NOVEL CYTOCIDAL COMPOUNDS, OXOPROPALINES FROM *Streptomyces* sp. G324 PRODUCING LAVENDAMYCIN

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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In the course of our investigation aimed at the discovery of novel antitumor antibiotics from microorganisms, *Streptomyces* sp. G324 was found to produce the antitumor antibiotic, lavendamycin, and also, to yield the novel β-carboline compounds, oxopropalines. We isolated five compounds as oxopropalines A, B, D, E and G. Oxopropalines B, D and G showed cytocidal activities against human or murine tumor cell lines *in vitro*.

In the course of our investigation aimed at the discovery of novel antitumor antibiotics from microorganisms, a Streptomycete, isolated from a soil sample was found to produce an antitumor antibiotic, lavendamycin. In addition, novel cytocidal compounds oxopropalines possessing characteristic UV spectra, were found. In this paper, we describe the taxonomic studies of the producing organism, fermentation, isolation and biological activities. Physico-chemical properties and structure elucidation will be described in a subsequent paper[1].

Materials and Methods

Microorganism

Strain G324 were isolated from a soil sample collected in Fujieda City, Shizuoka Prefecture, Japan. A subculture of the strain has been deposited at the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM BP-3948.

Taxonomic Studies

To investigate the morphological and physiological properties of the strain, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB were used[2]. Cell wall analysis was performed using the methods described by BECKER *et al.*[3] and YAMAGUCHI[4]. Cultures were observed after 2 weeks at 28°C. The color index was in accordance with the ISCC-NBS Color-Name chart. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[5].

Fermentation

The seed medium containing 2.0% dextrin, 1.0% glucose, 1.0% peptone, 0.5% corn steep liquor and 0.2% $CaCO_3$ was adjusted to pH 7.0 and then sterilized at 121°C for 15 minutes in 3×500-ml baffled Erlenmeyer flasks containing 100 ml of medium. The strain G324 on an agar slant was inoculated into the flasks and cultured at 35°C for 3 days on a rotary shaker at 100 rpm. Two ml of the seed culture was inoculated into each of the 80×500-ml Erlenmeyer flasks containing 100 ml of the previously described medium. The fermentation was carried out at 35°C for 5 days.

Fermentation Analyses

The fermentation was monitored daily for growth, pH and the production of oxopropaline and lavendamycin. Growth was evaluated using the packed cell volume of 10 ml of whole broth centrifuged at 3,000 rpm for 10 minutes. The production of oxopropaline and lavendamycin was analyzed by HPLC. Ten ml of the whole broth was adjusted to pH 10.0 (oxopropalines) or pH 2.0 (lavendamycin) and added to 10 ml of ethyl acetate. The mixture was stirred for 10 minutes and then centrifuged at 3,000 rpm for 10 minutes. The supernatant liquor was separated into 2 layers. The ethyl acetate layer was concentrated *in vacuo* and dissolved in 1.0 ml of CH_3OH for HPLC analysis. HPLC analysis was performed using a JASCO HPLC system consisting of 2×880-PU pumps, a 875-UV detector and Sic chromatocoder 12 integrator (System Instrument Co. Ltd.), a Wako sil-II 5C18 HG reverse-phase analytical column (4.6×250 mm, Wako Pure Chemical Industries Ltd.), elution with 0.15% KH_2PO_4 (pH 3.5)-acetonitrile (7:3 for 6 minutes, 7:3 to 3:7 linear gradient for 9 minutes, 3:7 to 15:85 linear gradient for 2 minutes and then isocratic with 15:85 for 13 minutes) used as the mobile phase at a flow rate of 1.0 ml/minute with detection at 380 nm. The HPLC profile of the mixture of oxopropalines and lavendamycin is shown in Fig. 1.

Cytocidal Activity

For the test of *in vitro* cytocidal activity, a microculture tetrazolium assay (MTT assay)[6] was employed with little modification. Murine P388[7] and human A549[8] cells were obtained from JCRB. Human MKN45 and MKN74 cells were gift from Dr. H. HOJO of Niigata University[9]. Human WiDr[10] cell was purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. The IC_{50} value was calculated with PROBIT's method.

Results and Discussion

Taxonomic Studies of the Producing Strain

Strain G324 has branched substrate mycelia from which aerial hyphae develop in the form of a tightly closed spiral and contained 10 to 50 spores per chain. Spores, ranging from $0.6 \times 1 \sim 1.2\,\mu m$ in size with a smooth surface, were cylindrical. Sclerotium, sporangium and zoospore were not observed, but a hygroscopic character was observed. The cultural characteristics of the strain are shown in Table 1. The aerial mass color showed that the strain belonged to the red color series. The physiological characteristics and the utilization of carbohydrate observed after incubation at 28°C for 14 days are shown in Tables 2 and 3. LL-Diaminopimelic acid was contained in the whole-cell hydrolysate of the strain and melanin was produced on ISP No. 6 agar. Hence, the strain was placed in cell-wall Type I. Based on the taxonomic observation already described, the producing strain G324 belonged to the genus *Streptomyces*, and was

Fig. 1. HPLC profile of oxopropalines and lavendamycin.

