ACTINOPYRONES A, B AND C, NEW PHYSIOLOGICALLY ACTIVE SUBSTANCES

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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A strain of *Streptomyces* was found to produce new physiologically active substances. The active compounds were purified and separated into three substances named actinopyrones A, B and C.

Actinopyrones exhibited coronary vasodilating activities in anesthetized dogs and weakly antimicrobial activities against some Gram-positive bacteria and dermatophytes.

In the course of our screening program for new physiologically active substances, a streptomycete, strain S12538, identified as *Streptomyces pactum* was found to produce new substances which exhibit vasodilating and antimicrobial activities.

In this report we provide the taxonomy of producing strain, and the fermentation, isolation and biological properties of actinopyrones.

Taxonomy of the Producing Strain

Strain S12538 was isolated from a soil sample collected at Satsukigaoka, Chiba City, Chiba Prefecture, Japan.

Taxonomic studies were carried out according to the methods described by SHIRLING and GOTTLIEB[1] and the color notations were from the ISCC-NBS Centroid Color Charts[2].

Morphological observation was made on the cultures grown at 27°C for 14 days on oatmeal agar

Fig. 1. Aerial mycelium of strain S12538.
Oatmeal agar, 14 days at 27°C, (×600).

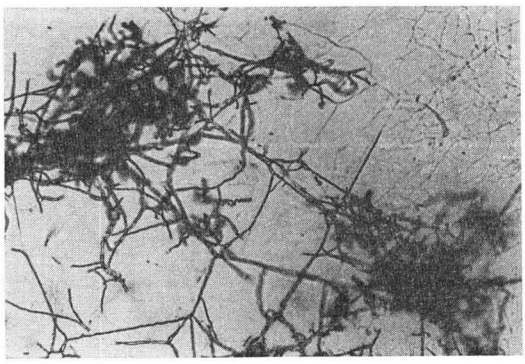


Fig. 2. Spores of strain S12538.
Oatmeal agar, 14 days at 27°C, (×28,000).

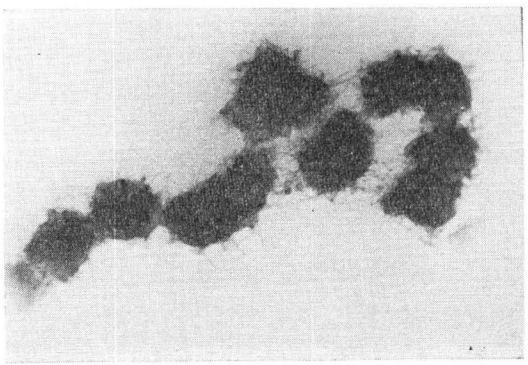


Table 1. Cultural characteristics of strain S12538.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Sucrose - nitrate agar	Poor	Thin, 63. l. br Gy	Colorless	None
Glucose - asparagine agar	Good	Thin, 263. white~264. l. gray	89. p. Y	None
Glycerol - asparagine agar	Good	Thin, 263. white~190. l. b gray	71. m. OY	None
Inorganic salts - starch agar	Good	Good, 265. med. Gy	71. m. OY	Faint, yellowish brown
Tyrosine agar	Good	Good, 263. white~264. l. gray	94. l. OlBr	None
Nutrient agar	Good	None	89. p. Y	None
Yeast extract - malt extract agar	Good	Moderate, 263. white~190. l. b gray	72. d. OY	None
Oatmeal agar	Good	Good, 265. med. Gy	89. p. Y	None
Glycerol - nitrate agar	Good	Thin, 92. y white	89. p. Y	None
Calcium - malate agar	Poor	Thin, 63. l. br Gy	Colorless	None

Table 2. Physiological characteristics of strain S12538.

Properties observed	Characteristics
Melanin formation	None
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Milk coagulation	Positive
Milk peptonization	Positive
Nitrate reduction	Positive

Table 3. Carbon utilization of strain S12538.

D-Glucose	+
L-Arabinose	−
Sucrose	−
D-Xylose	−
i-Inositol	−
D-Mannitol	−
D-Fructose	−
L-Rhamnose	−
Raffinose	+
D-Galactose	+
Salicin	−

+; Positive, −; negative.

and other agar media. Mature spore chains have 10 or more spores in the form of compact spirals (Fig. 1). The spores are 0.5~0.8×0.8~1.5 μm in size with a hairy surface (Fig. 2).

Cultural characteristics are shown in Table 1. Aerial mycelia developed on oatmeal agar, inorganic salts - starch agar and tyrosine agar. The aerial mass color was light bluish to medium gray (Gray color series). The color of substrate mycelia was pale to orange yellow. Faint yellowish brown pigment was found in the medium in inorganic salts - starch agar. This pigment was not pH sensitive when tested with 0.05 N NaOH or HCl.

Physiological characteristics of strain S12538 are presented in Table 2. Strain S12538 grows at 16~39°C with optimum temperature of 26~35°C. Melanoid pigments were not formed in peptone - yeast extract - iron agar, tyrosine agar and Tryptone - yeast broth.

