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Arisostatins A and B, New Members of Tetrocarcin Class of Antibiotics from *Micromonospora* sp. TP-A0316

I. Taxonomy, Fermentation, Isolation and Biological Properties

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Arisostatins A and B, new members of tetrocarcin class of antibiotics were isolated from the culture broth of an actinomycete strain. The producing strain, TP-A0316, was identified as *Micromonospora* sp. Arisostatins were obtained from the culture fluid by solvent extraction and chromatographic purification. They showed antibiotic activity against Gram-positive bacteria and antitumor activity.

In the course of screening of new antibiotics from microbial metabolites, we found that arisostatins A and B (Fig. 1) were produced in the culture broth of a strain TP-A0316 along with tetrocarcin A (TCA)[1]. Arisostatins, which have an *iso*-butanoyl substituent on one of the digitoxoses instead of the acetyl group in TCA, are new members of tetrocarcin class of antibiotics. In addition, we found that the previously reported stereostructure of TCA was incorrect with respect to its anomeric configurations. The structure of TCA has been revised as shown in Fig. 1. The producing strain was isolated from the seawater sample collected in Toyama Bay, Japan and identified as *Micromonospora* sp.

In this paper, we describe the taxonomy of the producing strain, fermentation, isolation and biological properties of arisostatins A and B. Structure elucidation of the arisostatins will be reported in the accompanying paper[2].

Materials and Methods

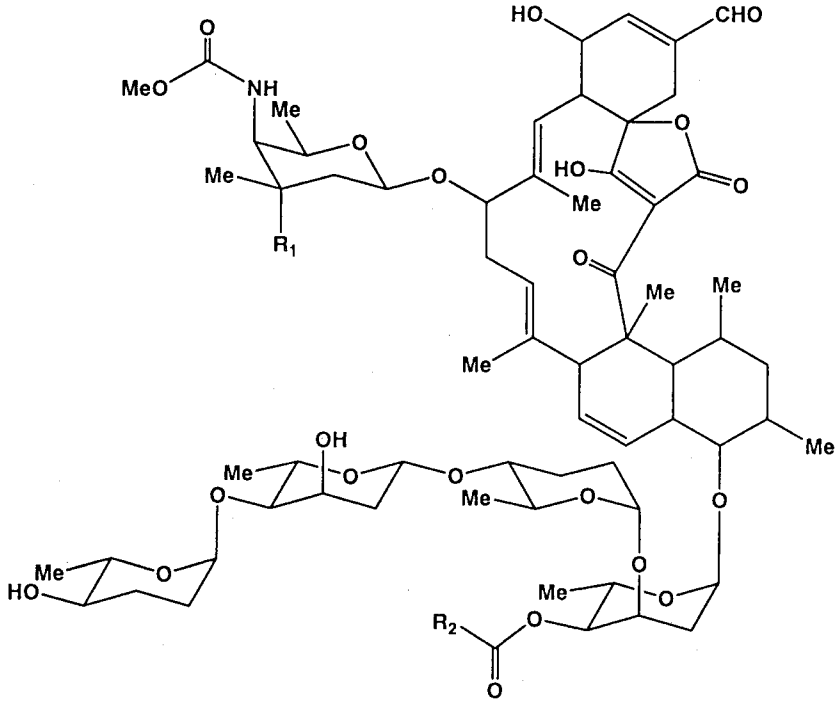
Microorganisms

Strain TP-A0316, arisostatin-producer, was isolated from the seawater sample collected in Toyama Bay, Japan. The spores of the strain were obtained by the membrane filter method and cultured on an agar plate. A pure culture of strain TP-A0316 was preserved in 20% glycerol at −80°C. It was also maintained at 10°C for laboratory use as a slant on BENNETT's agar.

Taxonomy

Taxonomic characteristics of strain TP-A0316 were determined by cultivation on various media described by SHIRLING and GOTTLIEB[3], WAKSMAN[4] and ARAI[5]. Morphological characteristics were observed after incubation of the culture at 30°C for 14 days on oatmeal agar (ISP. med. 3) supplemented with 0.2% yeast extract. Cultural and physiological characteristics were determined after growth at 30°C for 14 days. The color names and hue numbers were assigned using the Manual of Color Names (Japan Color Enterprises Co., Ltd., 1987). Temperature range for growth was determined using a temperature gradient incubator TN-2148 (Advantec Toyo Co.). The carbon utilization was determined by the method of SHIRLING and GOTTLIEB[3]. Cell wall composition was analyzed by the method of LECHEVALIER *et al.*[6], using thin

Fig. 1. Structure of arisostatins A and B and tetrocarcin A.



