SAPURIMYCIN, NEW ANTITUMOR ANTIBIOTIC PRODUCED BY *STREPTOMYCES*

PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

MITSUNOBU HARA, TOSHIMITSU TAKIGUCHI,† TADASHI ASHIZAWA,†
KATSUSHIGE GOMI† and HIROFUMI NAKANO

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
3-6-6 Asahimachi, Machida-shi, Tokyo 194, Japan
†Pharmaceutical Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
Nagaizumi, Shizuoka 411, Japan

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In screening actinomycetes for antitumor compounds, *Streptomyces* sp. DO-116 was found to produce a new antitumor antibiotic sapurimycin. It is structurally related to, but distinct from, kapurimycins. The antibiotic was produced in a fermentation medium supplemented with high porus polymer resin which adsorbs antibiotic in the culture and results in an increase of titer. Active material was separated from the polymer resin by a solvent extraction procedure and isolated by repeated solvent extraction, adsorption chromatography and HPLC. Sapurimycin was active against bacteria, particularly Gram-positive organisms. It exhibited antitumor activity against leukemia P388 and sarcoma 180 in mice. Sapurimycin caused single strand breaks in supercoiled plasmid DNA *in vitro*. These results are discussed in comparison with data for kapurimycins.

Antitumor antibiotics of a wide range of structural types have been reported to produce strand breaks in DNA. Bleomycin, neocarzinostatin, esperamicin/calicheamicin, leinamycin *etc.* cause DNA cleavage *via* different mechanisms and they appear to mediate antitumor activity as a results of this.[1~6] We have previously reported on the isolation and characterization of a new family of antitumor antibiotics called the kapurimycins, produced by *Streptomyces* sp. DO-115.[7,8] They are new type of polycyclic microbial metabolite with a tetrahydroanthrapyrone skeleton. They also contain the β,γ-unsaturated-δ-keto carboxylic acid structure which correspond to the C_5 unit composed of atoms 4, 4a, 5, 13 and its neighboring carboxyl carbon atom in the molecule (see the following paper in this issue). The kapurimycins exhibit single strand scission of plasmid DNA and the β,γ-unsaturated-δ-keto carboxylic acid moiety and epoxide in the side chain on the pyrone ring are necessary for the DNA cleaving activity.[9] Examination of the interaction of kapurimycin with calf thymus DNA indicated that single strand scission by kapurimycin is due to alkylation of guanine by ring-opening of the epoxide in the side chain on the pyrone ring, depurination of guanine and presumably subsequent hydrolysis of the apurinic sites.[9]

In the course of our continued screening program for new antitumor antibiotics produced by microorganisms, a newly isolated culture designated DO-116 was found to produce a new antitumor compound, sapurimycin. As described in an accompanying paper,[10] sapurimycin is structurally related to but distinct from kapurimycins. It has the structural moieties which is essential for DNA cleaving activity of kapurimycin, *i.e.* the anthrapyrone skeleton with the epoxide group in the side chain on the pyrone ring and a β,γ-unsaturated-δ-keto carboxylic acid moiety.

We report here the taxonomy of the producing strain, fermentation, isolation, biological activities

and DNA cleaving activity of sapurimycin. Physico-chemical properties and structure determination of the compound are reported in the following paper.[10]

Materials and Methods

Microorganism

Strain DO-116 was isolated from a soil collected at Hashimoto-cho, Kanagawa Prefecture, Japan.

Taxonomic Studies

Growth characteristics and carbohydrate utilization were determined by the methods of the International Streptomyces Project (ISP).[11] Color codes were assigned to the substrate and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago). The spores and mycelia were observed with a scanning electron microscope (Model S-570, Hitachi Co., Ltd.).

Diaminopimelic acid of the cell wall was analyzed on the hydrolysate of cultures grown in a medium (glucose 10 g, starch 10 g, beef extract 3 g, yeast extract 5 g, $CaCO_3$ 2 g per liter of tap water, pH 7.0) for 48 hours at 28°C.

