NOTES

HIKIZIMYCIN, A NEW ANTIBIOTIC

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During the course of our screening program for antibiotics, we have isolated a new antibiotic, named hikizimycin, from the fermentation broth of *Streptomyces* sp., strain A-5 which was obtained from a soil sample collected at the Hikizi river-side, Kanagawa Prefecture, Japan.

Hikizimycin is active against fungi, especially phytopathogenic fungi. This report describes the characteristics of the producing strain, the fermentation process, the isolation procedure and some of the physical, chemical and biological properties of the antibiotic.

Producing Organism

The morphological and physiological properties of *Streptomyces* A-5 strain were observed on a variety of media. According to the microscopic observation, the mycelia are well-branched and the aerial hyphae stretch straight and long without verticils, loops or spirals. The conidia form long globes 1.0~1.5 μ with a smooth surface.

In Table 1, the cultural characteristics of strain A-5 are reported. The optimum temperature for the development of the colonies was found to be 27~29°C and the optimum pH to be 6.0~8.0.

The results of the carbon utilization test performed according to PRIDHAM and GOTTLIEB[1] and other biochemical properties are shown in Table 2. As can be seen, culture A-5 was able to utilize practically all compounds tested except rhamnose, sorbitol, dulcitol, raffinose and cellulose.

Identification of the strain is now under investigation.

Table 1. Cultural characteristics of strain A-5

Medium	Growth	Aerial mycelium	Soluble pigment
CZAPEK's agar (27°C)	moderate creamy to orange	moderate cottony white to grayish pink	none
Starch ammonium agar (27°C)	moderate white to yellow	moderate cottony white to pink	none
Glucose asparagine agar (27°C)	moderate wrinkled, creamy to faint orange	poor white	none
Glucose nutrient agar (27°C)	good, wrinkled orange	poor white	none
Nutrient agar (27°C)	moderate creamy to orange	none or poor	none
Yeast extract-malt extract agar (27°C)	good creamy	good white to pink	none
Potato plug (27°C)	good orange	moderate white to pink	none
Gelatin stab (18~20°C)	moderate creamy	poor white	none
Milk (27°C)	poor on surface, white to creamy	none or poor	none
Ca malate agar (27°C)	moderate creamy to faint orange	moderate white	none
Tyrosine agar (27°C)	moderate creamy to faint orange	none or poor	none
Nutrient broth (27°C)	moderate, ring on surface creamy to faint orange	moderate white to faint pink	none
Bacto nitrate broth (27°C)	moderate, ring on surface creamy	poor white	none
Cellulose (27°C)	none	none	none

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Table 2. Biochemical properties of strain A-5

Utilization of carbon sources	Good growth : glucose, dextrin, glycerin, salicin, xylose, arabinose, fructose, galactose, saccharose, maltose, inulin, mannose
	Faint or no growth: lactose, mannitol, inositol, starch
	No growth: rhamnose, sorbitol, dulcitol, raffinose, cellulose
Melanin formation	negative
Nitrate reduction	negative
Utilization of collulose	negative
Milk coagulation	negative
Milk peptonization	positive (weak)
Liquefaction of gelatin	positive (strong)
Starch hydrolysis	positive (weak)

Fermentation

Streptomyces strain A-5 grows well under agitated or aerated submerged conditions at 27~29°C. The fermentation media for seed and production cultures were as follow; 3 % maltose, 1 % glucose, 2 % soybean meal, 1 % peptone, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.3 % NaCl, and 0.5 % soybean oil.

The media were adjusted to pH 7.0. Seed cultures were grown in flasks on a reciprocating shaker (145 strokes/minute, 7 cm amplitude) at 27°C for 44 hours. Jar fermentor containing 12 liters of fermentation media was inoculated with 2.5 % (v/v) of the shaking flask culture.

Main fermentation was run at 29°C, agitated at 180 r.p.m. and aerated with 1 v/v/min. Maximal antibiotic activity was usually obtained after eleven days.

A microbiological assay, using the paper disc method and *Pseudomonas fluorescens* as test organism on agar plate at pH 7.0, was developed to follow the activity of the antibiotic.

The production of hikizimycin, the pH of the broth and the volume of mycelia (after centrifugation for 10 minutes at 3,000 r.p.m.) are shown in Table 3.