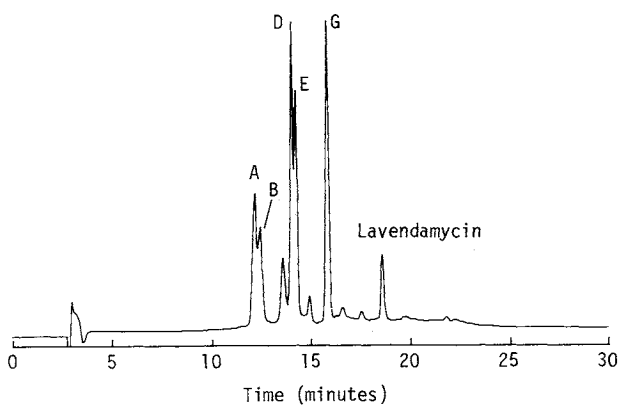

Table 1. Cultural characteristics of G324 strain.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	Abundant (Hygroscopic), moderate reddish brown (43)	Dark yellowish brown (78)	None
Oatmeal agar (ISP-3)	Good	Abundant (Hygroscopic), light reddish brown (42)	Deep yellowish brown (75)	None
Inorganic salt-starch agar (ISP-4)	Good	Abundant (Hygroscopic), dark reddish brown (44)	Colorless	None
Glycerin-asparagine agar (ISP-5)	Good	None	Black red (21)	None
Peptone-yeast extract agar (ISP-6)	Good	None	Colorless	Dark yellowish brown (78)
Tyrosine agar (ISP-7)	Good	Abundant, moderate reddish brown (43)	Black red (21)	None
Peptone-nitrate agar	Good	None	Colorless	None
Nutrient agar	Good	None	Colorless	None
CZAPEK's agar	Good	Abundant, grayish yellowish pink (32)	Strong yellowish brown (74)	None

The color index in the parenthesis ( ) is in accordance with ISCC-NBS Color-Name Chart and represents the result of observation on the second week at 28°C on each medium.

Table 2. Physiological properties of G324 strain.

Growth temperature range (ISP 2 or yeast extract-malt extract agar, incubation for 14 days)	20~38°C
Gelatin liquefaction	Negative
Hydrolysis of starch	Positive
Coagulation of skim milk	Negative
Peptonization of skim milk	Positive
Reduction of nitrate	Positive
Decomposition of cellulose	Negative

Table 3. Utilization of carbon sources by G324 strain.

Positive utilization:	D-glucose, D-xylose, D-arabinose, D-fructose, D-mannose, sucrose, trehalose
Negative utilization:	L-rhamnose, raffinose, D-mannitol, inositol, D-galactose, lactose, salicin

very similar to *Streptomyces katrae*[11]. However, by a direct comparison with *S. katrae* JCM4777, strain G324 differed from JCM4777 with regard to its hygroscopic character (strain G324 was hygroscopic), salicin utilization (JCM4777 is positive) and a sensitivity to copper ion (JCM4777 is sensitive). Therefore, the strain G324 was designated as *Streptomyces* sp. G324.

Fermentation

The time course of oxopropaline fermentation is shown in Fig. 2. The production of oxopropalines and lavendamycin was evaluated by HPLC analyses detected at 380 nm. The rate of each oxopropaline production changed with time. Oxopropalines D and G and lavendamycin were accumulated during the early stage of the fermentation followed by production of oxopropalines A, B and E in exchange for the compounds in the early stage.

Isolation

The isolation procedure from the cultured whole broth of *Streptomyces* sp. G324 is shown in Fig. 3.

Fig. 2. Time course of the oxopropaline fermentation.

▽ Growth (% packed cell volume), ▼ pH, ■ A, ● B, ▲ D, △ E, □ G, ○ Lavendamycin.

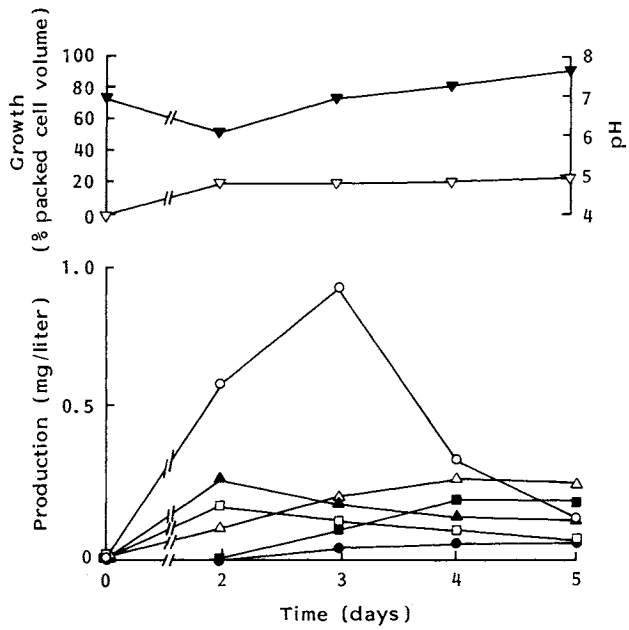

Fig. 3. Isolation of oxopropalines.