Fermentation

The media for seed and fermentation cultures are: SR III medium; glucose 10 g, soluble starch 10 g, Bacto-tryptone 5 g, yeast extract 5 g, beef extract 3 g, $CaCO_3$ 2 g per liter (pH 7.2 prior to sterilization), DF4D medium; glycerol 25 g, glucose 25 g, dry yeast 15 g, soybean meal 10 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 5 g and antifoam agents LG109 (Asahi Denka Kogyo) 0.3 ml and KM70 (Shinetsu Kagaku) 0.3 ml per liter (pH 7.0 prior to sterilization).

The 30-liter jar fermentation was performed as follows; A 300-ml Erlenmeyer flask containing 50 ml of SR III medium was inoculated with an loopful spores of strain grown on ISP4 agar slants. The flask was incubated at 28°C on a rotary shaker for 48 hours. Ten-ml of the seed culture was transferred to a 2-liter Erlenmyer flask containing 300 ml of the same medium. Following 24 hours of incubation at 28°C, the second stage seed culture (0.9 liter) was used as the inoculum to initiate the fermentation in 30-liter jar fermenter batched with 15 liters of a DF4D medium. The fermentation was carried out at 28°C with 1.8 liters of air per minute and agitation at 300 rpm. Diaion HP-20 resin (10 volume %) was added at 24 hours after inoculation.

Culture growth was evaluated by centrifuging untreated fermentation broth in 10 ml conical tubes at $1,200 \times g$ for 10 minutes. The packed cell volume was recorded as % of total broth volume.

Antibiotic production was monitored during the fermentation by the paper-disc method on nutrient agar using *Bacillus subtilis* as the test organism.

Antimicrobial and Antitumor Activity

The *in vitro* antimicrobial activity of sapurimycin was determined on nutrient agar by a 2-fold serial dilution method. The lowest concentration that inhibited growth of a bacterial strain after 18 hours incubation at 37°C was recorded as the MIC.

In vivo antitumor activity were measured and calculated as follows.[12] P388 cells (10^6) were transplanted ip into CDF_1 mice and ip administration of drug was started the day after tumor transplantation. Antitumor efficacy was expressed as a percentage of the mean survival time of the control group. Sarcoma 180 (5×10^6 cells/mouse) was inoculated sc at the axillary region in *ddY* mice. Drugs were administrated iv starting the day after tumor inoculation and the tumor volume was measured on day 10. The antitumor activity was evaluated by the T/C, where T and C represents the mean size of tumor of the treated animal and that of control animal, respectively.

DNA Nicking Activity *In Vitro*

The DNA cleavage was determined using purified pBR322 DNA and analyzed with agarose gel electrophoresis. Typical reaction mixtures include 20 μl of 20 mM Tris-HCl buffer pH 7.5, 0.3 μg of pBR322 DNA and sapurimycin. The reaction mixtures were incubated at 37°C for 60 minutes. After the addition

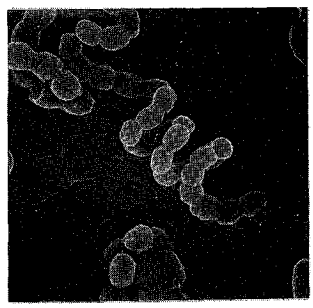
of 3.5 μl of 0.02% bromophenol blue and 50% sucrose, 20 μl of the mixture was placed in the well of agarose slab gel. Electrophoresis was carried out in 89 mM borate pH 8.3-2 mM EDTA buffer containing 0.01% SDS at 50 mV for 12 hours. Following electrophoresis, gels were stained in an aqueous solution of ethidium bromide (1 μg/ml). DNA bands were visualized by transillumination with UV light (300 nm) and photographed with Kodak Nos. 24A and 12 filters with Polaroid type 665 positive-negative film.

