AZINOMYCINS A AND B, NEW ANTITUMOR ANTIBIOTICS

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION, AND CHARACTERIZATION

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A strain of *Streptomyces griseofuscus* S42227 (FERM P-8443) was found to produce new antitumor antibiotics, called azinomycins A and B. The molecular formulas of azinomycins A and B were determined as $C_{30}H_{33}N_3O_{10}$ and $C_{31}H_{33}N_3O_{11}$, respectively.

They were active against Gram-positive bacteria, Gram-negative bacteria and L5178Y cells in tissue culture.

In the course of screening for new antitumor substances, azinomycins A and B (Fig. 1) were discovered in the culture broth of strain S42227. This paper reports the taxonomy of the producing organism, fermentation, isolation, physico-chemical properties, antimicrobial activity and *in vitro* cytotoxicity of azinomycins. The structures[1] and antitumor activity[2] of azinomycins will be published elsewhere.

Taxonomy

Strain S42227 was isolated from a soil sample obtained from Itakura, Oora, Gunma Prefecture, Japan.

The methods described by SHIRLING and GOTTLIEB[3] were principally employed for the taxonomic studies. Morphological observations were made with light and electron microscopes on cultures grown at 28°C for 14 days on yeast extract - malt extract agar or inorganic salts - starch agar. The mature spores occurred in chains of more than 10 spores forming spirals. The spores were oval or cylindrical and $0.7 \sim 0.9 \times 1.0 \sim 1.3$ μm in size. Spore surfaces were smooth (Fig. 2).

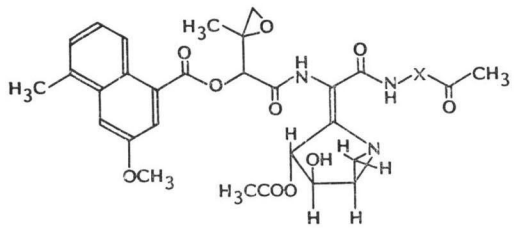
Cultural characteristics were observed on ten kinds of media described by SHIRLING and GOTTLIEB[3]. Results are shown in Table 1. Incubations were made at 28°C for 14 days. The color names used in this study were based on the Color Standard (Nihon Shikisai Co., Ltd.). Aerial mass color was in the Red or Gray-color series on various agar media. Faint brown soluble pigment was produced in some agar media.

Analysis of whole cell hydrolysates[4] of strain S42227 showed that it contained LL-diaminopimelic acid.

Physiological properties of strain S42227 are shown in Table 2. Utilization of carbon sources by strain S42227 was examined according to the methods of PRIDHAM and GOTTLIEB[5]. Results are summarized in Table 3.

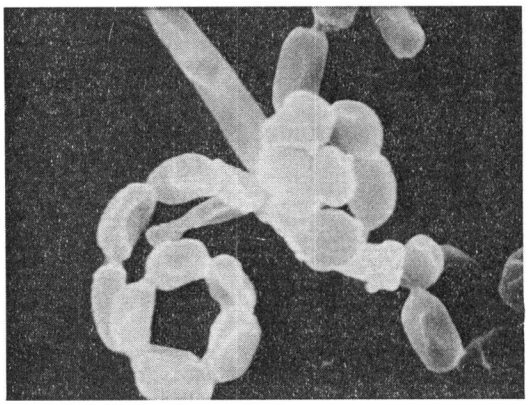
Microscopic studies and whole cell analysis of strain S42227 indicated that strain S42227 was classified in the genus *Streptomyces*. Accordingly a comparison of strain S42227 was made with the published descriptions[6~10] of various *Streptomyces* species. Strain S42227 was considered to

Fig. 1. Structures of azinomycins A and B.



Azinomycin A X = CH_2
Azinomycin B X = C=CHOH

Fig. 2. Electron micrograph of the spores of strain S42227 on oatmeal agar, 14 days culture at 28°C.



resemble *Streptomyces griseofuscus* SAKAMOTO *et al.*[11]. Good agreement was obtained by comparing strain S42227 with *S. griseofuscus* (ISP 5191), therefore, strain S42227 was identified as *S. griseofuscus* S42227.

