NOVEL ANTITUMOR ANTIBIOTICS, SAPTOMYCINS

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

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Streptomyces sp. HP530 was found to produce novel antitumor antibiotics, saptomycins, closely related to the pluramycin-group and was further found to mutate frequently. The natural mutant produced several new saptomycins as determined by HPLC analyses. We isolated saptomycins A, B, C_1, C_2 and F from the parent strain and saptomycins D, E, G and H from the mutant. The saptomycins showed antimicrobial activities and potent antitumor activities against human or murine tumor cell lines *in vitro* and against Meth A fibrosarcoma *in vivo*. In particular, saptomycin D was most effective component *in vivo* of all saptomycins.

In the course of our searching program for novel antitumor antibiotics from microorganisms, a Streptomycete, isolated from a soil sample collected in Ichikawa City, Chiba Prefecture, Japan, was found to produce potent antitumor antibiotics, saptomycins, closely related to the pluramycin-group. A further investigation resulted in an interesting observation that the producing strain, named *Streptomyces* sp. HP530, mutated frequently and the natural mutant produced several new compounds found in HPLC analysis. Saptomycins A, B, C_1, C_2, F and β-indomycinone were detected in and purified from the mycelial cake of the parent strain. On the other hand, saptomycins D, E[1], G and H were isolated from the mutant. In this paper, we describe the taxonomic studies of the producing organism, fermentation, HPLC analysis and biological activities. Isolation, physico-chemical properties and structure elucidation will be described in the following paper[2,3].

Materials and Methods

Taxonomic Studies

Morpholgical and physiological properties of the strain were examined according to Shirling and Gottlieb[4]. Cell wall analysis was performed by the methods of Becker *et al.*[5] and Yamaguchi[6]. The utilization of carbon sources was tested by growth on Pridham and Gottlieb's medium[7].

Fermentation

This strain mutated naturally and frequently. One of the natural mutants was used in this study.

Both the parent and the mutant strains were cultured under the same conditions in seed culture. The seed medium consisting of glucose 0.5%, oatmeal 3.0%, Pharma media 1.0%, $MgSO_4 \cdot 7H_2O$ 0.5%, $CoCl_2 \cdot 2H_2O$ 0.002% and $CaCO_3$ 0.3%, was adjusted to pH 7.0 and sterilized at 121°C for 15 minutes in a 500-ml Erlenmeyer flask containing 100 ml of medium. Freeze dried vegetative mycelium was inoculated into this flask, which was incubated at 30°C for 4 days as the primary seed culture. Twenty ml of the seed culture was inoculated into each of three 3 liter-Erlenmeyer flasks containing 500 ml of the above medium. After cultivating at 30°C for 4 days, 3% inoculum of the second seed was transferred to each

of three 30-liter jar fermenters containing 15 liters of the same medium. The fermentation was carried out with aeration and agitation for 72 hours (the mutant) or 96 hours (the parent) at 30°C, at an agitation rate of 250 rpm and an aeration rate of 1 v/v/m.

HPLC Analysis

Each 20 ml sample of the cultured broth, into which either the parent strain or the mutant was inoculated, was centrifuged at 3,000 rpm for 15 minutes. The mycelial cake, except for the supernatant liquid, was soaked in 20 ml of acetone for 30 minutes and then recentrifuged at 3,000 rpm for 15 minutes. The cultured supernatant was filtered and evaporated *in vacuo*. The concentrated acetone extract was dissolved in 20 ml of H_2O and then extracted with 40 ml of ethyl acetate. The ethyl acetate extract concentrated *in vacuo* was dissolved in 1.0 ml of methanol and used for HPLC analysis. HPLC analysis was performed using an analytical column Capcell pak C_{18} SG120 (4.6 × 250 mm, Shiseido Co. Ltd., Tokyo, Japan), elution with 0.15% KH_2PO_4 (pH 3.5) - methanol (6 : 4 to 2 : 8 linear gradient for 20 minutes and then isocratic elution with 2 : 8 for 40 minutes), at a flow rate of 1.0 ml/minute and detection at 395 nm.

Antimicrobial Activity

MIC values were determined by the agar dilution method. Heart infusion agar was used for bacteria and potato dextrose agar for yeasts.

Cytotoxic and Antitumor Activities

In vitro cytotoxicity tests using microculture tetrazolium assay (MTT assay)[8] were performed with 6 human and 2 murine tumor cell lines. Human MKN45 and MKN74 cells were gift from Dr. H. HOJO of Niigata University[9]. Human WiDr[10] and SW48[11] cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Human PC-3[12], A549[13] and murine P388[14] cells were obtained from JCRB. Meth A[15] cells were gift from Dr. H. SATO of Sasaki Institute. All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The IC_{50} value was calculated with PROBIT's method.

