STREPTOMYCES CITRICOLOR NOV. SP. AND A NEW ANTIBIOTIC, ARISTEROMYCIN*

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A new antibiotic, aristeromycin, was isolated from the culture filtrate of a new streptomyces, *Streptomyces citricolor* nov. sp. Aristeromycin is isolated using chromatography on active carbon, alumina and ion-exchange resin. Aristeromycin is isolated as colorless prism melting at 213~215°C (dec.), and has the molecular formula $C_{11}H_{15}O_3N_5$. Its ultraviolet absorption maximum is observed at 262 mμ ($E^{1\%}_{1cm}$ 555). It shows inhibitory activities against *Xanthomonas oryzae* and *Piricularia oryzae in vitro* as well as *in vivo*.

A new antibiotic, aristeromycin, has been obtained from the cultured broth of a new species of streptomyces, *Streptomyces citricolor* nov. sp., isolated from a soil sample collected in Nagoya City, Aichi Prefecture, Japan. Aristeromycin was found to be effective against *Xanthomonas oryzae* and *Piricularia oryzae in vitro*, and also in the control of bacterial leaf bright and blast disease of rice plants *in vivo*.

In this report, the characteristics of the organism, the medium for antibiotic production, its isolation and some of its physical, chemical and biological properties are presented.

I. *Streptomyces* sp. No. B-16575

1. Morphological and Cultural Characteristics

In general, aerial hyphae are relatively short. Orange to grayish aerial mycelia with simple branches are abundant, and form looped or hooked sporophores. Conidia

Plate 1. Aerial mycelia of *Streptomyces citricolor* strain No. B-16575 (×300)

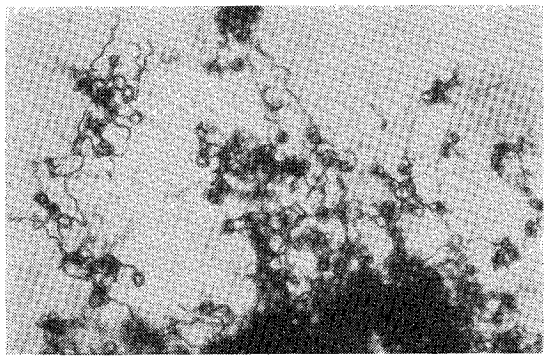

Plate 2. Electron micrograph of *Streptomyces citricolor* strain No. B-16575 (×4,667)

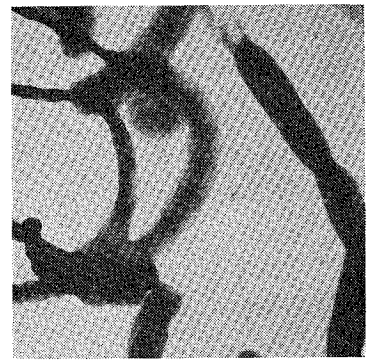

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Table 1. Characteristics of *Streptomyces* sp. No. B-16575

