New Cyclic Depsipeptide Antibiotics, Clavariopsins A and B, Produced by an Aquatic Hyphomycetes, Clavariopsis aquatica

1. Taxonomy, Fermentation, Isolation, and Biological Properties

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Clavariopsins were isolated from the fermentation broth of Clavariopsis aquatica AJ117363. Clavariopsins are cyclic depsipeptide antibiotics with the molecular weight of 1,153 and 1,139. Clavariopsins showed in vitro antifungal activity against not only Aspergillus fumigatus but also, although to a lesser extent, A. niger and Candida albicans.

In the course of our screening for antifungal antibiotics from various fungi, new compounds, clavariopsin A and clavariopsin B (Fig. 1), were obtained from the fermentation broth of Clavariopsis aquatica AJ117363. The producing organism, Clavariopsis aquatica AJ117363, was isolated from submerged decaying leaves collected from a mountain stream at Mt. Takao in Tokyo, Japan.

In this paper, we describe the isolation method and taxonomy of the producing organism, the production and isolation procedure of clavariopsins, and their physico-chemical properties and biological activities. The structural studies of clavariopsins will be described in the following paper1).

Materials and Methods

Isolation of Producing Strain

The aquatic fungal spores were trapped from decaying leaves collected from a mountain stream by air bubbles2).

We immersed decaying leaves in 300 ml water using a 500 ml beaker, and then bubbled air (1 liter/minute) to the bottom of the beaker to wash out the fungal spores from the

Fig. 1. Structures of clavariopsins A and B.

Clavariopsin A: R = Me
Clavariopsin B: R = H

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leaves. After 1 hour bubbling, we took the foam containing the spores from the surface of the water. The strain of AJ117363 was isolated from the foam by single cell manipulation according to the Skerman's method\(^3\)\(^4\) using Skerman's micromanipulator (Toyorikoki, Co., Inc., Japan).

Detection of Clavariopsins

Clavariopsins were detected by a bioassay, which was carried out by the paper disk diffusion method, based on their antifungal activity against Aspergillus niger AJ117364 grown in a medium consisting of Potato Dextrose Agar (Difco). The cell morphology of the fungus in the inhibitory zone, which appeared around the paper disk, was examined under a light microscope\(^5\).

Clavariopsin concentrations were measured by reversed phase HPLC (Capcell Pak C\(_{18}\), Shiseido) in linear gradient with a solvent of acetonitrile-water (containing 0.1% TFA) at the UV absorption of 277 nm.

Measurement of Antifungal Activity

The MICs against fungi were determined by microdilution method\(^6\) using the U-shaped 96 microplate (Nalgen Nunc). The fungal cell suspensions were prepared from a Potato Dextrose Agar (Difco) slant culture incubated for four days at 25°C, spores or conidia were harvested with saline and filtered through sterile gauze and used for the inoculums of \(1\times10^4\) CFU/ml. Cell suspensions were counted using a hemacytometer. The assay medium was RPMI1640 medium (Nissui Pharmaceutical, Japan) without sodium bicarbonate, supplemented with 2.5 mM L-glutamine (Sigma), 1.8% glucose, 0.165 M morpholinepropanesulfonic acid, 1.0% of the testing compound solution and brought to pH 7. The test compounds clavariopsins, as reference compounds amphotericin B (Sigma) and miconazole (Sigma) were dissolved and diluted in dimethyl sulfoxide. The plates were incubated at 37°C for 24 hours. The MICs were defined as the lowest concentration having no visible growth in broth wells.

### Results

Taxonomic Studies

The cultural characteristics of the strain AJ117363 after incubation on four agar media at 25°C for 14 days are summarized in Table 1. The strain showed good growth on the name agar media, LCA, CMA, PDA and MA. The surface of the strain was fluffy without production of pigments. The temperature permitting the growth of the strain was from 10°C to 27°C.

Since the spores were not formed on any of these plates tested, we induced the spore formation by immersing a part of the colonies cut by a knife 1 cm square in water\(^7\).

The spores were aleuro, colorless, tetrapot type (main part long (size 30–40 \(\mu\)m \(\times\) 10–15 \(\mu\)m), 2 celled, the three branches from the upper cell widely divergent (size 50–70 \(\mu\)m \(\times\) 1.5–2.5 \(\mu\)m)). (Fig. 2)

From the mycological characteristics described above, the strain AJ117363 was identified as Clavariopsis aquatica\(^7\). The strain AJ117363 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the name of Clavariopsis aquatica AJ117363 under the accession No. of FERM BP-6594.

### Fermentation

A loopful of the cells of a slant culture of the strain AJ117363 was inoculated in 150 ml of a liquid medium in a 500-ml flask and incubated on a rotary shaker 160rpm at

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Table 1. Cultural characteristics of *Clavariopsis aquatica* AJ117363.

<table>
<thead>
<tr>
<th>Media</th>
<th>Colony size (mm)</th>
<th>Color of mycelium</th>
<th>Color of reverse side</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCA</td>
<td>10</td>
<td>Greenish gray</td>
<td>Greenish gray</td>
</tr>
<tr>
<td>CMA</td>
<td>16</td>
<td>Greenish white</td>
<td>Greenish white</td>
</tr>
<tr>
<td>PDA</td>
<td>21</td>
<td>Gray</td>
<td>Greenish black</td>
</tr>
<tr>
<td>MA</td>
<td>19</td>
<td>Gray</td>
<td>Greenish black</td>
</tr>
</tbody>
</table>

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24°C for 7 days to give a seed culture. The seed cultures (2 ml) were transferred into each 150 ml of fermentation medium in 500-ml flask and the flasks were shaken at 24°C for 13 days. The seed and production media were composed of glucose 2.5%, Pharmamedia (Southern Cotton Oil Company, USA) 2.0%, Amino acids mixture for culture media (Ajinomoto Co., Inc., Japan) 0.1%, and CaCO₃ 0.5% at pH 7.0 prior to autoclaving.

