STUDIES ON A NOVEL ANTITUMOR ANTIBIOTIC, PHENAZINOMYCIN: TAXONOMY, FERMENTATION, ISOLATION, AND PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS

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A new antibiotic, phenazinomycin ($C_{27}H_{32}N_2O$, MW 400), was isolated from the cultural mycelium of *Streptomyces* sp. WK-2057. This antibiotic possesses antibacterial activities against Gram-positive bacteria *in vitro*, direct cytotoxic activities against HeLa S_3, P388 and P388 doxorubicin-resistant cells *in vitro* and antitumor activities against experimental murine tumors *in vivo*.

In the course of a screening program for new types of antibiotics showing cytocidal activity obtained from microorganisms, phenazinomycin was discovered from the fermentation broth of *Streptomyces* sp. WK-2057, which was isolated from a soil sample. The present paper deals with taxonomic studies of the producing strain, and the production, isolation and physico-chemical properties of the new antibiotic. The biological activity of phenazinomycin against tumor cells and microorganisms is also presented.

Materials and Methods

Taxonomic Studies

For taxonomic studies, most cultures were grown in accordance with methods adopted by the International Streptomyces Project (ISP)[1]. For experiments on cultural properties, all cultures were incubated at 27°C and were observed for 15~20 days. The color recorded for mature cultures was described according to the "Color Harmony Manual"[2]. Physiological properties including utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB[3]. The type of diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[4].

Fermentation

The stock culture of the producing organism was inoculated into three 500-ml Sakaguchi flasks, each containing 100 ml of the seed medium consisting of glycerol 2.0%, soybean meal powder 2.0% and NaCl 0.5% (adjusted to pH 7.0 before sterilization). The flasks were incubated at 27°C for 72 hours on a reciprocal shaker. The resulting culture (3×100 ml) was transferred to three 30-liter jar fermenters, each of which containing 20 liters of the producing medium consisting of glucose 2.0%, peptone 0.5%, meat extract 0.5%, dry yeast 0.3%, NaCl 0.5% and $CaCO_3$ 0.3% (adjusted to pH 7.0 before sterilization), and the fermentation was carried out at 27°C for 96 hours with an agitation rate of 160 rpm and an aeration rate of 20 liters/minute. Detection of the antibiotic in the fermentation broth was followed by a cytocidal assay using P388 doxorubicin-resistant (P388/ADM) cells described below or by HPLC analysis (column: Merck pre-packed column SI 60 (4×250 mm), Merck,

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solvent: CH_3Cl - MeOH (20 : 1), detection: UV 260 nm).

After 96 hours of fermentation, the amount of phenazinomycin in the mycelium reached a maximum (20 μg/ml).

Antimicrobial Activity

The antimicrobial activity of phenazinomycin was determined by the agar dilution method using Mueller-Hinton agar medium (Difco) for bacteria and potato agar medium for fungi and yeasts.

Antimicrobial activity was observed after 24 hours of incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Effect of Phenazinomycin on HeLa S_3, P388 and P388/ADM Cells

HeLa S_3 cells were maintained in monolayers in EAGLE's minimum essential medium supplemented with 10% bovine serum and antibiotics (100 U/ml of benzylpenicillin and 100 μg/ml of streptomycin) at 37°C. P388 and P388/ADM cells were kindly provided by Dr. M. INABA of the Japanese Foundation of Cancer Research and were maintained in RPMI medium.

To determine the cytotoxicity of phenazinomycin on tumor cells, HeLa S_3 (4×10^4), P388 (5×10^4) or P388/ADM (5×10^4) cells in 2 ml of the medium were placed in 2 cm^2-culture plate (Falcon 3047) and incubated for 48 hours at 37°C in a 5% CO_2 - 95% air atmosphere. Each culture dish was filled with fresh medium containing a different concentration of phenazinomycin and incubated for 72 hours. At the end of the incubation period, HeLa S_3 cells were trypsinized to form a single cell suspension, and HeLa S_3, P388 and P388/ADM cells were counted using a hemocytometer.

Antitumor Activity

For determination of antitumor activity of phenazinomycin, male ICR mice weighing 20~24 g were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). Sarcoma 180 ascites tumor cells were maintained by weekly ip passage in ICR mice. Seven to eight animals per group were used in this experiment.

Antitumor activity was evaluated by the increase in life span (ILS): $(T/C-1)\times100\%$, where "T" was the mean survival days (MSD) of the treated group and "C" the MSD of the control group.