Morphological characteristics		Spore-bearing hyphae : curling tips or loops Spore : cylindrical, 1~2×1.2~2 μ Surface of spore : smooth
Cultural characteristics	CZAPEK'S agar	(G) Colorless (R) Colorless (AM) Poor, white (SP) None
	Glucose CZAPEK'S agar	(G) Sulphin Yellow (Rdg. IV, 21-i) (R) Chamois (Rdg. XXX, 19″-b) or Citrine Drab (Rdg. XL, 19‴-i) (AM) Abundant, white to Dark Lavender (Rdg. XLIV, 61‴-b) (SP) Ochraceous Buff (Rdg. XV, 15′-b)
	Glycerol CZAPEK'S agar	(G) Reed Yellow (Rdg. XXX, 23″-b) (R) Picric Yellow (Rdg. IV, 23-d) to Yellow Ocher (Rdg. XV, 17′) (AM) Abundant, white to Grayish Lavender (Rdg. XLIII, 57‴-f) (SP) Chamois
	Glucose asparagine agar	(G) Amber Yellow (Rdg. XVI, 21′-b) (R) Court Gray (Rdg. XLVII, 29⁗-f) to Tea Green (Rdg. XLVII, 25⁗-b) (AM) Abundant. Pale Smoke Gray (Rdg. XLVI, 21⁗-f) (SP) Seafoam Yellow (Rdg. XXXI, 25″-f)
	Nutrient agar	(G) Good, colorless (R) Colorless (AM) None (SP) Sayal Brown (Rdg. XXIX, 15″-i)
	Glucose nutrient agar	(G) Good, wrinkled, colorless to Pale Ochraceous Buff (Rdg. XV, 15′-f) (R) Colorless to Light Ochraceous Buff (Rdg. XV, 15′-d) (AM) Poor, white patches (SP) Orange Cinnamon (Rdg. XXIX, 13″)
	Glucose nutrient broth	(G) Colorless, good growth at bottom (AM) None (SP) Pinkish Buff (Rdg. XXIX, 17″-d)
	Glycerol nutrient agar	(G) Very good, Isabella Color (Rdg. XXX, 19″-i) with Aniline Yellow (Rdg. IV, 19-i) patches (R) Light Ochraceous Buff (AM) White patches (SP) Mikado Brown (Rdg. XXIX, 13″-i)
	Starch agar	No growth
	Whole-egg	(G) Very good, Dark Varley's Gray (Rdg. XLIX, 57⁗-k) (AM) None (SP) Black
	Yeast extract agar	(G) Wrinkled, colorless (R) Colorless (AM) Rich, white (SP) Snuff Brown (Rdg. XXIX, 15″-k)
	Potato plug	(G) Wrinkled, Straw Yellow (Rdg. XVI, 21′-d) (AM) Abundant, white to Olive Gray (Rdg. LI, 23⁗-b) (SP) Dark Olive Gray (Rdg. LI, 23⁗-i)
	Carrot plug	(G) Empire Yellow (Rdg. IV, 21-b) (AM) Abundant, white to Olive Gray (SP) Mouse Gray (Rdg. LI, 15⁗)
	Milk	(G) Ring, Pale Pinkish Cinnamon (Rdg. XXIX, 15″-f) (AM) None (SP) Deep Brownish Drab (Rdg. XLV, 9⁗-i) Milk was peptonized and coagulated
	Gelatin	No growth
	Nutrient gelatin	(G) Surface-ring, Pale Grayish Vinaceous (Rdg. XXXIX, 9‴-f) (AM) None (SP) Dark Vinaceous Brown (Rdg. XXXIX, 5‴-k) Partial liquefaction
	Peptone solution with 0.2 % $NaNO_3$	(G) Surface-ring, colorless growth at bottom (AM) Abundant, white (SP) Chamois to Walnut Brown (Rdg. XXVIII, 9″-k) No reduction

(To be continued)

Table 1 (Continued)

Cultural characteristics	LÖFFLER'S serum	(G) Good, Pale Pinkish Buff (Rdg. XXIX, 17″-f) to Aniline Yellow (R) Honey Yellow (Rdg. XXX, 19″) (SP) Mikado Brown No liquefaction
	Cellulose	No growth
	Calcium malate agar	(G) Good, Pale Lemon Yellow (Rdg. IV, 23-b) to Pale Chalcedony Yellow (Rdg. XVII, 25′-f) (R) Light Cadmium (Rdg. IV, 19) (AM) Abundant, white (SP) Colonial Buff (Rdg. XXX, 21″-d)
	Tyrosine agar	(G) Colorless to Antimony Yellow (Rdg. XV, 17′-b) (R) Colorless to Pale Yellow Orange (Rdg. III, 15-f) (AM) None (SP) None
	Tryptone yeast extract agar	(G) Poor, colorless (R) Colorless (AM) None (SP) Ivory Yellow (Rdg. XXX, 21″-f)

(G) : Growth. (R) : Reverse. (AM) : Aerial mycelium. (SP) : Soluble pigment.

with smooth surface arranged in chains, are oval to spherical (1~2×1.2~2 μ) (Plates 1 and 2). Globular sporangia and flagellated spores are not observed.

Cultural characteristics on various media are shown in Table 1. Colors of the culture refer to Ridgway's "Color Standard and Nomenclature."

2. Physiological Properties

The tyrosinase reaction is negative or almost negative. Nitrate is not reduced to nitrite in CZAPEK's solution or in peptone solution with 0.2% $NaNO_3$, while coagulation and peptonization of skimmed milk and chromogenicity are positive. Hydrolysis of starch is observed only on the medium containing soluble starch 1%, K_2HPO_4 0.03%, $MgCO_3$ 0.1%, NaCl 0.05%, $(NH_4)_2SO_4$ 0.2%, and agar 2%. The strain does not grow on gelatin, but grows well with weak liquefaction on nutrient gelatin (Table 2). The strain is aerobic and grows at 15~38°C and at pH 5~10, but the optimum temperature and pH ranges are at 25~30°C and pH 5~7.