The typical time course of the production of clavariopsin A is shown in Fig. 3. The maximum production was observed at 13 day and its productivity reached at 480 μg/ml.

Isolation and Purification

The fermentation broth (6 liters) was centrifuged to separate the supernatant and mycelia cake. The mycelia cake was extracted with 3 liters of acetone. The acetone extract was evaporated under reduced pressure to remove acetone and the residue was extracted twice with ethyl acetate. The ethyl acetate extract was concentrated to dryness under reduced pressure, and the residue (10 g) was dissolved in chloroform. The chloroform solution (10 ml) was put onto a column of silica gel (250 ml). The column was eluted with 500 ml of chloroform-methanol (9:1). The active fraction eluted was condensed under reduced pressure to give 3.0 g of residue. The residue was dissolved in acetonitrile, applied on a C18 HPLC column Capcell Pak (20×250 mm) and eluted with acetonitrile-water (19:1). The fractions containing elude clavariopsin A and clavariopsin B were collected separately and concentrated under reduced pressure. The residues were dissolved in methanol, applied on C18 HPLC column Capcell Pak (20×250 mm) and eluted with methanol-water (9:1) to give pure clavariopsin A (50 mg) and clavariopsin B (2 mg).
Table 2. Physico-chemical properties of clavariopsins.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Clavariopsin A</th>
<th>Clavariopsin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Colorless powder</td>
<td>Colorless powder</td>
</tr>
<tr>
<td>UV λ max nm</td>
<td>277, 288</td>
<td>277, 288</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₅₉H₉₅N₉O₁₄</td>
<td>C₅₈H₉₃N₉O₁₄</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>1153</td>
<td>1139</td>
</tr>
<tr>
<td>HR FABMS</td>
<td>calcd 1176.6966 (M+Na)</td>
<td>1162.6740 (M+Na)</td>
</tr>
<tr>
<td></td>
<td>found 1176.6924 (M+Na)</td>
<td>1162.6740 (M+Na)</td>
</tr>
<tr>
<td>Amino acid analysis</td>
<td>Gly, Val, Tyr</td>
<td>Not tested</td>
</tr>
<tr>
<td>Solubility</td>
<td>soluble MeOH, EtOH, EtOAc, DMSO</td>
<td>MeOH, EtOH, EtOAc, DMSO</td>
</tr>
<tr>
<td></td>
<td>insoluble H₂O</td>
<td>H₂O</td>
</tr>
</tbody>
</table>

Table 3. Antifungal activity of clavariopsins.

Minimum inhibitory concentration (MIC, µg/ml).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clavariopsin A</th>
<th>Clavariopsin B</th>
<th>Amphotericin B</th>
<th>Miconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans IFO 0583</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Candida albicans IFO 0583</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Candida albicans ATCC 10231</td>
<td>8</td>
<td>8</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Aspergillus niger AJ117374</td>
<td>16</td>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aspergillus fumigatus AJ117190</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aspergillus fumigatus JCM1739</td>
<td>4</td>
<td>4</td>
<td>0.25</td>
<td>2</td>
</tr>
</tbody>
</table>

Physico-chemical Properties

Some properties of clavariopsins are summarized in Table 2. Clavariopsins showed the maxima at 277 (ε 1,900) and 288 nm (ε 1,700) in the UV absorption spectrum in methanol. They were soluble in ethanol, chloroform, ethyl ether, and dimethylsulfoxide and insoluble in water. They contained glycine, valine and tyrosine in common based on the amino acid analysis. Their molecular formulas were determined by HR-FABMS as C₅₉H₉₅N₉O₁₄ (MW 1153) and C₅₈H₉₃N₉O₁₄ (MW 1139).

Biological Properties

The antifungal activity of clavariopsins is shown in Table 3. Clavariopsins showed antifungal activity against C. albicans, A. fumigatus and A. niger. No activities were found against bacterial taxon such as Escherichia coli and Staphylococcus aureus in the concentration up to 100 µg/ml (data not shown). Clavariopsins induced hyphae swelling of A. niger at 2 µg/ml after 24 hour incubation (Fig. 4.).

Clavariopsin A showed no signs of toxicity when...
administered once to mice intraperitoneally at the dose of 100 mg/kg.

Discussion

In our screening program for new antifungal compounds, we targeted the unusual microbial groups in the hope that we could find novel structures and mode of actions. We selected aquatic hyphomycetes as one of such unexplored microorganisms since there are only few reports describing new antibiotic compounds from such a fungal group8-10).

We isolated new antifungal compounds, clavariopsins A and B, from *Clavariopsis aquatica*, a typical species of aquatic hyphomycetes. Other four strains of the same species, which had been isolated from different areas in Japan, were also found to produce clavariopsin A (data not shown). These data suggest that the compound is common to this aquatic species.

Clavariopsins have the unique activity inducing the swelling of fungal hyphae. Since several inhibitors, which disrupt the fungal cell wall constructions, were known to cause the similar hyper-swelling phenomenon11,12), it is suggested that the compounds may inhibit the synthesis of fungal cell walls. This notion is also supported by recent reports, which show cyclopeptide compounds inhibit the cell wall glycan synthesis12,13). However, further investigation is needed to clarify the mode of actions of clavariopsins against the target microorganisms.

Acknowledgement

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References