Fermentation

Seed culture was prepared by inoculating a loopful of mature spores of *S. griseofuscus* S42227 into each of 500-ml Sakaguchi flask, each containing 100 ml of following sterile medium; soluble starch 0.5%, Pharmamedia (cotton seed meal) 0.5%, (pH 7.0). The seed culture was incubated at 28°C for 48 hours. This culture (160 ml) was transferred into a 30-liter jar fermentor containing 16 liters of the same seed medium. The fermentation was carried out at 28°C for 3 days with an agitation rate of 450 rpm and an air flow of 16 liters/minute. The progress of the fermentation was monitored by

Table 1. Cultural characteristics of strain S42227.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Sucrose - nitrate agar	Moderate	Lt. gy. br.	Pale yellow	None
Glucose - asparagine agar	Moderate	White-lt. gy. br.	Pale yellow	None
Glycerol - asparagine agar (ISP medium 5)	Moderate	White-lt. gy. br.	Pale yellow-yellowish br.	Faint brownish
Inorganic salts - starch agar (ISP medium 4)	Moderate	White-lt. gy. br.	Pale yellow-yellowish br.	Faint yellowish
Tyrosine agar (ISP medium 7)	Moderate	White-lt. gy. br.	Yellowish br.	Faint brownish
Nutrient agar	Moderate	None	Lt. yellowish br.	None
Yeast extract - malt extract agar (ISP medium 2)	Abundant	White-gy. br.	Yellowish br.	None
Oatmeal agar (ISP medium 3)	Abundant	Lt. gy. br.	Pale yellow	Faint yellowish
Glycerol - nitrate agar	Moderate	White	Pale yellow	Faint brownish
Calcium - malate agar	Poor	White-lt. brownish gray	Colorless	None

Symbols: Lt.; light, gy.; grayish, br.; brown.

Table 2. Physiological properties of strain S42227.

Temperature range for growth	11~41°C
Optimum temperature	24~29°C
Nitrate reduction	+
Starch hydrolysis	+
Milk coagulation	−
Milk peptonization	+
Melanin production	−
Gelatin liquefaction	+

Symbols: +; Positive, −; negative.

Table 3. Carbon sources utilization by strain S42227.

No-carbon	−
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	−
Inositol	−
L-Rhamnose	−
Raffinose	−
D-Mannitol	+
Sorbitol	−
D-Galactose	+
D-Mannose	+
Inulin	−
Salicin	±

Symbols: +; Utilization, ±; doubtful utilization, −; no utilization.

the antibacterial activity using *Bacillus subtilis* PCI 219.

Isolation

After the fermentation was carried out, the culture broth (16 liters) was centrifuged (10,000 rpm) to separate the mycelium from the broth. The supernatant was extracted one time with an equal volume of chloroform. The chloroform extract was concentrated to 30 ml, and diluted with *n*-hexane (300 ml). The resultant precipitate was collected by centrifugation (3,000 rpm), and extracted with diethyl ether (50 ml) to afford diethyl ether soluble fraction and insoluble fraction. The diethyl ether soluble fraction was concentrated and chromatographed on silica gel (Kiesel Gel 60, 230~400 mesh; Merck) using chloroform - methanol (50: 1). The major antibacterial fraction was concentrated to dryness and azinomycin A (20 mg) was obtained from ethyl acetate as colorless plates. The diethyl ether insoluble fraction was extracted with chloroform (50 ml), and *n*-hexane was added to this solution gradually. The precipitate initially obtained was discarded and that obtained secondly was collected by centrifugation to give azinomycin B (130 mg) as colorless amorphous solid.

Physico-chemical Properties

The physico-chemical properties of azinomycins A and B were summarized in Table 4. Both azinomycins A and B gave positive reaction to Dragendorff reagent and were acid-labile. Azinomycin A was soluble in diethyl ether, chloroform, ethyl acetate, acetone and methanol, but was insoluble in *n*-hexane and water. Azinomycin B was soluble in chloroform, ethyl acetate, acetone and methanol, but was insoluble in diethyl ether, *n*-hexane and water. The molecular formulas of azinomycins A and B were determined as $C_{30}H_{33}N_3O_{10}$ and $C_{31}H_{33}N_3O_{11}$, respectively, by mass spectra and elemental analysis. The IR spectra of azinomycins A and B are shown in Figs. 3 and 4.

