a ubiquinol-cytochrome c oxidoreductase inhibitor and a succinate-quinone oxidoreductase dehydrogenase inhibitor, respectively (Jagow et al., 1986; Matthe, 1970; Mori et al., 1983). Although trichopolyns have already shown immunosuppressant and antimicrobial activities, it has not been reported that trichopolyns act on mitochondrial function. Our findings show that I has a similar profile to mitochondrial respiration inhibitors, such as strobilurin B and carboxin. So, we hypothesized that I might inhibit mitochondrial function. In addition, strobilurin and carboxin derivatives have already been used as agricultural pesticides or fungicides (Balba, 2007; Newcombe and Thomas et al., 2000).

In conclusion, I was isolated from a culture broth of the fungus Trichoderma brevicompactum (strain FK1-6324) and its structure was elucidated, with the new compound deemed to be a new peptaibol. Our data demonstrated that I might inhibit the mitochondrial respiratory system in S. cerevisiae. Further comprehensive chemical and biological studies of trichopolyn I and its analogues, including I, might lead to the development of much-needed potent new insecticides.

Acknowledgments

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