Results and Discussion

Taxonomy of the Producing Strain

The appearance of strain DO-116 on nine solid media is shown in Table 1. The vegetative mycelia grew on both synthetic and complex media. The aerial mycelium was gray, white or pink colored and well developed, branched but not fragmented. It bore chains of 10 to 30 or more spores, which were spiral, belonging to type *Spira*. Scanning electron micrographs indicated that the spore was oval in shape, 0.5×0.7 μm in size, and smooth-surfaced (Fig. 1). The substrate mycelium was branched but not fragmented. Analysis of the whole cell hydrolysate by TLC revealed the presence of the L,L-isomer of diaminopimelic acid, indicating that the cell wall belongs to type I.

Fig. 1. Scanning electron micrographs of strain DO-116.



In Table 2, the physiological properties of strain DO-116 are presented. DO-116 grew at temperature ranging from 16 to 37°C with optimum range from 28 to 32°C, peptonized milk and decomposed

Table 1. Cultural characteristics of strain DO-116.

Sucrose-nitrate agar	G: Good	Nutrient agar	G: Good
	AM: Fair, shell pink (5ba)		AM: Abundant, dark covert gray (2ih)
	SM: Shell pink (5ba)		SM: Old wine (8ng)
	P: None		P: None
Glucose-asparagine agar	G: Good	Yeast extract-malt extract agar	G: Good
	AM: Abundant, dark covert gray (2ih)		AM: Abundant, dark covert gray (2ih)
	SM: Covert gray (2fe)		SM: Black
	P: None		P: None
Glycerol-asparagine agar	G: Good	Oatmeal agar	G: Good
	AM: Abundant, ashes (5fe)		AM: Abundant, silver gray (3fe)
	SM: Taupe gray (7ih) to slate (10ih)		SM: Black
	P: None		P: Brown
Inorganic salts-starch agar	G: Good	Peptone-yeast extract-iron agar	G: Good
	AM: Abundant, silver gray (3fe)		AM: Abundant, white
	SM: Mauve taupe (8li) to rose taupe (6ig)		SM: Amber (3pe)
	P: None		P: None
Tyrosine agar	G: Good		
	AM: Abundant, silver gray (3fe)		
	SM: Old wine (8ng)		
	P: None		

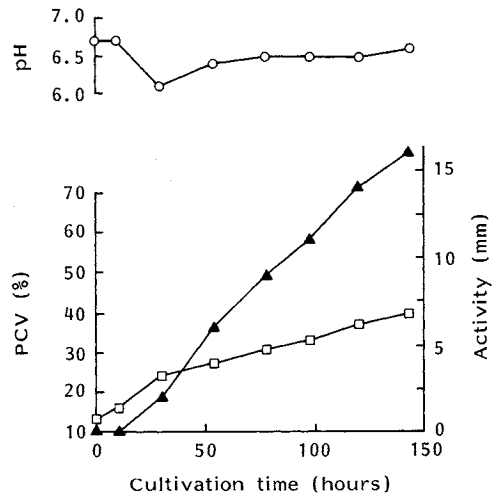
Abbreviations: G, degree of growth; AM, formation of aerial mycelium and its color; SM, color of substrate mycelium; P, formation of soluble pigment and its color.

Table 2. Physiological properties of strain DO-116.

Liquefaction of gelatine (28°C)	Negative
Coagulation of milk (28°C)	Negative
Peptonization of milk (28°C)	Positive
Decomposition of cellulose	Positive
Hydrolysis of starch	Positive
Formation of melanoid pigment	Positive
Optimum growth temperature	28~32°C
Optimum growth pH	6.8~7.5

Fig. 2. Time course of sapurimycin fermentation in a 30-liter jar fermenter.

pH (○), packed cell volume (PCV, □), and antibacterial activity (▲) were determined.



cellulose (Table 2). The strain grew well on various carbon sources tested, including L-arabinose, D-xylose, D-glucose, D-mannitol, sucrose, *m*-inositol, raffinose, L-rhamnose, lactose, and D-galactose.