The antitumor activities of these compounds were evaluated in an experimental tumor system in mice bearing Meth A. Cells were implanted intraperitoneally into CDF_1 mice (female, 6 weeks old, purchased from Charles River Japan, Inc., Atugi, Japan) at an inoculum size of 1×10^6 cells per mouse. Drugs were

Table 1. Cultural characteristics of HP530 strain.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract - malt extract agar (ISP-2)	Good	Abundant, pale yellow (89)	Deep orange yellow (69)	None
Oatmeal agar (ISP-3)	Good	Abundant, yellowish white (92)	Deep grayish yellow (91)	None
Inorganic salt - starch agar (ISP-4)	Good	Abundant, yellowish white (92)	Strong yellow (84)	None
Glycerin - asparagin agar (ISP-5)	Moderate	Abundant, yellowish white (92)	Vivid deep reddish purple (243)	None
Peptone - yeast extract agar (ISP-6)	Good	Abundant, pale orange yellow (73)	None	Brownish orange (54)
Tyrosine agar (ISP-7)	Moderate	Abundant, yellowish white (92)	Vivide deep reddish purple (243)	None
Peptone - nitrate agar (ISP-8)	Moderate	Abundant, yellowish white (92)	Vivide deep purplish red (257)	None
Nutrient agar	Good	Abundant, yellowish white (92)	Deep brown (56)	None
CZAPEK's agar	Moderate	Moderate, pale greenish yellow (104)	Vidid deep purplish red (257)	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.

given ip daily from day 1 to day 4. Each group consisted of 6 mice. Negative and positive control mice received physiological saline and 5FU, respectively. Survival or death of the treated mice was recorded during the experimental period of 4 weeks. The mediam survival time of each test group was calculated.

Results and Discussion

Taxonomic Studies of the Producing Strain

The strain HP530 producing saptomycins was cultured in various ISP media and the characteristics are summarized in Table 1. Substrate mycelia were well developed in the media and tested without fragmentation. Aerial mycelia formed abundantly on various madia appeared yellow to white. A spore chain with 10 to 50 spores was observed to be straight to flexuous. Spores were cylindical in shape with a size of $0.6 \times 1.2\,\mu m$ and their surface was smooth. Sclerotia, sporangia and zoospores were not observed. The physiological characteristics and the utilization of carbohydrates observed after incubation at 28°C for 14 days are shown in Tables 2 and 3, respectively. The soluble pigment was brown to orange on peptone-yeast extract agar. Whole cell hydrolysates of the strain HP530 contained L,L-diaminopimelic acid. The strain was placed in the type I cell wall group. On the basis of the observations described above, the producing strain HP530 belonged to the genus *Streptomyces* and was closely similar to *S. fluorescens*[16] and *S. puniceus*[17]. However, the HP530 strain was different from *S. fluorescens* JCM4373 in the color of the reverse side and from

Table 2. Physiological properties of HP530 strain.

Property	Result
Growth temperature range (ISP-2 or yeast extract-malt extract agar, incubation for 14 days)	15~37°C
Growth pH range (ISP-2 or yeast extract-malt extract agar, incubation for 14 days)	pH 5.5~11.4
Gelatin liquefaction	Positive
Hydrolysis of starch	Positive
Coagulation of skim milk	Positive
Peptonization of skim milk	Positive
Reduction of nitrate	Positive
Decomposition of cellulose	Negative

Table 3. Utilization of carbon sources by HP530 strain.

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

Fig. 1. Time course of saptomycins production (parent strain).

■ pH, ○ C_1, △ C_2.

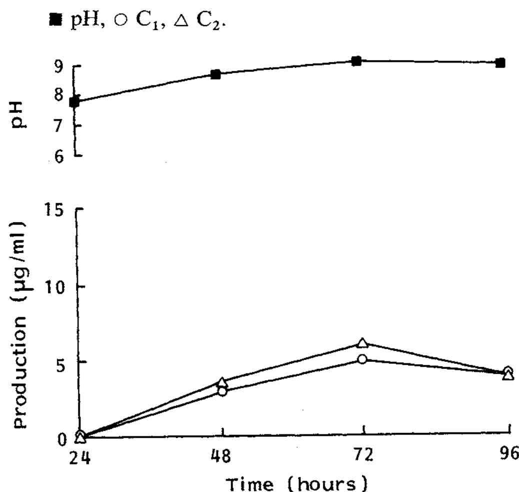
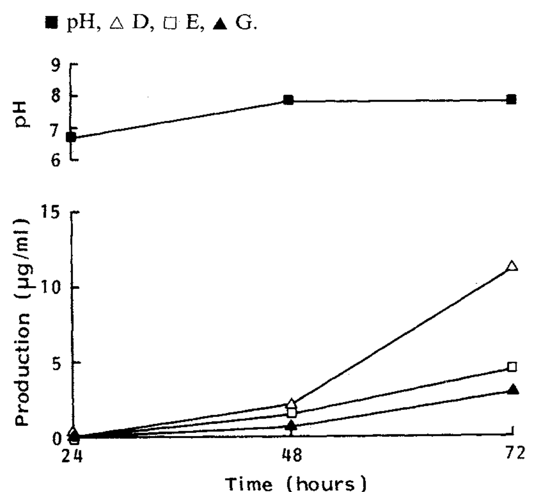