Results and Discussion

Taxonomic Studies

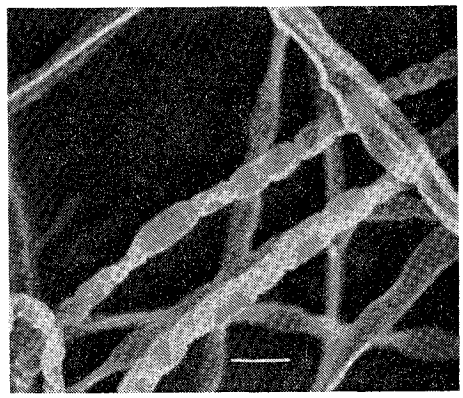
An electron micrograph of strain WK-2057 is shown in Fig. 1. The aerial mycelia were well developed on yeast extract - malt extract agar and were long, with no vertical formation.

Culture characteristics, physiological properties and utilization of carbon sources of strain WK-2057 are shown in Tables 1~3, respectively. Cell wall analysis showed the presence of LL-diaminopimelic acid.

Microscopic studies and the cell wall type indicated that strain WK-2057 belongs to the genus *Streptomyces*.

Fig. 1. Scanning electron micrograph of aerial mycelia of *Streptomyces* sp. WK-2057 on inorganic salts - starch agar.

Bar represents 1 μm.



Isolation and Purification

The harvested broth (3×20 liters) of *Streptomyces* sp. WK-2057 was centrifuged, and the resulting mycelium was extracted with acetone (20 liters). The acetone extract was concentrated to *ca.* 2 liters *in vacuo* and then extracted

Table 1. Cultural properties of strain WK-2057.

Medium	G	AM	R	SP
Glucose - asparagine agar[a]	Good, biscuit (2ec)	Moderate, white (a)	Light wheat (2ec) to mustard (2le)	None
Glycerol - asparagine agar[a]	Good, bamboo (2fb) to light mustard tan (2ie)	Moderate, white (a)	Bamboo (2fb) to light mustard tan (2ie)	None
Inorganic salts - starch agar[a]	Good, light wheat (2ea)	Good, alabaster tint (13ba)	Covert tan (2ge) to golden olive (1 1/2lg)	None
Tyrosine agar[a]	Moderate, light ivory (2ca) to mustard tan (2lg)	None	Bamboo (2fb) to camel (3ie)	None
Oatmeal agar[a]	Good, mustard (2le)	Moderate, alabaster, tint (13ba)	Light ivory (2ca)	None
Yeast extract - malt extract agar[a]	Good, dull gold (2ng)	None	Mustard gold (2pg)	None
Nutrient agar[a]	Poor, putty (1 1/2ec)	None	Dusty yellow (1 1/2gc)	None
Peptone - yeast extract - iron agar[a]	Moderate, bamboo (2fb)	None	Light ivory (2ca) to bamboo (2fb)	None
Glucose - nitrate agar[b]	Poor, light ivory (2ca)	None	Bamboo (2gc)	None
Sucrose - nitrate agar[b]	Moderate, cream (1 1/2ca) to mistletoe green (24 1/2ig)	None	Cream (1 1/2ca) to sage green (24ig)	None
Glycerol - calcium malate agar[b]	Good, pearl pink (3ca) to topaz	Very poor, pearl (2ba)	Bamboo (2gc) to light mustard tan (2ie)	None
Glucose - peptone agar[b]	Good, bamboo (2gc)	None	Mustard (2le)	None

G: Growth of vegetative mycelium, AM: aerial mycelium, R: reverse, SP: soluble pigment.
[a] Medium recommended by ISP.
[b] Medium recommended by S. A. WAKSMAN.

with EtOAc (2 liters). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a greenish oil (*ca.* 12 g).

The oily residue was chromatographed on a silica gel column (Kieselgel 60, Merck, 500 ml) using a $CHCl_3$ - MeOH mixture as the developing solvent. Fractions exhibiting cytocidal activity on P388/ADM cells were collected and rechromatographed on a silica gel column using $CHCl_3$ - MeOH to give crude phenazinomycin (*ca.* 400 mg).

The antibiotic was further purified by preparative HPLC (column: Asahipack GS-310H, Asahi Chemical Co., Japan, solvent: $CHCl_3$ - MeOH (20 : 1), detection: UV 260 nm) to give phenazinomycin as dark blue needles.

Table 2. Physiological characteristics of strain WK-2057[a].

Nitrate reduction	Negative
Liquefaction of gelatin (21~22°C)	Negative
Starch hydrolysis	Positive
Coagulation of milk (37°C)	Negative
Peptonization of milk (37°C)	Positive
Melanin formation	Negative
Tyrosinase reaction	Negative
Production of H_2S	Negative
Cellulolytic activity	Negative

[a] Temperature range for growth 15~36°C.