Utilization of carbon sources by strain S12538 is shown in Table 3. D-Glucose, raffinose and D-galactose were utilized for growth of strain S12538 but other carbon sources were not.

The whole-cell analysis of strain S12538 according to the method of STANECK *et al.*[3] revealed the presence of the L-isomer of diaminopimelic acid.

Microscopic studies and cell wall pattern indicated that strain S12538 belongs to the genus *Streptomyces*. Among known species of *Streptomyces*,[4~8] strain S12538 was considered to be closely related to *S. pactum* BHUYAN *et al.*[9] The differences observed between both organisms were nitrate reduction and utilization of raffinose. Namely, strain S12538 reduces nitrate and utilizes raffinose while

S. pactum IFO 13433 does not.

Nevertheless, the only difference cited above does not seem to us sufficient to make a distinction between both organisms. Therefore strain S12538 was identified as *Streptomyces pactum* BHUYAN *et al.* and designated *Streptomyces pactum* S12538.

Fermentation

A well-sporulated slant of *S. pactum* S12538 was inoculated into 500-ml flasks each containing 100 ml of a medium composed of glycerol 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%. The pH of the medium was adjusted to pH 7.0 before sterilization. The flasks were incubated on a reciprocal shaker at 27°C for 48 hours.

The fermentation was carried out in a 30-liter jar fermentor containing 16 liters of the same medium as described above. After inoculation of 300 ml of a seed culture the fermentation was conducted at 27°C for 96 hours under aeration of 16 liters/minute and agitation of 400 revolution/minute. The production of active compounds during fermentation was monitored by HPLC (Table 4).

A typical time course of fermentation in a 30-liter jar fermentor is shown in Fig. 3. The amount of actinopyrones reached a maximum after 4 days fermentation, respectively.

Isolation and Purification

The isolation and purification procedure of actinopyrones A, B and C is shown in Fig. 4.

The culture broth was centrifuged to separate the mycelium from the broth. The supernatant was extracted three times with an equal volume of ethyl acetate. The mycelium was extracted twice

Table 4. Chromatographic behavior of actinopyrones A, B and C on HPLC.

Solvent system	Retention time (minutes)		
	A	B	C
CH_3CN - H_2O (8: 2)	10.1	9.0	11.8
CH_3CN - H_2O (7: 3)	16.0	12.6	19.2

Column; Nucleosile 5C-18 4.6×250 mm. Detection; UV absorption at 239 nm. Flow rate; 1 ml/minute.

Fig. 3. Time course of actinopyrone A production by *Streptomyces pactum* S12538.

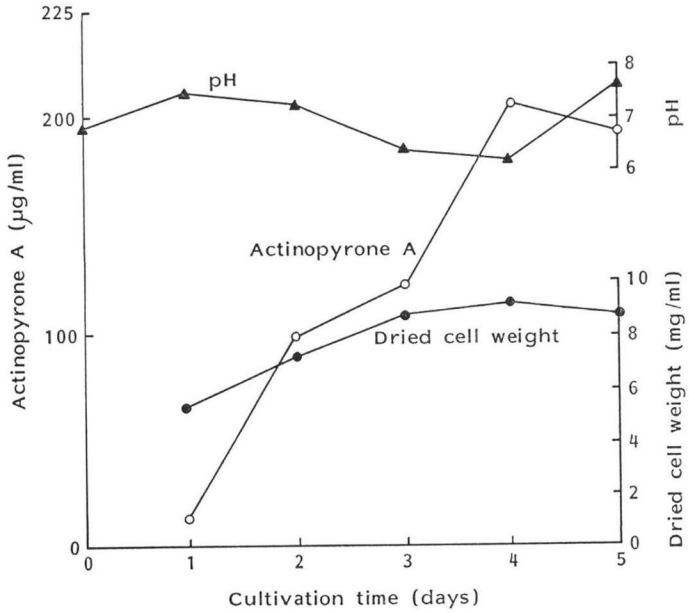
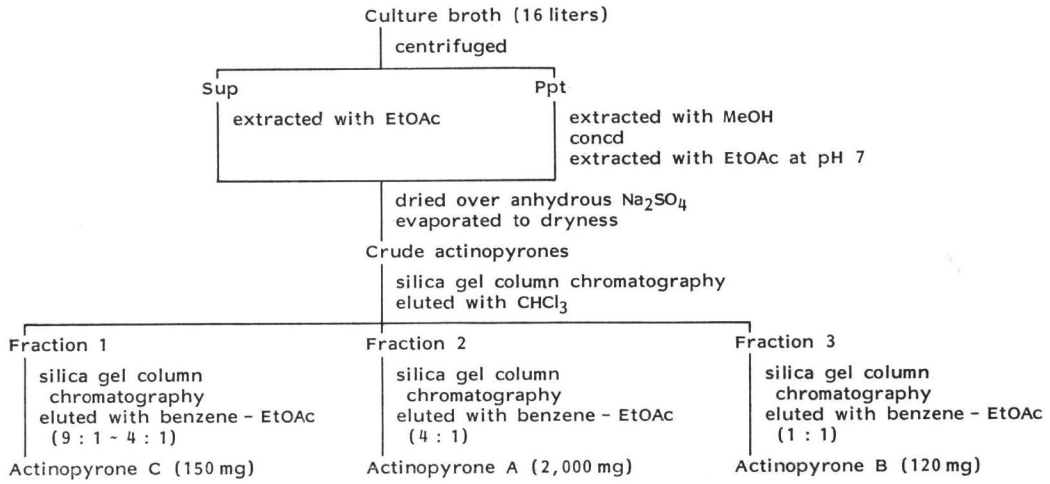