Arisostatin A: R_1=NO_2, R_2=$CH(CH_3)_2$
B: R_1=NH_2, R_2=$CH(CH_3)_2$
Tetrocarcin A: R_1=NO_2, R_2=CH_3

layer chromatography plates as described by STANECK *et al.*[7] Phospholipids and fatty acids compositions were determined by the methods of LECHEVALIER[8] and SUZUKI *et al.*[9], respectively.

Biological Assay

Antibiotic acitivity in fermentation broths and purification samples were evaluated by the conventional paper disc assay using *Bacillus subtilis* ATCC 6633 as an indicator strain. MIC values were determined by the conventional serial two-fold dilution method against laboratory strains. Cytotoxicity against human myeloid leukemia U937 cells was determined by MTT method according to the supplier's protocol (Promega Corp.). IC_{50} values against other cancer cell lines were determined by colorimetric analysis of the cells stained with sulforhodamine B.

Results and Discussion

Taxonomy of the Producing Strain

On observation with light microscope, spores were singly formed on substrate mycelium. By scanning electron microscope, the spore was oval in shape and 0.8~1.2 μm in size (Fig. 2). The aerial mycelium was not visible on ISP med. Nos. 2 and 3, yeast-starch agar and BENNETT's agar. The cultural characteristics are summarized in Table 1. Color of colony was dark olive gray. Color of reverse side was light orange to dark olive gray. Diffusible pigments were not formed. As summarized in Table 2, milk coagulation and peptonization gave a positive reaction. The temperature range for growth was 21~43°C and the optimum temperature for growth was 25~31°C. L-Arabinose, D-xylose, D-glucose, sucrose, L-rhamnose, D-mannose and raffinose were utilized by strain TP-A0316 for growth. D-Fructose, inositol and D-mannitol were not utilized.

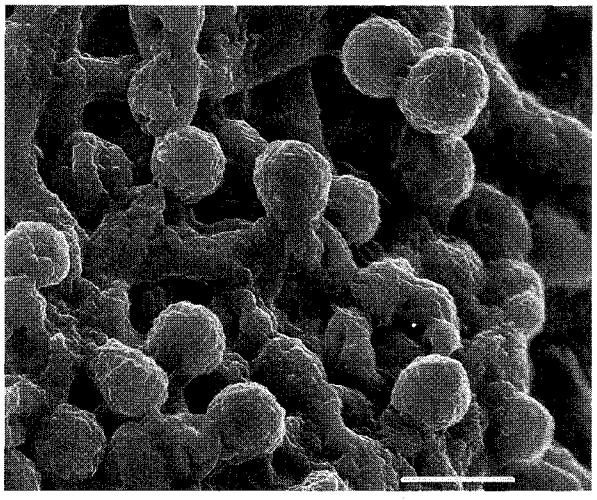
Whole cell hydrolysates contained *meso*-diaminopimelic

acid with no LL isomer present. Strain TP-A0316 has type PII phospholipid (presence of phosphatidylethanolamine and phosphatidylinositol). Fatty acids consisted of 46% 13-methyltetradecanoic acid (*i*-14), 16% heptadecanoic acid (17 : 0), 15% 12-methyltridecanoic acid (*i*-13), 9% 15-methylhexadecanoic acid (*i*-16), 8% hexadecanoic acid (16 : 0) and other minor fatty acids.

The taxonomic results of this strain described above coincided with the characteristics of the genus *Micromonospora* Orskov 1923. Thus it is concluded that the culture TP-A0316 represents a species of *Micromonospora*.

Fig. 2. Scanning electron micrograph of *Micromonospora* sp. TP-A0316.

Bar represents 1 μm.



Strain TP-A0316 grown on oatmeal agar supplemented with 0.2% yeast extract at 30°C for 10 days, showing the formation of single spores with warty surface on substrate mycelium.