Table 3. Utilization of carbon sources by strain WK-2057.

Responses	Carbon sources
Positive	D-Glucose, L-arabinose, D-xylose, raffinose, melibiose, D-mannitol, D-fructose, L-rhamnose, *i*-inositol, sucrose

Table 4. Antimicrobial spectrum of phenazinomycin.

Organism	MIC (μg/ml)
Staphylococcus aureus KB 210	12.5
S. aureus FDA 209P	25.0
Bacillus subtilis KB 211	6.3
B. cereus KB 143	1.6
Micrococcus luteus KB 212	3.1
Mycobacterium smegmatis KB 42	>100
Escherichia coli NIHJ	>100
E. coli NIHJ JC-2	>100
Klebsiella pneumoniae KB 212	>100
Salmonella typhimurium KB 20	>100
Proteus vulgaris KB 127	>100
Pseudomonas aeruginosa KB 115	>100
Candida albicans KF 1	>100
Saccharomyces cerevisiae KF 237	>100
Cryptococcus neoformans 802 KF 33	100
Microsporum gypseum KF 64	50.0
Trichophyton mentagrophytes T-5 KF 213	100
Penicillium herquei KF 227	>100
Botrytis cinerea KF 241	100
Sclerotinia cinerea KF 181	25.0
Piricularia oryzae KF 180	12.5
Mucor racemosus KF 223	>100

Table 5. Antitumor activity of phenazinomycin on Sarcoma 180 tumor.

Dose (mg/kg/day)	MSD (range)	ILS (%)	Mean body weight (g) (day-9)
Saline	10.1 (9~12)	0	35.2±2.4
22.2	24.2(21~33)	140	26.1±0.9
11.1	20.3(13~25)	101	27.9±2.3
5.6	13.5 (9~20)	34	31.1±4.3
2.8	14.4 (9~19)	43	32.5±3.0

Sarcoma 180 cells (1×10^6) were inoculated into ICR mouse.
Mice were administered with phenazinomycin ip on days 1~9.

Biological Properties of Phenazinomycin

The antimicrobial activity of phenazinomycin was determined by the agar dilution method. The antibiotic was active against Gram-positive bacteria but inactive or only weakly active against Gram-negative bacteria, yeast and fungi. MICs of phenazinomycin are listed in Table 4.

The cytocidal activity of phenazinomycin was investigated using a strain of human tumor cells and two strains of murine tumor cells *in vitro*. When the cells were exposed to the antibiotic for 3 days, the growths of HeLa S_3, P388 and P388/ADM cells were inhibited at concentrations of 0.78, 25 and 3.1 μg/ml, respectively.

Table 6. Physico-chemical properties of phenazinomycin.

Appearance	Dark blue needle
Molecular formula	$C_{27}H_{32}N_2O$
EI-MS *m/z* (M^+)	400
MP	113~118°C
$[\alpha]_D^{25}$ (*c* 0.45, MeOH)	−49°
UV λ_{max}^{MeOH} nm (ε)	240 (24,500), 321 (27,200), 745 (6,100)
IR ν_{max} cm^{-1}	1633, 1501, 1467, 1446
TLC (SiO_2) Rf:	
$CHCl_3$ - MeOH (40 : 1)	0.33
EtOAc - MeOH (10 : 1)	0.28

Fig. 2. IR spectrum of phenazinomycin (KBr).