Table 2. Physiological characteristics of *Streptomyces* sp. No. B-16575

Melanin formation	±~+
Nitrate reduction	−
Utilization of cellulose	−
Milk coagulation	+
Milk peptonization	+
Liquefaction of gelatin (contained in bouillon)	±~+
Tyrosinase reaction	−
Hydrolysis of starch (with $(NH_4)_2SO_4$)	+
Liquefaction of serum	−

3. Utilization of Carbon Sources

Utilization of various carbon sources by the strain (Table 3) is investigated by PRIDHAM's method[1]. The strain assimilates sucrose, D-fructose, maltose, D-galac-

Table 3. Utilization of carbon sources by *Streptomyces* sp. No. B-16575

+++ (heavy growth)	D-glucose, D-fructose, sucrose, salicin, D-galactose, D-mannose, maltose, Na-acetate, Na-succinate
++ (good growth)	melibiose, raffinose, trehalose, Na-citrate
+ (poor growth)	dextran
± (faint growth)	control
− (no growth)	erythritol, D-sorbitol, D-mannitol, D-xylose, L-sorbose, inulin, adonitol, *i*-inositol, dulcitol, L-arabinose, L-rhamnose, lactose, esculin, Ca-2-ketogluconate

tose, D-glucose, salicin, D-mannose, Na-acetate and Na-succinate, weakly assimilates raffinose, trehalose, Na-citrate, melibiose and dextran, but does not assimilate erythritol, D-sorbitol, dulcitol, inulin, adonitol, L-rhamnose, esculin, Ca-2-ketogluconate, *i*-inositol, D-mannitol, L-arabinose, D-xylose, lactose and D-sorbitol.

4. Comparison of *Streptomyces* sp. No. B-16575 with Related Species

Some remarkable characteristics of *Streptomyces* sp. No. B-16575 are as follows: The strain has simple branches and forms no verticills, but rather looped or hooked sporophores. Spores with smooth surfaces are arranged in chains. The strain produces a brown pigment in the protein-containing media and generally shows gray to dark aerial mycelia and yellow growth.

The above-mentioned characteristics of *Streptomyces* sp. No. B-16575 resembled to those of *St. culvus*, *St. flavogriseus*, *St. flavus*, *St. cacaoi* and *St. aureus*[2,3,4]. But, the strain differs from *St. calvus*, *St. flavogriseus* and *St. flavus* which produce no soluble pigment on nutrient agar and grow on gelatin. *St. cacaoi* forms long open spirals and liquefies gelatin rapidly, but *Streptomyces* sp. No. B-16575 has looped or hooked sporophores and does not grow on gelatin. *St. aureus* shows light brown aerial mycelia and cream-colored growth with an almost black surface on malate-glycerol agar, grows well with rapid liquefaction on gelatin, and forms a black ring on the surface of milk. On the other hand, the aerial mycelia of *Streptomyces* sp. No. B-16575 are white, turning later to a cream color, this strain shows pale lemon yellow growth on malate-glycerol agar and no growth on gelatin. Moreover it forms a pale pinkish cinnamon ring growth on the surface of milk. These properties differentiated the strain from *St. aureus*. Furthermore, the strain produces a new antibiotic, aristeromycin.

Judging from the above-mentioned properties, the strain was recognized as a new species and named *Streptomyces citricolor* nov. sp.

II. Fermentation and Isolation of Aristeromycin

The medium employed for fermentation of aristeromycin was as follows (in g/1,000 ml tap water): glucose 20, soluble starch 30, soy bean flour 10, corn steep liquor 10, peptone 5 and NaCl 3, and, after pH adjustment to 7 with 2N NaOH, $CaCO_3$ 5 was added. *Streptomyces citricolor* No. B-16575 was inoculated into this medium and incubated under shaking at 28°C for 4~6 days. After optimum antibiotic production, the cultured broth was filtered.