Fermentation

A loopful of a mature slant culture of *Micromonospora* sp. TP-A0316 was inoculated into a 500-ml K-1 flask containing 100 ml of the seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case (Humco Scheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flask was incubated at 30°C for 4 days on a rotary shaker (200 rpm). Three-ml aliquots of the seed culture were transferred into thirty 500-ml K-1 flasks each containing 100 ml of the production medium consisting of glucose 2.0%, Protein S 1.5%, $CaCO_3$ 0.3%, NaI 0.00025% and L-leucine 0.2%. The pH of the medium was adjusted to 7.0 before sterilization. Fermentation was carried out for 9 days at 30°C on a rotary shaker (200 rpm). The production of arisostatins in the fermentation was monitored by HPLC using an ODS column (Cosmosil 5C18-AR-II, 4.6×250 mm, Nacalai Tesque Inc.) with the eluent of acetonitrile - 0.15% phosphate buffer, pH 3.5 (75 : 25) at a flow rate of 0.7 ml/minute with 230 nm detection. The retention times of arisostatins A and B were 19.5 and 5.0

Table 1. Cultural characteristics of strain TP-A0316.

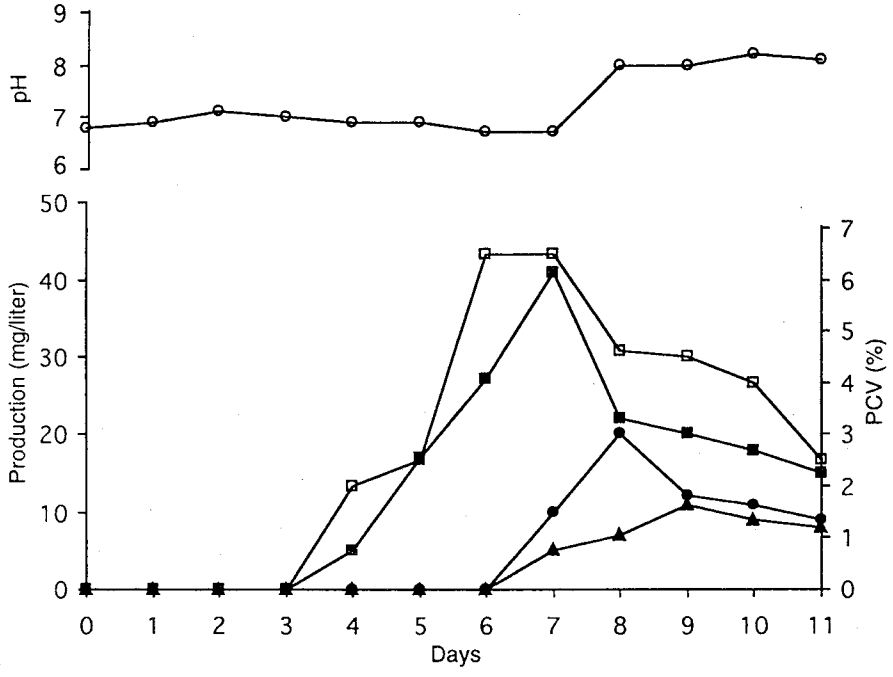
Medium	Growth	Reverse side	Diffusible pigment
Yeast extract - malt extract agar (ISP med. 2)	Grayish brown (124), good	Dark grayish brown (119)	None
Oatmeal agar (ISP med. 3)	Dark olive gray (419), good	Dark olive gray (419)	None
Inorganic salts - starch agar (ISP med. 4)	Light orange (66), moderate	Light orange (66)	None
Glycerol asparagine agar (ISP med. 5)	Colorless, poor	Coloress	None
Tyrosine agar (ISP med. 7)	Colorless, poor	Coloress	None
Nutrient agar (Waksman med. 14)	Deep orange (81), moderate	Deep orange (81)	None
Yeast starch agar	Light orange (66), good	Dark grayish brown (124)	None
Bennett's agar (Waksman med. 30)	Light orange (66), good	Dark yellowish brown (106)	None

Table 2. Physiological characteristics of strain TP-A0316.

Test	Results
Nitrate reduction	Negative
Milk (Difco, 10% skimmed milk)	
Coagulation	Positive
Peptonization	Positive
Cellulose decomposition (sucrose nitrate solution with a paper strip as the sole carbon source)	Negative (Growth: good)
Gelatin liquefaction	
on plain gelatin	Negative
on glucose peptone gelatin	Negative
Melanine formation (on ISP med. 7)	Negative
Temperature range for growth (on Yeast starch agar)	21~43°C
Optimum temperature (on ISP med. 2)	25~31°C
NaCl tolerance (on ISP med. 2)	
0%	Positive
>4%	Negative

Fig. 3. Typical time course of arisostatin production.