The spectral data of azinomycins A and B were similar to those of carzinophilin[12], but the molecular formulas of azinomycins A and B were different with that of carzinophilin[13], which showed that azinomycins were new antibiotics[1].

Antimicrobial Activity

The antimicrobial spectra of azinomycins A and B determined by the agar dilution method are shown in Table 5.

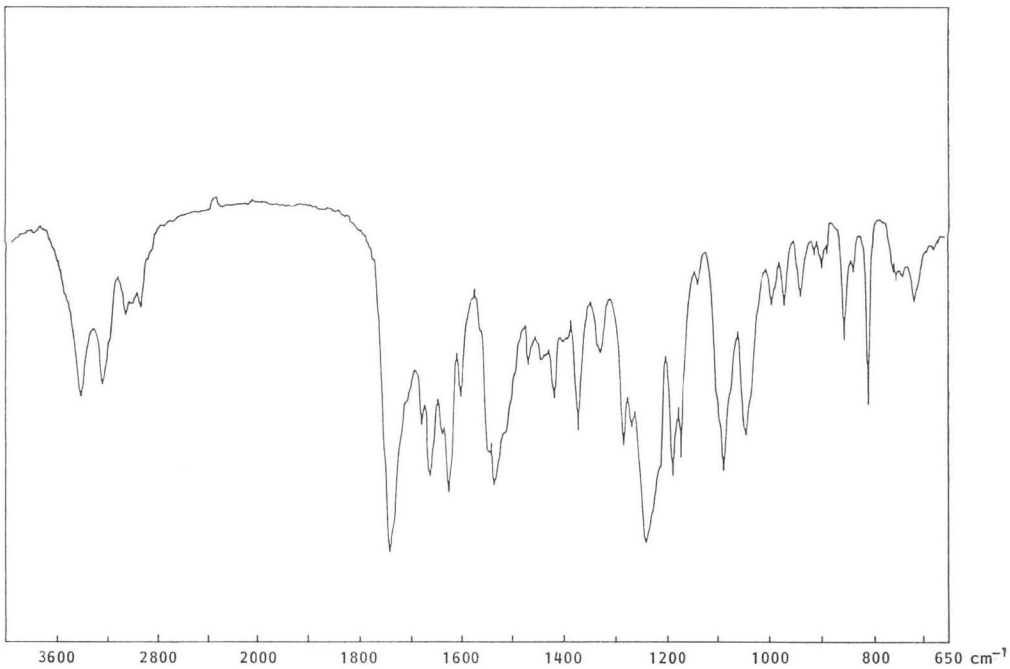
Azinomycins A and B were active against Gram-positive and Gram-negative bacteria but inactive against yeast and fungi.

Table 4. Physico-chemical properties of azinomycins A and B.

	Azinomycin A	Azinomycin B
Appearance	Colorless plates	Colorless amorphous solid
MP (°C)	140 (dec)	190 (dec)
Molecular formula	$C_{30}H_{33}N_3O_{10}$	$C_{31}H_{33}N_3O_{11}$
FAB-MS (positive) M+H, *m/z*	596	624
FAB-MS (negative) M−H, *m/z*	594	622
Analysis		
Calcd for	$C_{30}H_{33}N_3O_{10}\cdot CH_3COOC_2H_5$	$C_{31}H_{33}N_3O_{11}$
	C 59.73, H 5.78, N 6.15	C 59.76, H 5.33, N 6.74
Found	C 59.77, H 5.78, N 6.22	C 59.23, H 5.56, N 6.72
UV λ_{max}^{MeOH} nm (ε)	217 (52,000), 248 (sh), 344 (5,000)	217 (65,500), 250 (30,400), 290 (sh), 340 (8,500)
$[\alpha]_D^{20}$	—	+48° (*c* 0.48, $CHCl_3$)
Rf value*	0.34	0.21

* Silica gel TLC (Merck 5715), solvent; $CHCl_3$ - Me_2CO (2: 1).