Fig. 4. Flow diagram for the isolation and purification of actinopyrones.



with 5 liters of methanol. The methanol extract was concentrated under reduced pressure and then the residual solution was extracted three times with 1 liter of ethyl acetate. After combining both of the ethyl acetate extracts, they were concentrated under reduced pressure to dryness.

The crude product was dissolved in chloroform, applied to a column (3×30 cm) with Silica gel 60 (Merck) and eluted with chloroform.

The active fraction was concentrated separately and applied to a column (2×30 cm) with silica gel. Each fraction was eluted separately with the solvent composed of benzene and ethyl acetate.

Actinopyrones A (2,000 mg), B (120 mg) and C (150 mg) were obtained from each fraction respectively.

Biological Properties

Coronary vasodilating activities of actinopyrones A, B and C were estimated by the blood flow increasing action. Male mongrel dogs (15~25 kg) were anesthetized with sodium pentobarbital (30 mg/kg iv), and after thoracotomy under artificial respiration, the heart was exposed and the left circumflex coronary artery was isolated free from the surrounding tissue by blunt dissection. A flow prove was attached around the artery to measure the blood flow by the electromagnetic flow meter. Systemic blood pressure was measured directly from right femoral artery through a pressure transducer, and heart rate was also measured by the cardiotachometer from RR intervals of electrocardiogram.

Actinopyrones A, B and C, dissolved in aqueous solution contained less than 5% of dimethyl sulfoxide, were administered intravenously.

Coronary vasodilating activities of actinopyrones A, B and C are shown in Table 5.

Actinopyrones A and B increased coronary blood flow in a dose-related manner. The responses observed in higher dose level (more than 3 or 10 μg/kg) were long-lasting. The relative potencies of both compounds were approximately 100-hold greater than that of papaverine, a standard vasodilating drug. These compounds exhibited biphasic or triphasic responses in systemic blood pressure, that is, in lower dose, delayed hypertention followed by initial hypotention, and in higher dose, thereafter, long-lasting hypotention appeared. However, in any case the increase in coronary blood flow was always accompanied by the initial hypotensive phase. The heart rate decreased mildly.

Table 5. Effects of actinopyrones on CBF, SBP and HR in anesthetized dogs.

Compounds	Dose (μg/kg iv)	Changes in (%)		
		CBF	SBP[a]	HR
Actinopyrone A	3	62.1	−4.1	−2.0
	10	79.4	−14.9	−10.5
	30	196.2	−7.8	−35.1
Actinopyrone B	3	137.7	−12.3	−2.6
	10	215.8	−7.5	−17.7
Actinopyrone C	30	9.5	−3.6	1.7
	100	107.9	−3.2	−12.7
Papaverine	300	30.6	−15.4	8.0
	1,000	59.2	−23.9	16.3

Each value indicates the mean value of two to six experiments.
[a] Represents the initial hypotensive response.
CBF: Coronary blood flow, SBP: systemic blood pressure, HR: heart rate.

Table 6. Antimicrobial spectra of actinopyrones A, B and C.

Organism	MIC (μg/ml)		
	A	B	C
Staphylococcus aureus ATCC 6538P	>100	25	100
S. epidermidis ATCC 12228	<6.25	25	25
Micrococcus lysodeikticus IFO 3333	25	25	12.5
Bacillus subtilis ATCC 6633	>100	50	>100
B. cereus IID 871	12.5	50	25
Escherichia coli O-1	>100	>100	>100
Klebsiella pneumoniae ATCC 10031	>100	>100	>100
Pseudomonas aeruginosa IFO 13736	>100	>100	>100
Candida albicans ATCC 10231	>100	>100	>100
Saccharomyces cerevisiae ATCC 9763	>100	>100	>100
Aspergillus niger ATCC 9642	>100	>100	>100
Trichophyton mentagrophytes QM 248	>100	25	50
Microsporum gypseum IFO 8231	<6.25	100	>100

Agar dilution method (Mueller-Hinton agar for bacteria, Sabouraud agar for fungi and yeasts).

Actinopyrone C showed almost similar profiles with these two compounds, though the vasodilating potency was ten-fold reduced.

Acute toxicities of the actinopyrones were examined with mice. An intravenous dosage of 1 mg/kg or more sacrificed all the mice, but at 0.3 mg/kg all survived.

Antimicrobial Spectrum

Minimal inhibitory concentrations (MICs) of actinopyrones A, B and C against some microorganisms are shown in Table 6.

Actinopyrones showed weakly antimicrobial activities against some Gram-positive bacteria and dermatophytes but no activities against Gram-negative bacteria and yeasts.

Acknowledgment

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References

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