AUREOBASEIDINS, NEW ANTIFUNGAL ANTIBIOTICS

TAXONOMY, FERMENTATION, ISOLATION, AND PROPERTIES

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(Received for publication January 30, 1991)

Aureobasidins A to R were isolated from the fermentation broth of Aureobasidium pullulans
R106. Aureobasidins are cyclic depsipeptide antibiotics with MW's ranging from 1,070 to 1,148.
Aureobasidins showed high in vitro antifungal activity against Candida albicans.

In the course of our screening for antifungal substances from various microorganisms, new antifungal
antibiotics that we named aureobasidins were obtained from the fermentation broth of a strain of
Aureobasidium pullulans, a species of black yeast.1,2) The producing organism, strain R106, was isolated
from a leaf collected at Tsushima in Nagasaki, Japan.

In this paper we describe the taxonomy of the producing organism, the production and isolation
procedures of aureobasidins A to R, and their physico-chemical and biological properties. The structural
studies of aureobasidins A to R will be described in the following papers.3,4)

Materials and Methods

General

FAB-MS were recorded on a Jeol JMS DX-302 spectrometer. Optical rotations were measured with
a Jasco DIP-181. The antibiotics were hydrolyzed with 6N HCl at 110°C and the hydrolysates were
examined in an amino acid autoanalyzer (Jeol JCL-300).

Detection of Aureobasidins

Aureobasidins were detected by a bioassay, which was carried out by the paper disc diffusion method,
based on their in vitro antifungal activity against Candida albicans TIMM 0136 grown in a medium
consisting of yeast nitrogen base (Difco) 0.67% and glucose 2%.

Aureobasidin A concentrations were measured by reversed phase HPLC (Capcell Pak C18, Shiseido)
with a solvent of acetonitrile-water (7:3) at the UV absorption of 230 nm.

Measurement of Antifungal Activity

The MICs against fungi were determined by an agar dilution method on Casitone agar medium.
Aureobasidins were dissolved in methanol and the solution was diluted with 50% aqueous methanol. A
loopful of a fungal cell suspension in sterile water (5 x 10^7 cells/ml) prepared from the slant culture was
streaked on the surface of the agar plates that contained a drug. After incubation of the plates at 30°C
for 3 days, the lowest concentration of the drug causing virtually complete growth inhibition was determined as the MIC.

Results

Taxonomic Studies

The cultural characteristics of strain R106 on various media after incubation for 4, 7 and 14 days at 25°C are shown in Table 1. The temperature permitting growth of the strain was 12.5 to 29°C, and optimum temperature for growth was 23 to 29°C. The strain showed good growth in a vitamin-free medium.

Strain R106 showed good growth on potato-dextrose (PD) agar, CZAPEK agar, and yeast extract-malt extract agar media. The surface of colonies of the strain was usually mucoid or pasty, and were sometimes velvety; with time, the colonies became leathery. The color of colonies was white to creamy or light pink, then changing olive green to light brown or brown, and often finally changing to black with production of dark brown pigments, which were insoluble. Rhizoid-like structures often formed around the colonies. Hyphae sized 2 to 15 μm i.d. elongated into the agar medium without forming aerial mycelia. Blastic

<table>
<thead>
<tr>
<th>Medium</th>
<th>Color of colonies after cultivation for</th>
<th>Growth characteristics</th>
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<tbody>
<tr>
<td></td>
<td>4 days</td>
<td>7 days</td>
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<tr>
<td>Yeast extract-malt extract agar</td>
<td>Creamy</td>
<td>Olive green</td>
</tr>
<tr>
<td>Potato-dextrose (PD) agar</td>
<td>Creamy or light pink</td>
<td>Olive green</td>
</tr>
<tr>
<td>CZAPEK agar</td>
<td>Creamy</td>
<td>Brown</td>
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<tr>
<td>Sabouraud-dextrose agar</td>
<td>Creamy or light pink</td>
<td>Olive green</td>
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<tr>
<td>Oatmeal agar</td>
<td>Creamy gray</td>
<td>Dark brown</td>
</tr>
<tr>
<td>YpSs agar</td>
<td>Creamy</td>
<td>Light olive green</td>
</tr>
</tbody>
</table>

Fig. 1. Microscopic features of strain R106.

Grown on PD agar for 21 days. Bar represents 20 μm.
conidia measuring 1~5 x 2~10 μm, were often formed in an intercalary or terminal position on hyphae like fingers, and sometimes formed ball-like clusters (Fig. 1). Vegetative cells in the early stage of growth were yeast-like, ellipsoidal or lemon-shaped measuring 3~5 x 8~15 μm and multiplied by polyblastic budding. The strain formed arthrospores sized 4~10 x 10~25 μm. No ascospores were observed.

From the mycological characteristics described above, strain R106 was identified as belonging to the genus *Aureobasidium*. Among the known species of *Aureobasidium*, strain R106 was most like a strain of *A. pullulans*. So we designated the strain *A. pullulans* R106. The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession No. of FERM BP-1938.

Fermentation

A loopful of cells of a slant culture of strain R106 was inoculated in 100 ml of a liquid medium composed of yeast nitrogen base (Difco) 0.67% and glucose 2% in a 500-ml Erlenmeyer flask and the flask was shaken at 25°C for 2 days to give a seed culture. The seed culture (1,000 ml) was transferred into a 200-liter fermenter containing 100 liters of a medium consisting of glucose 2%, (NH₄)₂SO₄ 0.5%, KH₂PO₄ 0.15%, MgSO₄·7H₂O 0.05%, CaCl₂·2H₂O 0.01%, NaCl 0.01%, FeCl₃·6H₂O 0.5 μg/ml and ZnSO₄·7H₂O 0.5 μg/ml. Fermentation was carried out at 25°C for 56 hours with aeration (100 liters/minute) and agitation (100 rpm). To the culture, 20 liters of a medium containing glucose 10%, (NH₄)₂SO₄ 2.5%, Polypepton 5%, KH₂PO₄ 0.75%, MgSO₄·7H₂O 0.25%, CaCl₂·2H₂O 0.05%, NaCl 0.05%, FeCl₃·6H₂O 2.5 μg/ml and ZnSO₄·7H₂O 2.5 μg/ml was supplemented, and fermentation was further continued at 25°C for 78 hours with aeration (120 liters/minute) and agitation (100 rpm).