Fig. 3. IR spectrum of azinomycin A (KBr).



In Vitro Cytotoxicity

The *in vitro* cytotoxicities of azinomycins A and B against L5178Y cells were determined.

L5178Y cells (4×10^4) were incubated with azinomycin A or B in 200 μl of RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 - 95% air and the cell number was counted by a Coulter counter after 48 hours incubation.

The concentrations of 50% inhibition of cell growth (IC_{50}) for azinomycins A and B were 0.07

Fig. 4. IR spectrum of azinomycin B (KBr).

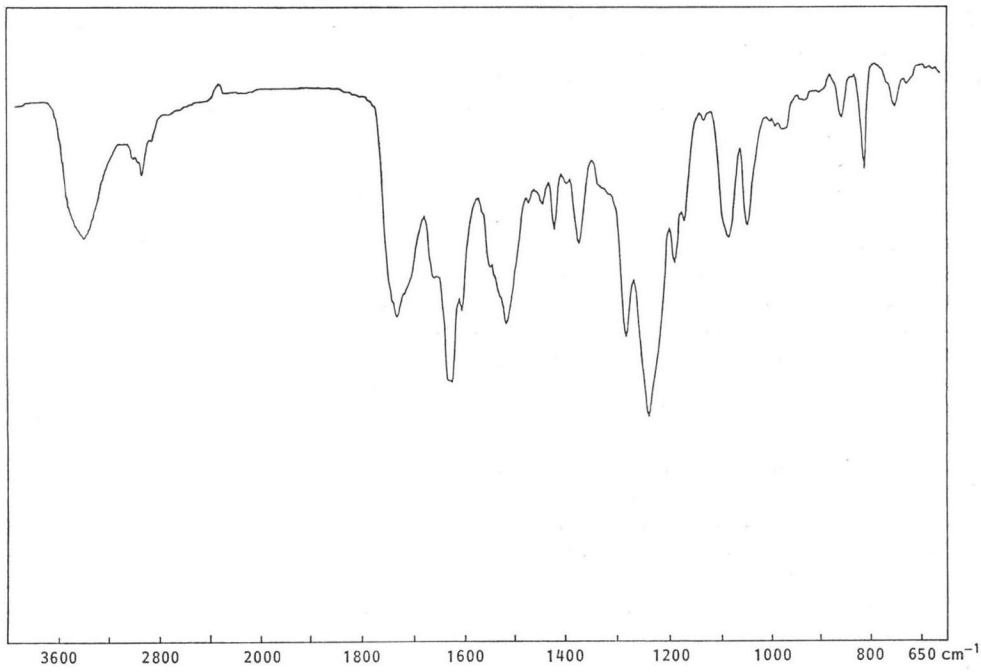


Table 5. Antimicrobial spectra of azinomycins A and B.

Organism	MIC (μg/ml)	
	Azinomycin A	Azinomycin B
Bacillus subtilis ATCC 6633	0.78	50
Staphylococcus aureus FDA 209P	6.25	50
S. aureus Terajima	6.25	50
S. aureus Smith	1.56	1.56
S. epidermidis ATCC 12228	6.25	100
Micrococcus luteus ATCC 9341	<0.20	<0.20
Enterococcus faecalis IFO 12964	0.78	0.78
Micrococcus lysodeikticus IFO 3333	<0.20	<0.20
Escherichia coli O-1	>100	>100
Shigella flexneri 2b	<0.20	0.78
Pseudomonas aeruginosa IFO 13736	>100	>100
Klebsiella pneumoniae ATCC 10031	3.13	50
Providencia rettgeri	100	>100
Serratia marcescens NHL	100	>100
Candida albicans NHL 4019	>100	>100
Aspergillus niger ATCC 9642	>100	>100
Trichophyton tonsurans IFO 5928	>100	>100
Piricularia oryzae IAM 5019	100	>100

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

μg/ml and 0.11 μg/ml, respectively. Azinomycins A and B possessed potent cytotoxicities, so that studies on the antitumor activities of these two antibiotics against murine transplantable tumors are under way.

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