From the morphological, cultural and physiological characteristics observed, strain DO-116 is considered to belong to the genus *Streptomyces* Waksman and Henrici 1943.[13~17] Compared with the published descriptions of *Streptomyces* species,[13~17] strain DO-116 resembled *Streptomyces nigra*. The strain was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the name of *Streptomyces* sp. DO-116 under the accession No. FERM BP-2559.

Fermentation

As was observed for utilization of many carbon sources for agar plate culture of strain DO-116, it also grew well in liquid media containing various carbon sources. However, the maximum titer of antibiotic depends upon the nature of the carbon and nitrogen sources. Glycerol and glucose were preferable carbon sources and dry yeast as well as soybean meal were good nitrogen sources for production of sapurimycin. From these results, DF4D medium was found to support the higher titers. Examination in shake flasks indicates that the addition of high porus polymer resin Diaion HP-20 (10 volume %) to the production medium adsorbs sapurimycin, which resulted in a significant increase in accumulation of sapurimycin. Therefore, Diaion HP-20 resin was added at 24 hours of incubation. Fig. 2 shows a typical time course for production of the antitumor antibiotic in a 30-liter jar fermentation under optimum culture conditions. Antibiotic production started at 25 hours concomitantly with gradual increases of mycelium volume and reached a maximum at 140 hours.

Isolation

Sapurimycin was isolated from 30 liters of culture broth by the following procedure. Sapurimycin accumulated in the filtered cake containing Diaion HP-20. The filtered cake was suspended in 50% aqueous MeOH and mycelium containing Diaion HP-20 was recovered by filtration and then resuspended in EtOAc to elute sapurimycin. The eluate containing sapurimycin was filtered and concentrated. The concentrate was diluted with an equal volume of water and extracted with EtOAc. The active EtOAc layer was concentrated and was applied to a column of Diaion HP-20. The column was washed with 10 mM ammonium acetate, pH 4.5-MeOH (1:1) and sapurimycin was eluted with 10 mM ammonium acetate, pH 4.5-MeOH (1:9). The fractions containing sapurimycin were concentrated, extracted with

EtOAc and concentrated to dryness. Sapurimycin was then dissolved with a small volume of MeOH and applied to a column of Sephadex LH-20. The column was developed with MeOH. The active fractions were collected and subjected to HPLC purification. HPLC was carried out on YMC SH 363-5 ODS, 30 (i.d.) × 250 mm and monitored at 254 nm. The mobile phase used was a mixture of MeOH and 10 mM ammonium acetate, pH 4.5 (8 : 2). The active fraction had a Rt of 19 minutes at a flow rate of 20 ml/minute. Fractions containing sapurimycin were collected and concentrated to a small volume. To desalt, the concentrated solution was adsorbed on Diaion HP-20 column and eluted with MeOH after washing 50% aqueous MeOH. The eluate was concentrated *in vacuo* to give sapurimycin (20 mg) as a red powder.

Biological Activities

Antimicrobial Activity

The antimicrobial activity of sapurimycin is shown in Table 3. It exhibited antimicrobial activity against Gram-positive bacteria but was inactive against Gram-negative bacteria and fungi.

Antitumor Activity

Antitumor activity of sapurimycin against murine experimental tumors are summarized in Tables 4 and 5. Data for kapurimycin A3, which is a structurally related antibiotic with an anthrapyrone moiety and a β,γ-unsaturated-δ-keto carboxylic acid,[7,8] are also included. The LD_{50} of sapurimycin was 6.9 mg/kg by single intraperitoneal administration. Sapurimycin prolonged the life span (ILS 32%) of mice bearing P388 leukemia at the dose of 1 mg/kg, which is a comparable effect to that obtained for kapurimycin A3. Although kapurimycin A3 was not effective against sarcoma 180, sapurimycin at a single

Table 3. Antimicrobial activity of sapurimycin.