Fig. 2. Time course of saptomycins production (mutant strain).

■ pH, △ D, □ E, ▲ G.



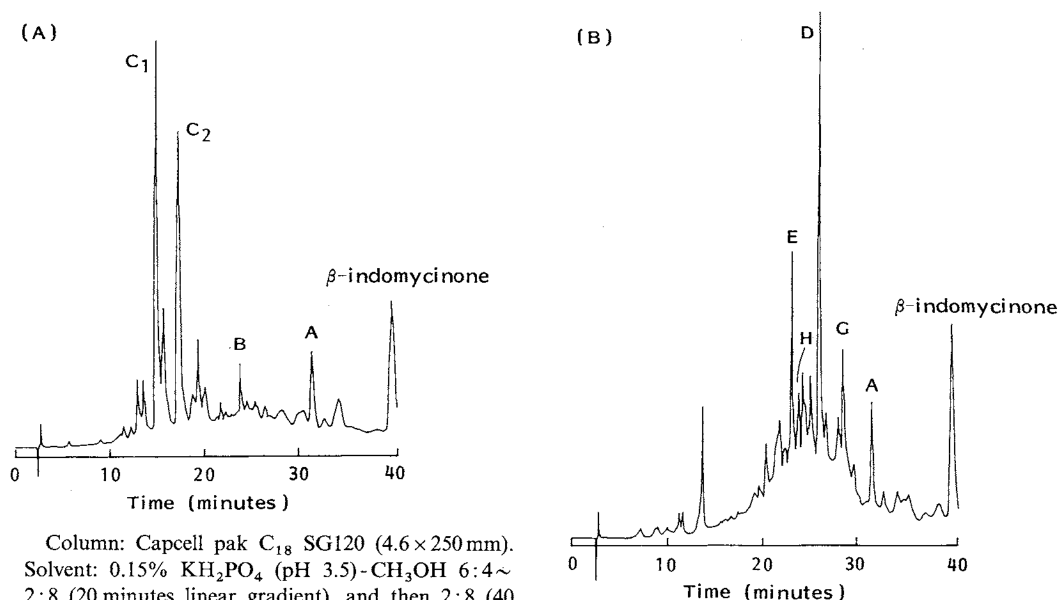
S. puniceus JCM4406 in the utilization of L-arabinose and the color of their soluble pigment. The strain, named *Streptomyces* sp. HP530, was deposited in the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of industrial Science and Technology, Japan, as No. FERM BP-2786.

Fermentation

The fermentations of saptomycins were carried out as described in Materials and Methods. A typical

Fig. 3. HPLC profile of products.

(A) Parent strain, (B) mutant strain.



Column: Capcell pak C_{18} SG120 (4.6×250 mm). Solvent: 0.15% KH_2PO_4 (pH 3.5)-CH_3OH 6:4～2:8 (20 minutes linear gradient), and then 2:8 (40 minutes). Flow rate: 1.0 ml/minute. Detection: UV 395 nm.

Saptomycin F was eluted at 50 minutes in both analyses, thus the peak of this compound did not appear on these charts.

Table 4. Antimicrobial activities of saptomycins.

Test organisms	MIC (μg/ml)							
	A	B	C_1	C_2	D	E	F	G
Bacillus subtilis M45 (Rec$^-$)[a]	3.13	12.5	3.13	6.4	0.4	0.2	1.6	3.2
B. subtilis H17 (Rec$^+$)	>100	50	25	12.5	3.2	12.5	>100	100
Staphylococcus aureus JCM 2151	>100	>100	>100	25	1.6	6.3	25	50
S. epidermidis JCM 2414	>100	50	100	25	3.2	>100	6.3	25
Micrococcus luteus JCM 1464	50	>100	>100	3.2	1.6	0.8	>100	12.5
Escherichia coli JCM 1649	>100	>100	>100	>100	>100	>100	>100	>100
Klebsiella pneumoniae JCM 1662	>100	>100	>100	>100	>100	>100	>100	>100
Proteus vulgaris JCM 1668	>100	>100	>100	>100	>100	>100	>100	>100
Xanthomonas maltophilia JCM 1975	50	50	25	12.5	3.2	>100	>100	>100
Salmonella typhimurium TA 1535	>100	>100	6.25	12.5	1.6	0.8	>100	100
Candida albicans JCM 1542	>100	>100	>100	>100	100	>100	>100	>100
Saccharomyces cerevisiae JCM 1499	>100	>100	>100	>100	6.3	>100	>100	>100

