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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.

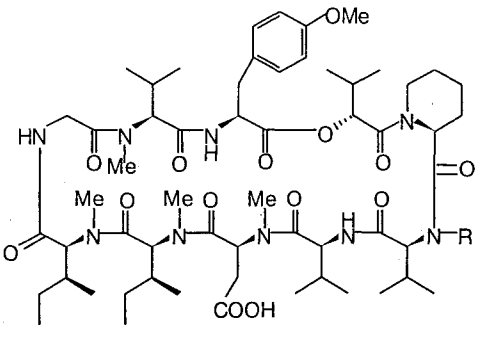
In this paper, we describe the isolation method and taxonomy of the producing organism, the production and isolation procedure of clavariopsins, and their physicochemical properties and biological activities. The structural studies of clavariopsins will be described in the following paper[1].

Materials and Methods

Isolation of Producing Strain

The aquatic fungal spores were trapped from decaying leaves collected from a mountain stream by air bubbles[2]. 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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Table 1. Cultural characteristics of *Clavariopsis aquatica* AJ117363.

Media	Colony size (mm) 25℃ 14days	Color of mycelium	Color of reverse side
LCA	10	Greenish gray	Greenish gray
CMA	16	Greenish white	Greenish white
PDA	21	Gray	Greenish black
MA	19	Gray	Greenish black

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).

General

HR-FABMS were measured by a JEOL Mstation JMS-700 spectrometer. The antibiotics were hydrolyzed with 6 N HCl at 110°C and the hydrolysates were examined by an amino acid auto analyzer (Hitachi L8500).

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 1×10^4 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 morpholine-propanesulforic 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 μm×10~15 μm), 2 celled, the three branches from the upper cell widely divergent (size 50~70 μm×1.5~2.5 μm)). (Fig. 2)

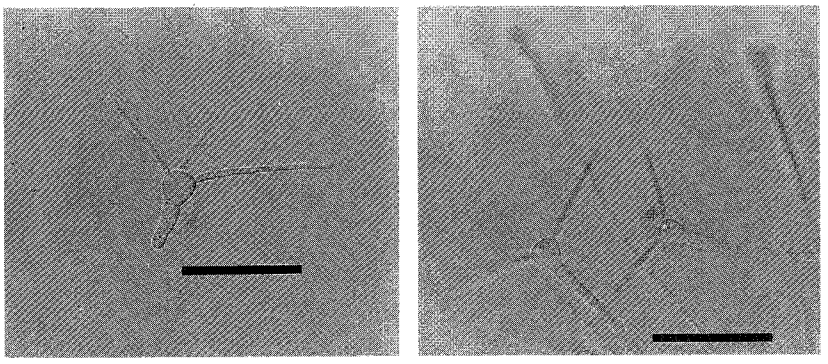
From the mycological characteristics described above, the strain AJ117363 was identified as *Clavariopsis aquatica*[7]. The strain AJ117363 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the name of *Clavariopsis aquatica* 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 160 rpm at

Fig. 2. Microscopic features of strain AJ117363.

Bar represents 50 μm.



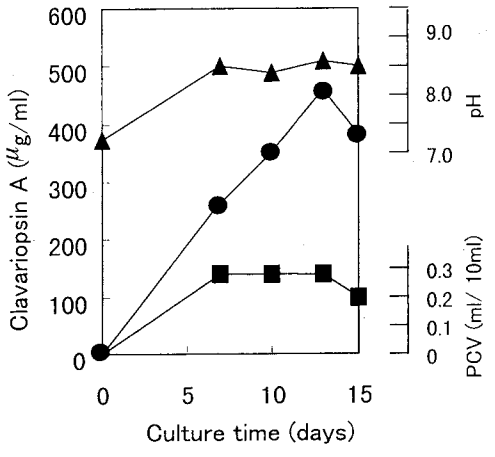
24°C for 7 days to give a seed culture. The seed cultures (2 ml) were transferred into each 150 ml of fermentation medium in 500-ml flask and the flasks were shaken at 24°C for 13 days. The seed and production media were composed of glucose 2.5%, Pharmamedia (Southern Cotton Oil Company, USA) 2.0%, Amino acids mixture for culture media (Ajinomoto Co., Inc., Japan) 0.1%, and $CaCO_3$ 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.

Fig. 3. Time course of production of clavariopsin A.

● Clavariopsin A (μg/ml), ▲ pH, ■ packed cell volume (ml/10 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 acetonitorile, applied on a C18 silica gel column (200 ml), and developed with acetonitorile - water (8 : 2). The fractions containing clavariopsins were collected and concentrated under reduced pressure to give 0.6 g of residue. The residue was dissolved in acetonitorile, applied on C18 HPLC column Capcell Pak (20×250 mm) and eluted with acetonitorile - water (19 : 1). The fractions containing clude 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.

Properties		Clavariopsin A	Clavariopsin B
Appearance		Colorless powder	Colorless powder
UV λ_{max}^{MeOH} nm		277, 288	277, 288
Molecular formula		$C_{59}H_{95}N_9O_{14}$	$C_{58}H_{93}N_9O_{14}$
Molecular weight		1153	1139
HR FABMS	calcd	1176.6896 (M+Na)	1162.6740 (M+Na)
	found	1176.6924 (M+Na)	1162.6740 (M+Na)
Amino acid analysis		Gly, Val, Tyr	Not tested
Solubility	soluble	MeOH, EtOH, EtOAc, DMSO	MeOH, EtOH, EtOAc, DMSO
	insoluble	H_2O	H_2O

Table 3. Antifungal activity of clavariopsins.

Minimum inhibitory concentration (MIC, μg/ml).

Strain	Clavariopsin A	Clavariopsin B	AmphotericinB	Miconazole
Candida albicans IFO 0583	8	8	1	0.25
Candida albicans IFO 0583	8	8	2	0.25
Candida albicans ATCC 10231	8	8	0.5	0.5
Aspergillus niger AJ117374	16	16	1	1
Aspergillus fumigatus AJ117190	2	4	1	1
Aspergillus fumigatus JCM1739	4	4	0.25	2

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 $C_{59}H_{95}N_9O_{14}$ (MW 1153) and $C_{58}H_{93}N_9O_{14}$ (MW 1139).

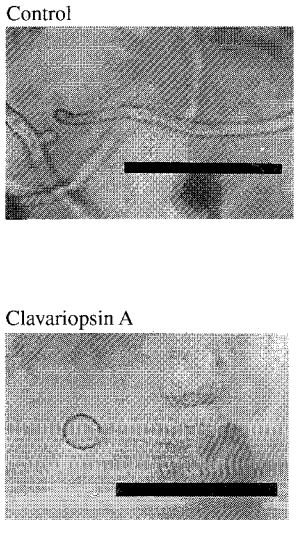
Biological Properties

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

Fig. 4. Hyphal morphology change of *Aspergillus niger* induced by clavariopsin A.

Bar represents 50 μm.



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 group[8~10].

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 phenomenon[11,12], 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 synthesis[12,13]. However, further investigation is needed to clarify the mode of actions of clavariopsins against the target microorganisms.

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