Organisms	MIC (μg/ml)
Staphylococcus aureus ATCC 6538P	1.3
Enterococcus faecium ATCC 10541	0.7
Bacillus subtilis No. 10707	10
Klebsiella pneumoniae ATCC 10031	>100
Escherichia coli ATCC 26	>100
Pseudomonas aeruginosa Bin H No. 1	>100
Salmonella typhi ATCC 9992	>100
Proteus vulgaris ATCC 6897	>100
Shigella sonnei ATCC 9290	>100
Candida albicans ATCC 10231	>100

Table 4. Antitumor activity of sapurimycin against P388 leukemia.

Compounds	Dosage (mg/kg)	MST[a]	ILS (%)
Sapurimycin	8	11.6±5.7	16
	4	11.8±2.0	18
	2	12.8±1.3	28
	1	13.2±0.8	32
	0.5	12.2±1.3	22
	0.25	12.2±0.4	22
	0.13	11.0±0.7	10
Kapurimycin A3	1	13.2±1.6	32
	0.5	13.0±1.2	30
Mitomycin C	4	18.0±3.0	80
	2	16.4±1.8	64
Control		10.0±0.9	0

[a] MST of deceased mice. Mean±SD.

Table 5. Antitumor activity of sapurimycin on murine sarcoma 180.

Compounds	Dosage (mg/kg)	T/C	WBC[a]	BW (g)
Sapurimycin	80	Toxic		
	40	0.27	20.6	+3.5
	20	0.54	43.6	+6.6
	10	0.76	46.8	+5.4
	5	0.83	49.8	+8.1
Kapurimycin	10	Toxic		
A3	5	0.28 (1/5)[b]	27.3	−1.5
	2.5	0.61	40.0	+3.7
Mitomycin C	6	0.28	17.0	+0.5
	4	0.44	19.4	+3.5
Control[c]		1.00	83.8	+7.2

Sarcoma 180 cells (5×10^6/mouse) were inoculated sc on day 0. Drugs were administrated iv on day 1.

[a] Number of white blood cells (WBC) in peripheral blood ($\times 10^2/mm^3$).

[b] Number of toxic death mouse.

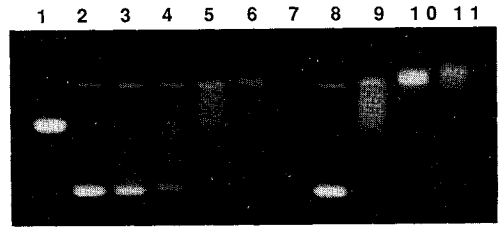
[c] Mean±SD of tumor volume was 1,787.9±137.9 mm^3.

dose of 40 mg/kg induced the regression of sarcoma 180 (T/C 0.27). Thus, sapurimycin possesses broader spectrum of antitumor activity than kapurimycin A3. Further studies on the antitumor activity of sapurimycin are in progress.

Effect of Sapurimycin on Plasmid DNA

Since sapurimycin contains the same structural elements as that essential for DNA cleavage by kapurimycins, we examined the effect of sapurimycin on plasmid DNA. Fig. 3 shows that conversion of form I supercoiled DNA to form II (nicked circular) DNA is apparent with increasing concentrations of sapurimycin. Form III (linear duplex) DNA was not observed, indicating that sapurimycin causes single strand breaks but not double strand breaks in supercoiled circular DNA. It is to be noted that DNA cleaving activity of sapurimycin is greater than kapurimycin A3. Neither metal cations nor reducing agents were necessary for DNA strand scission by sapurimycin. Oxygen radical scavengers did not suppress the DNA cleaving activity of sapurimycin (data not shown). These DNA cleaving profiles as well as structural similarities between the kapurimycins and sapurimycin suggest that sapurimycin cleaves DNA by the same mechanism as that of the kapurimycins.[9]

Fig. 3. Agarose electrophoresis of pBR322 DNA treated with sapurimycin.



Lane 1: Form III (linear) pBR322 DNA, lanes 2 and 8: pBR322 DNA alone, lanes 3~7: pBR322 DNA plus sapurmycin at 0.19, 0.78, 3.13, 12.5 and 50 μM, lanes 9~11: pBR322 DNA plus kapurimycin A3 at 6.3, 25 and 100 μM.

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