Table 3. Production of hikizimycin, pH of the broth and volume of mycelia after the fermentation in jar fermentor

	Incubation period (days)				
	3	5	7	9	11
Hikizimycin (mcg/ml)	0	490	1,500	2,650	3,500
pH	7.5	7.5	7.5	7.7	7.8
Volume of mycelia (%)	18	37	37	33	32

Table 4. Elution of hikizimycin from IRC-50 column

Fraction No.	Volume (ml)	pH	Content of hikizimycin (mg)
1	1,500	7.0	0
2	700	7.0	420
3	1,000	8.5	24,000
4	500	>10.0	5,500
5	500	>10.0	0

Isolation

Hikizimycin exists mainly in the liquid part of the fermentation broth. It is extracted from the aqueous solution by cation-exchange resins or activated carbon, but not by organic solvents such as *n*-butanol, ether, ethyl acetate or benzene. For example, 12 liters of culture broth obtained by the fermentation in the jar fermentor were adjusted to pH 7.0 with hydrochloric acid, and centrifuged at 3,000 r.p.m. for 15 minutes. Ten liters of the supernatant solution containing 3.4 mg/ml of hikizimycin were passed through a column (8.0 cm in diameter) of Amberlite IRC-50 in NH_4^+ form (1.5 liters).

After washing this column with 2 liters of water, elution was made with 0.5 N ammoniacal water. The elution pattern of hikizimycin is shown in Table 4. The fractions Nos. 2~4 were combined, neutralized with hydrochloric acid and concentrated to $^1/_3$~$^1/_2$ volume under reduced pressure below 50°C.

The concentrated solution was passed through a column (8.0 cm in diameter) of activated carbon (Wako Activated Charcoal for chromatography, 1.0 liter). After washing the column with 2 liters of distilled water, elution was carried out with 0.1 N hydrochloric acid. The active fractions were combined and passed through a column of Amberlite IR-45 in OH^- form. The effluent

Fig. 1. U.V. absorption spectra

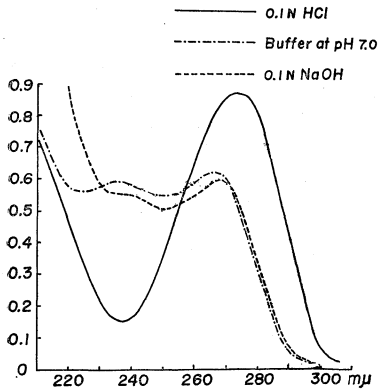


Fig. 2. N.M.R. absorption spectrum

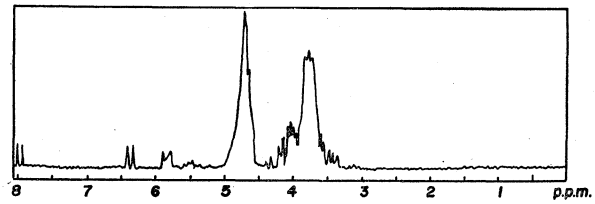
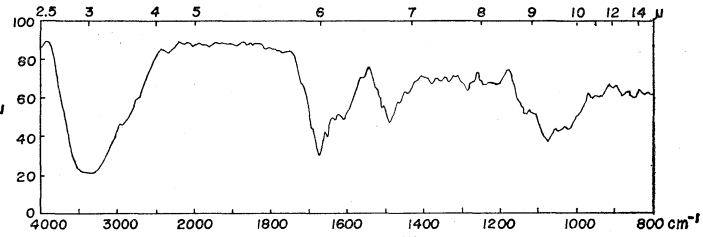


Fig. 3. I.R. absorption spectrum



was adjusted to pH 4.0 with hydrobromic acid and concentrated to dryness under reduced pressure at 50°C. The yellow powder thus obtained was dissolved in methanol, the methanol solution concentrated to half volume and was allowed to stand in a refrigerator for 2~3 days to yield plate crystals. Recrystallization was done in methanol. Hikizimycin hydrobromide usually crystallizes more easily than the hydrochloride.

Physical and Chemical Properties

Hikizimycin hydrobromide was obtained as colorless crystals. A clear melting point was not observed. When the antibiotic was heated, the color changed to brown at about 214~215°C and darkened at 230°C without melting up to 300°C. It is optically active: $[\alpha]_D^{23.5}$ −40.6 (*c* 1, H_2O).

Hikizimycin hydrobromide is highly soluble in water, slightly soluble in methanol, and practically insoluble in other organic solvents such as acetic acid, pyridine, ethanol, ethyl acetate, benzene and chloroform. Hikizimycin base is also highly soluble in water but insoluble in organic solvents.