(1) After being adjusted to pH 9 with 2N NaOH, the filtered broth was mixed with active carbon (3%, w/v) (Shirasagi*) and agitated for 30 minutes. The active carbon was collected by filtration and washed with water. The biologically active component was eluted with 80% aqueous acetone from a column packed with the aforesaid carbon. The eluate was concentrated under reduced pressure. To the concentrate 10 volumes of acetone was added to give crude aristeromycin as a precipitate, which was further washed with acetone. This powder (5 g, 60 mcg/mg) was dissolved in hot 55% aqueous methanol and absorbed on an acid-washed alumina column (100 g)

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packed with absolute methanol. The column was then washed with 100 % methanol and developed gradiently with 90~70 % aqueous methanol. The biologically active fractions were collected and concentrated under reduced pressure. The concentrated solution was kept in a refrigerator overnight to give the crystal of aristeromycin (250 mg, 600 mcg/mg). It was then recrystallized from hot water (83 mg).

(2) The above-mentioned filtered broth (200 mcg/ml, 390 liters) was absorbed on active carbon (2 %, w/v) at pH 8.0. The carbon was washed with water and eluted with 80 % aqueous acetone. After concentration of the eluate under reduced pressure to 37 liters, the concentrate was passed through an Amberlite IR-120 (H^+, 10 liters) column. The column was then washed with water and eluted with 1 N NH_4OH. The biologically active fractions were collected and concentrated under reduced pressure to 2.2 liters. After chilling the concentrate in a refrigerator, a pale brown precipitate was obtained. The crude powder (140 g, 450 mcg/mg) was collected by filtration and washed with acetone. The crude aristeromycin was further purified by chromatography on active carbon. The crude powder (10 g) dissolved in hot water (400 ml) was adsorbed on a column of active carbon (100 g). The column was washed with water and eluted with 30 % acetone. The biologically active fractions were collected and concentrated under reduced pressure. When the concentrate was kept in a refrigerator, it gave crystals of aristeromycin (4.3 g). Recrystallization from hot water gave pure aristeromycin (3.5 g) as colorless prisms.

(3) The above mentioned filtered broth (75 liters, 200 mcg/ml) was absorbed on an Amberlite IRC-50 (H^+, 7.5 liters) column at pH 7.0. The column was washed with water and eluted with 1 N NH_4OH. The biologically active fractions were collected and concentrated to 0.3 liters. The crude powder (45 g, 300 mcg/mg) was collected by filtration after addition of acetone (1 liter). The pure crystals of aristeromycin (5.4 g) were obtained from the crude powder (25 g) by chromatography on active carbon and recrystallization from water.

III. Properties of Aristeromycin

1. Physical and Chemical Properties

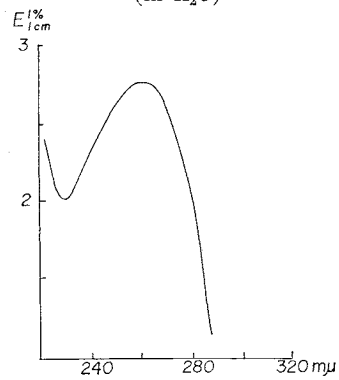
Aristeromycin is isolated as colorless prisms melting at 213~215°C (with decomposition), $[\alpha]_D^{25}$ −52.5 (c, 1, DMF). It is easily soluble in acetic acid, dimethylsulfoxide, dimethylformamide and ethyleneglycol, soluble in water, aqueous methanol and aqueous acetone, and slightly soluble or insoluble in absolute methanol, ethanol, ethyl acetate, chloroform, diethyl ether and benzene. By means of mass spectral and X-ray methods, the molecular weight of aristeromycin is found to be 265 and 269.7 respectively.

Analysis. Found : C 49.88, H 5.65, N 26.46, O 18.59 %,
Calcd. for $C_{11}H_{15}O_3N_5$: C 49.80, H 5.70, N 26.40, O 18.09 %.

The ultraviolet absorption spectrum of aristeromycin exhibits almost the same maximum at 262 mμ ($E_{1cm}^{1\%}$ 555) in water, 0.002 N HCl and 0.002 N NaOH (Fig. 1). Its infrared absorption spectrum (KBr disk) is shown in Fig. 2. It is negative to ninhydrin, EHRLICH, BARTON and SAKAGUCHI reactions. Three solvent systems and the

Rf values of aristeromycin in them by paper chromatography (Whatman No. 1) are as follows: (1) *n*-BuOH sat. with water, 0.35; (2) AcOH - *n*-BuOH - H_2O (1 : 4 : 5), 0.38; and (3) pyridine - *n*-BuOH - H_2O (3 : 4 : 7), 0.61. The Rf value of aristeromycin on thin-layer chromatography (silica gel, Merck G) using AcOEt - MeOH (2 : 1) is 0.25. After being heated at 120°C for 15 minutes or irradiated with ultraviolet rays (3,100 Å) for 48 hours, aqueous solutions of aristeromycin show no reduction of activity.