Production of arisostatin A (●) and B (▲) and tetrocarcin A (■), packed cell volume (□) and pH (○).



minutes, respectively, and that of tetrocarcin A was 10.3 minutes. For preparation of the assay sample, a 10 ml portion of the whole broth was extracted with the same volume of ethyl acetate at pH 8.0. The extract was concentrated *in vacuo*, diluted with 1 ml of methanol and 20 μl of the diluted extract was used for HPLC analysis. The production of tetrocarcin A in shake flask culture reached a maximum of 41 mg/liter after 7 days. With the decrease of tetrocarcin A production, the production of arisostatins A and B reached a maximum of 22 mg/liter

Table 3. *In vitro* antibacterial activities of arisostatins A (**1**) and B (**2**) and tetrocarcin A (TCA).

Organism	MIC (μg/ml)		
	1	**2**	TCA
Staphylococcus aureus 209P JC-1	100	50.0	50.0
Bacillus subtilis ATCC 6633	0.39	25.0	0.10
Micrococcus luteus ATCC 9341	12.5	3.1	1.6
Escherichia coli NIHJ JC-2	>100	>100	>100
Pseudomonas aeruginosa A3	>100	>100	>100
Saccharomyces cerevisiae S-100	100	100	100
Candida albicans A9540	>100	>100	>100

after 8 days and 13 mg/ml after 9 days, respectively (Fig. 3).

Isolation

The fermented whole broth (3 liters) was centrifuged (8,000 rpm, 15 minutes) to separate the mycelia and the supernatant. The supernatant was extracted with 1.5 liters of ethyl acetate at pH 8.0. The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a brown oil (376 mg). The oily substance was purified by reverse phase HPLC using Cosmosil 5C18-AR-II (Nacalai Tesque Inc., 20×250 mm) column with the eluent of acetonitrile - 0.15% phosphate buffer, pH 3.5 (75 : 25) at a flow rate of 9.0 ml/minute. With monitoring the antibacterial activity against *Bacillus subtilis* ATCC 6633 three active fractions I (Rt. 4~8 minutes), II (Rt. 9.5~10.5 minutes) and III (Rt. 18.0~18.5 minutes) were obtained. Fraction III was evaporated under reduced pressure to remove acetonitrile. The resultant aqueous solution was adjusted to pH 7.0 with 0.1 N NaOH and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give arisostatin A (27.1 mg). Fraction I was further purified by preparative HPLC under the same conditions described above except for the solvent system, acetonitrile - 0.15% phosphate buffer, pH 3.5 (65 : 35). The active fraction thus obtained was evaporated and extracted in the same manner as described above to give arisostatin B (6.6 mg). Tetrocarcin A (16.6 mg) was obtained from fraction II in the same manner as described for arisostatin A.

Table 4. Cytotoxicity of arisostatins A (**1**) and B (**2**) and tetrocarcin A (TCA) against U937 cell line.

IC_{50} (μg/ml)		
1	**2**	TCA
0.4	4.0	0.6

Biological Properties

The antimicrobial activities of arisostatins A and B are shown in Table 3 in comparison with tetrocarcin A. Arisostatins exhibited antibacterial activity against Gram-positive bacteria and no activities against Gram-negative bacteria and yeasts. *In vitro* cytotoxic activity of arisostatins A and B was tested against human myeloid leukemia U937 cells (Table 4). Arisostatin A was most potent among the congeners with an IC_{50} value of 0.3 μM. Arisostatin A also showed *in vitro* antitumor activity against cancer cell lines derived from organs such as breast, brain, colon and lung with IC_{50} values of 0.059~0.26 μM as shown in Table 5. The antitumor activity of tetrocarcin A was reported in previous papers without any discussion of the mechanism[1,10,11]. We have found that arisostatin A inhibits the neuritogenesis of NGF-stimulated PC12 cells at less than 1 μM by inhibiting tubulin polymerization. The inhibition of tubulin polymerization by arisostatin A was

Table 5. Antitumor activity of arisostatin A.

		IC_{50} (μM)
Cell line	Origin	IC_{50} (μM)
HBC-4	Breast cancer	0.26
HBC-5	Breast cancer	0.059
SF-539	Brain cancer	0.21
HCC2998	Colon cancer	0.22
NCI-H522	Lung cancer	0.22
DMS114	Lung cancer	0.23

detected at 1~10 μM by the immunostaining experiment using anti-tubulin antibody and Swiss 3T3 cells on a laser microscope. Recently, the inhibition of the anti-apoptotic function of Bcl-2 by tetrocarcin A was reported by Kyowa Hakko's group[12]. This multifunctional mode of action makes the tetrocarcin class compound a pharmacologically intriguing molecule.

Acknowledgments

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