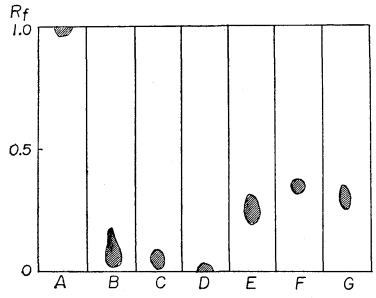
The ultraviolet absorption spectra of hikizimycin in 0.1 N HCl, in buffer solution of

Table 5. Antimicrobial spectra of hikizimycin

Test organism	MIC (mcg/ml)	Medium	Test organism	MIC (mcg/ml)	Medium
Bacillus subtilis	>200	Nutrient broth	*Trichophyton rubrum*	150	Sabouraud's agar
Bacillus megaterium	>200	〃	*Alternaria tenius*	2	Potato glucose agar
Escherichia coli	>200	〃	*Alternaria kikuchiana*	1	〃
Proteus vulgaris	>200	〃	*Ascochyta sojaecola*	2	〃
Sarcina lutea	>200	〃	*Botrytis cinerae*	20	〃
Staphylococcus aureus	>200	〃	*Botrytis tulipae*	10	〃
Pseudomonas aeruginosa	100	〃	*Curvularia lunata*	2	〃
Pseudomonas tabaci	40	〃	*Carvularia geniculata*	10	〃
Pseudomonas fluorescens	10	〃	*Colletotrichum linicolum*	20	〃
Pseudomonas syringiae	2	〃	*Fusarium oxysporum*	>200	〃
Xanthomonas campestri	200	〃	*Helminthosporium sativum*	10	〃
Xanthomonas oryzae	50	〃	*Helminthosporium oryzae*	10	〃
Candida albicans	>200	Malt agar	*Piricularia oryzae*	100	〃
Candida utilis	>200	〃	*Rhizoctonia solani*	10	〃
Saccharomyces cerevisiae	>200	〃	*Sclerotium bataticola*	2	〃
Aspergillus oryzae	150	〃	*Sclerotium rolfsii*	20	〃
Aspergillus niger	100	〃	*Stemphylium lotii*	5	〃
Penicillium chrysogenum	80	〃	*Trichoderma viridi*	>200	〃

Fig. 4. Paper chromatogram of hikizimycin.

Paper: Toyo Roshi No. 51
Development: ascending technique
Bioautogram: on *Pseudomonas fluorescens* plate
A: 3%-NH_4Cl
B: 50% acetone
C: 80% phenol
D: wet butanol
E: butanol-methanol-water (40 : 20 : 20)+1.5 g methyl orange
F: butanol-acetic acid-water (1 : 1 : 1)
G: propanol-pyridine-acetic acid-water (15 : 10 : 3 : 12)



pH 7.0 and in 0.1 N NaOH are shown in Fig. 1 with following absorption maxima: 0.1 N HCl, λ_{max} 274 mμ (ε 8,000); pH 7.0, λ_{max} 266 mμ (ε 5,600), 237 mμ (ε 5,300); 0.1 N NaOH, λ_{max} 268 mμ (ε 5,500), $\lambda_{shoulder}$: 238 mμ (ε 5,200).

These spectra suggest that the antibiotic contains a N_1-substituted cytosine as a component. The nuclear magnetic resonance absorption spectrum in deuterium oxide at 60 Hz is shown in Fig. 2. The spectrum confirms the presence of a cytosine nucleus with two doublets at δ=6.30, and δ=7.95, and also indicates the absence of methyl groups.

The infrared absorption spectrum in KBr shows bands at 3400 cm^{-1} and at 1675 cm^{-1}, indicating the existence of hydroxyl and amide carbonyl groups in hikizimycin (Fig. 3).

Hikizimycin gives positive cerium nitrate, periodic acid tests and slightly positive ninhydrin test, but gives negative biuret, MOLISCH, FEHLING, EHRLICH and ferric chloride tests. Titration shows that the antibiotic is a diacidic base (pK=3.9, 7.7) with an approximate molecular weight of 694 (856 as the dihydrobromide).

The elementary analysis show the following results: C 32.18, H 6.33, O 32.18, N 8.54, Br 20.77.

The paper chromatogram of hikizimycin hydrobromide is shown in Fig. 4.

On paper electrophoresis, hikizimycin moves to the cathode in buffer solution at pH 5.0, 7.0 and 9.0. For example, it moves 4.1 cm to the cathode under the condition: 300 V/35 cm, 2.0~2.5 mA/5 cm, 1.5 hours, 15°C, M/30 SÖRENSEN phosphate buffer (pH 5.0), Toyo Roshi No. 51.

Degradation experiments performed with hikizimycin (unpublished data) have shown the presence of cytosine and 3-amino-3-deoxy-D-glucose (kanosamine) in the antibiotic. Details of these experiments as well as other structural details of the antibiotic will be published elsewhere.

It is interesting to note here that hikizimycin is the first example of an antibiotic having cytosine and kanosamine as its constituents.

Reference

1) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bact. 56 : 107~114, 1948