The time course of the production of aureobasidin A is shown in Fig. 2.

Isolation and Purification

The fermentation broth was centrifuged to separate the supernatant and the mycelial cake. The mycelial cake was extracted with 10 liters of ethanol. The ethanol extract was concentrated under reduced pressure.

Fig. 2. Time course of production of aureobasidin A by fermentation.

○ Aureobacandin A (μg/ml), ● pH, ▲ glucose (mg/ml), ▲ packed cell volume (ml/10 ml).

The glucose concentration was determined by the glucose oxidase method.
to remove ethanol and the residue was extracted twice with ethyl acetate. The ethyl acetate extract was
concentrated to dryness under reduced pressure, and the residue was dissolved in chloroform. The
chloroform solution was put onto a column of silica gel (1.5 liters). After the column was washed with
3 liters of hexane, it was developed and eluted with 6 liters of hexane - 2-propanol (7:3). The active fraction
eluted with hexane - 2-propanol was condensed under reduced pressure to give 15 g of a residue.

The residue was dissolved in acetonitrile. HPLC analysis of the solution suggested that at
least 10 related compounds were contained in the residue (Fig. 3).

The solution was applied on a preparative
ODS-silica gel column of Soken Pak/C18 (5 cm x
50 cm, 15/30 μm) and developed with acetonitrile-
water (7:3). The fractions containing aureobasidins
A to R were collected separately and concentrated
under reduced pressure to give residues of crude
aureobasidins A to R. The residues containing A,
D, E, F, G, H, I, J, P, Q and R were each dissolved
in acetonitrile, applied on a column of Capcell
Pak C18 (2 x 25 cm) and eluted with acetonitrile-
water (7:3) to give pure aureobasidins (A: 3,500 mg,
D: 96 mg, E: 81 mg, F: 72 mg, G: 1,380 mg, H:
32 mg, I: 60 mg, J: 24 mg, P: 15 mg, Q: 14 mg, and
R: 1,101 mg).

Fig. 3. HPLC of a mixture of aureobasidins.

Table 2. Physico-chemical properties of aureobasidins A to R.

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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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<td>1,087</td>
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<td>1,117</td>
<td>1,087</td>
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<td>c 1.0</td>
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<td>c 0.8</td>
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<th>K</th>
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<tr>
<td>FAB-MS (M+H, m/z)</td>
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Table 3. Antifungal activity of aureobasidins A to R.

<table>
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<th>Organisms</th>
<th>TIMM No.</th>
<th>MIC (µg/ml)</th>
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<tr>
<td></td>
<td></td>
<td>A</td>
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<tr>
<td>Candida albicans</td>
<td>0136</td>
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<td>C. albicans</td>
<td>0171</td>
<td>&lt;0.05</td>
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<td>C. albicans</td>
<td>1768</td>
<td>&lt;0.05</td>
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<td>C. kefyr</td>
<td>0301</td>
<td>0.20</td>
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<td>C. glabrata</td>
<td>0354</td>
<td>0.78</td>
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<tr>
<td>Cryptococcus neoformans</td>
<td>0354</td>
<td>0.78</td>
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R: 18mg). The residue containing aureobasidins B and C was dissolved in chloroform and chromatographed on a silica gel HPLC column (Nucleosil-5, 10x250mm) with a solvent of hexane - 2-propanol - acetonitrile (85:6:9) to obtain purified aureobasidins (B: 55mg, and C: 54mg). The residue containing aureobasidins K, L and M was purified in the same way by silica gel HPLC column chromatography to obtain pure aureobasidins (K: 107mg, L: 42mg, and M: 78mg). The residue containing aureobasidins N and O was also purified by silica gel HPLC to obtain pure aureobasidins (N: 18mg, and O: 18mg).

**Physico-chemical Properties**

Some properties of aureobasidins A to R are summarized in Table 2. Aureobasidins showed maxima at 258 (ε 3,850) and 264 nm (ε 2,660) in UV absorption spectrum in methanol. Aureobasidins were soluble in ethanol, chloroform, ethyl ether and insoluble in water. Aureobasidins contained phenylalanine, proline, and leucine in common, and contained five different amino acids, including N-methylated amino acids. Aureobasidins were cyclic depsipeptide antibiotics containing eight amino acids and a hydroxy acid3,4 with MW’s of 1,070 ~ 1,148.

**Biological Properties**

The antifungal activity of aureobasidins is shown in Table 3. Aureobasidins showed strong antifungal activity, especially against C. albicans. Aureobasidins A, B, C and E showed higher activity than the others. Aureobasidin A, B, E or G showed no signs of toxicity when administered once to mice intraperitoneally at the dose of 200 mg/kg.

Details of the biological properties of aureobasidin A, including its in vitro and in vivo antifungal activity, will be described elsewhere.
Discussion

There are some peptide antibiotics with antifungal activity, including the aculeacin/echinocandin family and lipopeptin.8-12 These antibiotics all contain fatty acids, which are important for their activity. Aureobasidins are new antifungal peptide antibiotics without fatty acids, but which have high activity and low toxicity.

References