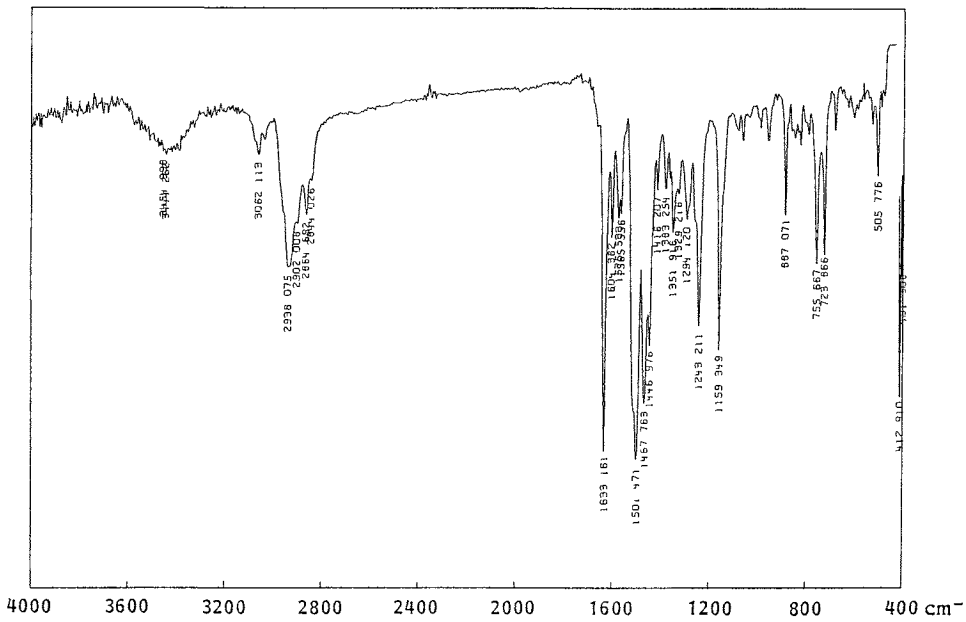
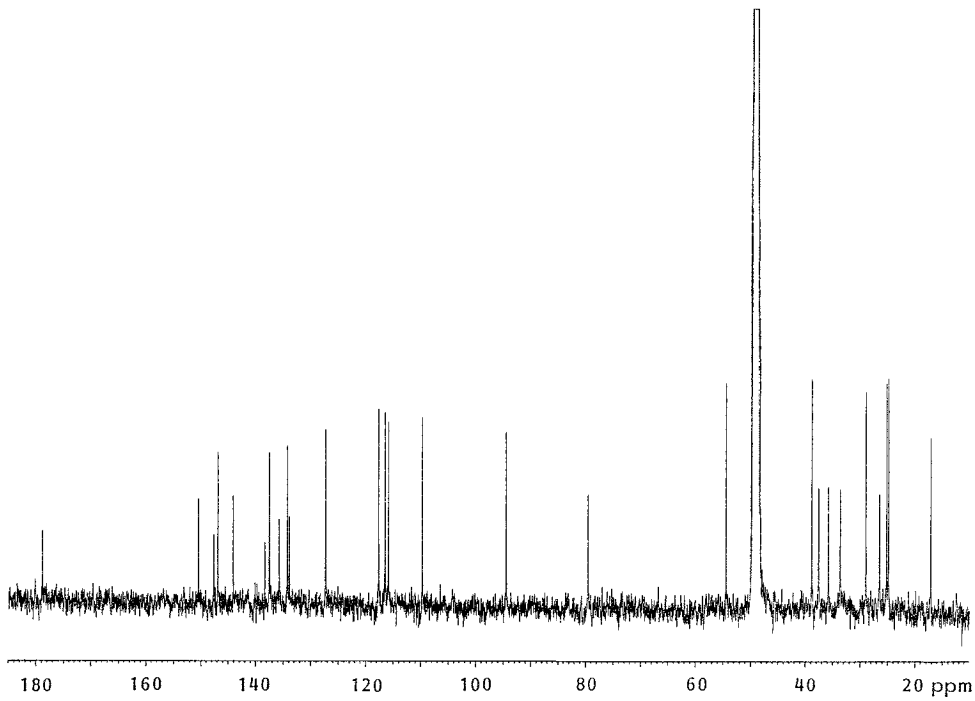


Fig. 3. ^{13}C NMR spectrum of phenazinomycin (125 MHz, CD_3OD).

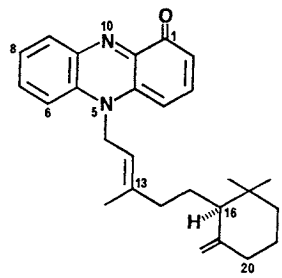


The antitumor activity of phenazinomycin on Sarcoma 180 is shown in Table 5. The antibiotic caused a prolongation of survival time for tumor-bearing animals. The antitumor spectrum on a variety experimental tumors of phenazinomycin will be reported elsewhere.

Physico-chemical Properties of Phenazinomycin

Physico-chemical properties of phenazinomycin are summarized in Table 6. This antibiotic is soluble in MeOH, EtOH, CH_2Cl_2, $CHCl_3$, EtOAc, BuOAc and acetone, but practically insoluble in H_2O and *n*-hexane. The IR and ^{13}C NMR spectra of phenazinomycin are shown in Figs. 2 and 3, respectively. In the electron impact mass spectrum (EI-MS) of this compound, a molecular ion peak was observed at *m/z* 400. The molecular formula of phenazinomycin was elucidated by high resolution mass spectrometry; observed: *m/z* 400.2510; calcd for $C_{27}H_{32}N_2O$: 400.2516. The structure of phenazinomycin (Fig. 4) was established on the basis of spectral analyses and chemical degradation studies and details of the structure elucidation of phenazinomycin will be reported in a separate paper[5].

Fig. 4. Structure of phenazinomycin.



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