Fig. 1. Ultraviolet absorption spectrum of aristeromycin (in H_2O)



2. Biological Properties

The antibacterial and antifungal spectra obtained by the agar dilution method are shown in Tables 4 and

Fig. 2. Infrared absorption spectrum of aristeromycin (KBr)

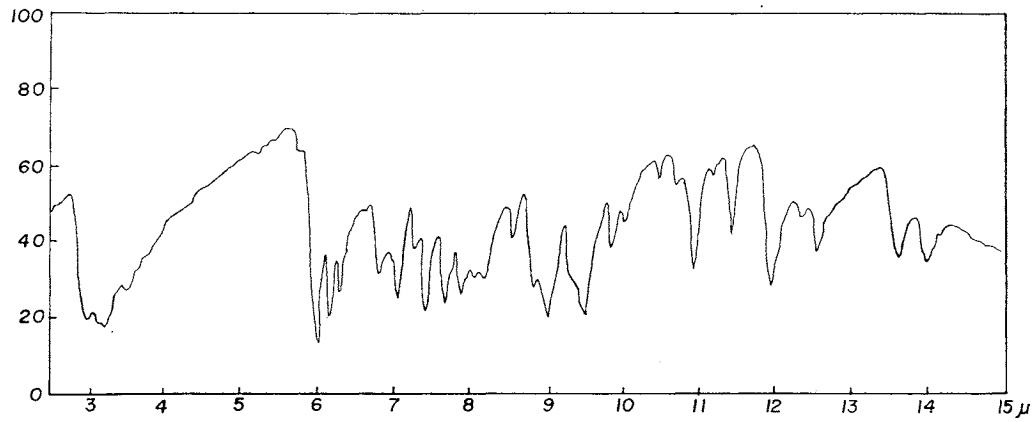


Table 4. Antimicrobial spectra of aristeromycin. I

Test organisms	Minimum inhibitory concentration (mcg/ml)			Condition for pre-incubation	Assay condition
	pH 6	pH 7	pH 8		
Bacillus subtilis	>100	>100	>100	Bouillon agar 37°C 1~2 days	Bouillon agar 37°C 18 hours
Bacillus brevis	>100	>100	>100		
Bacillus cereus	>100	>100	>100		
Staphylococcus aureus	>100	>100	>100		
Micrococcus flavus	>100	>100	>100		
Escherichia coli	>100	>100	>100		
Proteus vulgaris	>100	>100	>100		
Pseudomonas aeruginosa	>100	>100	>100		
Serratia marcescens	—	>100	—		
Aerobacter aerogenes	—	>100	—		
Mycobacterium avium	100	100	100	Glycerol bouillon agar 37°C 2~3 days	Glycerol bouillon agar 37°C 42 hours
M. avium, streptomycin-resistant	50	50	50		
Mycobacterium smegmatis	100	100	50		
Mycobacterium phlei	50	50	100		
Mycobacterium ATCC 607	50	100	50		

5. As shown in these tables, aristeromycin displays strong inhibitory activities against some of phytopathogenic bacteria and fungi, *Xanthomonas oryzae*, *Piricularia oryzae etc.*, but no inhibitory activity against yeast, pathogenic fungi and bacteria except acid-fast bacteria.

The curative effect of aristeromycin (400 mcg/ml) against the blast disease of rice plants in green houses and the inhibiting effect against spore formation of *Piricularia oryzae* on leaves of rice plants were found to be of the same degree as that of blasticidin S (20 mcg/ml).

Mice survived without any change for more than a week, after receiving 50 mg/kg of aristeromycin intravenously. Fish (killifish) in an aqueous solution of 10 mcg/ml of aristeromycin were not killed after 6 days.