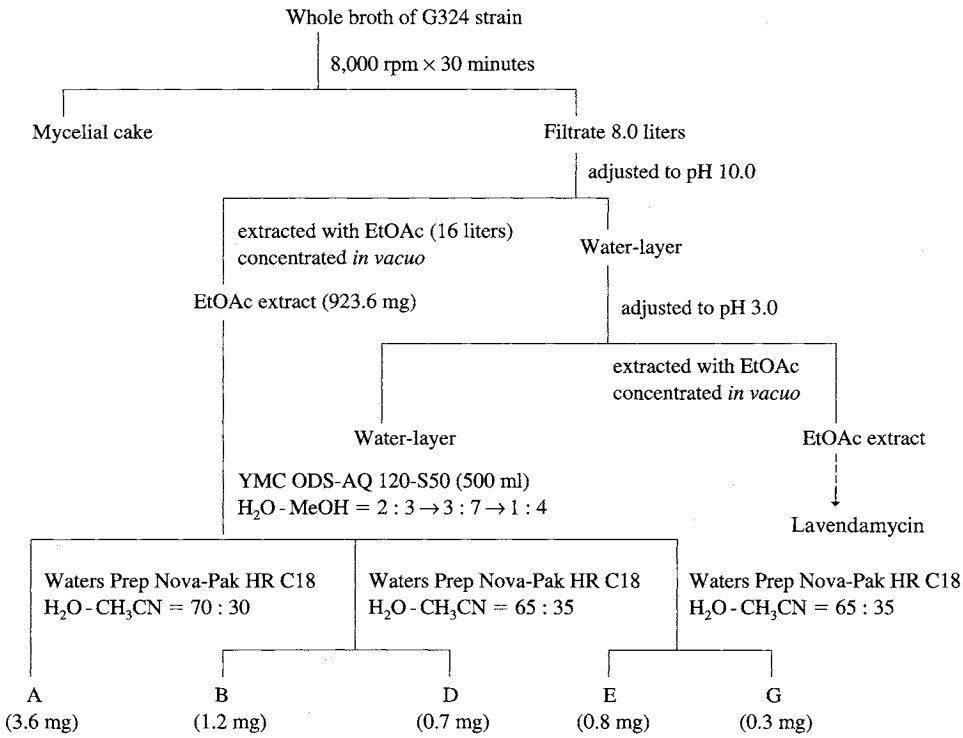

Table 4. *In vitro* cytocidal activities of oxopropalines A, B, D, E and G and lavendamycin.

Cell lines	IC_{50} value (μg/ml)					
	A	B	D	E	G	Lavendamycin
P388 (Leukemia)	>50	41	31	>50	43	0.06
A549 (Lung carcinoma)	>50	>50	17	>50	32	NT
MKN45 (Gastric carcinoma)	>50	>50	39	>50	33	0.10
MKN74 (Gastric carcinoma)	>50	>50	>50	>50	>50	NT
WiDr (Colon adenocarcinoma)	>50	49	38	>50	45	0.09

NT: Not tested.

Oxopropalines A, B, D, E and G displayed lipophilic properties mainly associated with the filtrate. The filtrate was separated from 8 liters of the cultured broth by centrifuging at 8,000 rpm for 30 minutes and was then adjusted to pH 10.0. To this was added 16 liters of ethyl acetate and the mixture was then stirred for 1 hour. Ethyl acetate extract was removed *in vacuo* and dissolved in a small volume of methanol. The methanol solution was charged on the column shielded against light with ODS-AQ 120-S50 and was then developed stepwise with a solvent system of H_2O-methanol, 2:3 to 1:4. The three fractions including the oxopropalines were given. Each fraction was purified by using a reversed-phase HPLC column (Waters Nova-Pak HR C18 25 × 100 mm, flow rate 11.8 ml/minute) with a mobile phase of H_2O-acetonitrile (70:30 or 65:35). The first fraction gave 3.6 mg of oxopropaline A as a pale yellow powder. The next fraction yielded two components, oxopropalines B (1.2 mg) and D (0.7 mg). Further, oxopropalines E (0.8 mg) and G (0.3 mg) were purified from the third fraction.

Cytocidal Activities

The *in vitro* cytocidal activities of oxopropalines and lavendamycin against various tumor cell lines are shown in Table 4. Oxopropalines D and G exhibited growth inhibitory activity in a dose-dependent manner.

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