[a] Recombination deficient.

time course of the fermentation is shown in Fig. 1 and Fig. 2. The monitoring of saptomycin production was performed using HPLC analysis. The production of saptomycins reached a maximum at 72 hours after inoculation with the parent or the mutant strain.

HPLC Analysis

The HPLC profile of saptomycin production is shown in Fig. 3. The parent strain yielded saptomycins A, B, C_1, C_2, F and β-indomycinone, while the mutant gave saptomycins A, D, E, F, G, H and β-indomycinone as products. The resulting profile of the analysis indicated the different patterns of production between the parent and the mutant.

Table 5. *In vitro* cytocidal activities of saptomycins.

Cell lines	IC_{50} value (μg/ml)								
	A	B	C_1	C_2	D	E	F	G	H
P388 (Leukemia)	>100	3.4	0.30	0.32	0.035	0.042	1.7	0.11	0.025
Meth A (Fibrosarcoma)	NT	NT	NT	0.43	0.12	0.047	3.4	0.58	0.032
A549 (Lung carcinoma)	100	9.5	0.85	0.52	0.075	0.058	2.4	0.69	0.035
PC-3 (Lung carcinoma)	>100	13	>1.0	0.46	0.10	0.11	2.5	0.73	0.049
MKN45 (Gastric carcinoma)	100	5.3	0.48	0.33	0.047	0.042	1.9	0.14	0.020
MKN74 (Gastric carcinoma)	>100	6.0	0.85	0.54	0.064	0.079	2.5	0.20	0.036
SW48 (Colorectal carcinoma)	>100	6.0	0.57	0.91	0.069	0.052	1.9	0.21	0.024
WiDr (Colorectal carcinoma)	80	5.6	0.51	0.67	0.057	0.052	2.3	0.11	0.021

NT: not tested.

Table 6. Antitumor activities of saptomycins against mice bearing Meth A tumor.

Drug	Dose (mg/kg)	Survival		Survivor[b]	Drug	Dose (mg/kg)	Survival		Survivor[b]
		MST[a]	Mean±SD				MST[a]	Mean±SD	
Control		13	13.3±0.6		D	2	28	27.4±1.2	2/5
A	50	14	13.8±0.6			1	25	25.0±1.8	
B	10	14	14.0±0.9			0.5	16	15.6±1.5	
	5	13	13.4±1.0			0.25	14	14.2±1.0	
	2.5	13	13.4±0.5		E	20	25	21.0±7.8	2/6
	1.25	12	12.0±0.8			10	26	25.7±1.5	1/6
C_1	10	23	22.2±4.7	1/5		5	17	17.3±2.4	
	5	24	24.2±2.5			2.5	14	13.5±1.3	
	2.5	23	22.8±4.3		F	50	19	19.3±4.7	
	1.25	27	25.4±2.9	1/5	G	20	16	15.8±0.7	
C_2	5	28	27.2±1.1	3/6		10	15	15.3±0.5	
	2.5	27	27.2±0.9	3/6		5	16	17.0±3.3	
	1.25	27	26.5±1.6	3/6		2.5	15	14.3±0.7	
	0.63	26	26.0±3.2	1/6					

[a] MST: mean survival time (day).
[b] No. of survival mice at day 28.

Antimicrobial Activities

The MICs saptomycins A~G are shown in Table 4. The growth of *Bacillus subtilis* M45 a recombination deficient strain was inhibited by all saptomycins. Interestingly saptomycin D was a weak growth inhibitor of a yeast, *Saccharomyces cerevisiae* JCM1499.

Antitumor Activities

In vitro antitumor activities of saptomycins against various cell lines are shown in Table 5. All saptomycins except saptomycin A exhibited growth inhibitory activity in a dose dependent manner (data not shown). Saptomycins D, E and H showed especially strong activity. The effects of saptomycins on Meth A fibrosarcoma implanted in mice are shown in Table 6. Saptomycins C_1, C_2, D and E were remarkable for their clear increase in life span.

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