The ultraviolet absorption spectrum of aristeromycin is similar to those of angustmycin A[5] and C[6], cordycepin[7], nebularine[8], canarius[9], and 3′-amino-3′-deoxyadenosine[10], but elemental analysis, optical rotation, melting point, infrared absorption spectrum and antimicrobial activity differentiated aristeromycin from these antibiotics. Tubercidin[11], which inhibits the growth of *Piricularia oryzae*, seems to resemble aristeromycin, but it differs in melting point, thin-layer chromatography, ultraviolet and infrared absorption spectra, and elemental analysis. Therefore it is concluded that aristeromycin is a new antibiotic.

Table 5. Antimicrobial spectra of aristeromycin. II

Test organisms	Minimum inhibitory concentration (mcg/ml)				Condition for pre-incubation	Assay condition
	1 day	2 days	3 days	7 days		
Agrobacterium tumefaciens		10	20		Potato sucrose agar 27°C, 1~2 weeks	Glucose bouillon agar 27°C
Corynebacterium sepedonicum	>100	>100	>100			
Erwinia aroideae	>100	>100	>100			
Pseudomonas fluorescens	>100	>100	>100			
Ps. solanacearum		50	100			
Xanthomonas citri		>100	>100		Glucose bouillon 27°C 24-hour shake culture	
X. malvacearum		>100				
X. oryzae N 5804	5	5				
// N 5843	5	5				
// N 5801	5	5				
// N 5841	5	5				
// N 5803	5	5				
// N 5810	5	5				
// N 5860	5	5				
// Beniya	5	5				
X. pruni	5	20	100			
Candida albicans	>100(2)		>100(50)		Glucose bouillon agar 37°C 1~2 weeks	Modified PFEFFER'S agar 27°C
C. krusei	>100(5)		>100			
C. parakrusei	>100(50)		>100			
C. pseudotropicalis	>100		>100			
C. tropicalis	>100(10)		>100			
Cryptococcus neoformans	>100(50)		>100			
Trichophyton mentagrophytes	>100		>100			
T. rubrum			>100			
T. tonsurans	>100		>100			

(To be continued)

Table 5 (Continued)

Test organisms	Minimum inhibitory concentration (mcg/ml)				Condition for pre-incubation	Assay condition
	1 day	2 days	3 days	7 days		
Alternaria bataticola			>100	>100	Potato rose agar 27°C, 1~2 weeks	Potato sucrose agar 27°C
A. kikuchiana			2(0.2)	10(0.2)		Modified PFEFFER's agar, 27°C (sucrose 3%, asparagine 0.2%, $(NH_4)_2SO_4$ 0.3%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, Versenol 0.001%, pH 7)
Aspergillus niger			>100	>100		
Asp. oryzae			>100	>100		
Botrytis cinerea			>100	>100		
Cephalosporium gramineum			2	>100		
Diaporthe citri			>100(5)	>100		
Elsinoe fawcetti			<0.1	0.5		
Fusarium oxysporum f. *lycopersici*			>100	>100		
Fusicladium levieri			0.5	>100		
Gibberella fujikuroi			>100	>100		
Glomerella cingulata			>100	>100		
Helminthosporium sigmoideum var. *irregulare*			5	>100(5)		
Hormodendrum compactum			<0.1	>100(5)		
Ophiobolus miyabeanus			>100(<0.1)	>100(0.5)		
Ophiostoma fimbriata			2	>100(10)		
Pellicularia filamentosa f. sp. *sasakii*			>100(<0.1)	>100(<0.1)		
〃 f. sp. *solani*			0.2	(<0.1)		
Penicillium chrysogenum			>100	>100		
P. citrinum			>100	>100		
Pestalotia diospyri			>100	>100		
Phaeoisariopsis vitis			>100(10)	>100		Potato sucrose agar 27°C
Phoma citricarpa			>100	>100		
Piricularia oryzae			5(0.5)	50(2)		Modified PFEFFER's agar, 27°C
Pyrenophora graminea			10(<0.1)	50(0.2)		
Rhizopus nigricans			>100	>100		
Sclerotinia sclerotiorum			>100	>100		
Stemphylium botryosum			10(<0.5)	>100(5)		
Trichoderma viride			>100	>100		
Ustilago zeae			>100(5)	>100		
Venturia pirina			>100	>100		
Verticillium albo-atrum			>100	>100		
Debaryomyces globosus	>100		>100			
Endomyces magnusii	50		>100			
Hansenula anomala	>100		>100			
Rhodotorula glutinis	>100		>100			
Saccharomyces cerevisiae	>100		>100			

( ) : Partial inhibitory concentration.

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