STREPTOMYCES NOVOGUINEENSIS SP. NOV., AN AMIPURIMYCIN PRODUCER, AND ANTIMICROBIAL ACTIVITY OF AMIPURIMYCIN

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A taxonomic study of *Streptomyces* strain T-36496, which produces an antibiotic effective against rice blast, revealed that it represented a new taxon and it was named *Streptomyces novoguineensis* sp. nov. The antibiotic, which was named amipurimycin, showed antifungal activity *in vitro* and considerable curative effect on leaf blast both in green house and field tests at concentrations ranging from 10 to 20 ppm. It was also effective against neck and panicle blast at the same concentration range.

In the course of our screening program for new agricultural antibiotics, *Streptomyces* strain T-36496, isolated from soil collected in New Guinea, was found to secrete an antibiotic effective against rice blast. The antimicrobial spectrum of the filtered broth indicated that this substance was not identical with known antiblast antibiotics such as blasticidin S and kasugamycin. The antibiotic was isolated, found to be a new nucleoside antibiotic, and named amipurimycin.[1]

This report deals with taxonomical studies of the organism, production and *in vitro* antimicrobial activity of amipurimycin and its antiblast activity on rice plants.

Materials and Methods

Taxonomic studies

Strain T-36496 was isolated from a soil sample collected at Rae, Papua, New Guinea. The taxonomic characterization was carried out according to the methods described by WAKSMAN,[2] SHIRLING and GOTTLIEB,[3] and PRIDHAM and GOTTLIEB.[4] The color names used in this study were based on Color Harmony Manual.[5]

Fermentation procedure

A loopful from a slant culture of *S. novoguineensis* was used to inoculate 500 ml of the seed medium in a 2-liter SAKAGUCHI flask. The medium (pH 7) consisted of 3 % glucose, 2.2 % soybean flour, 0.3 % peptone and 0.4 % $CaCO_3$ in tap water. The inoculated flask culture was incubated on a shaker (120 reciprocations per minute) at 28°C for 48 hours and the culture was transferred to 100 liters of a medium of the same composition in a 200-liter fermentor. After incubation at 28°C for 48 hours with an air-flow rate of 50 liters per minute and agitation at 100 rev./min., the whole culture was added to a 2,000-liter fermentor containing 1,000 liters of the following production medium: 5.0 % glucose, 2.0 % cotton seed meal, 2.0 % soybean flour, 0.2 % peptone, 0.6 % $CaCO_3$, 0.05 % Antifoam (Dai-ichi Kogyo Seiyaku Co.) in tap water. The medium was adjusted to pH 7. Cultivation was carried out at 28°C with an air-flow rate of 500 liters per minute and an agitation at 70 rev./min. during first 42 hours

and 125 rev./min. during the remainder of the fermentation.

Antimicrobial activity *in vitro*

Antimicrobial activity of amipurimycin against various microorganisms was examined by the agar dilution and paper disk methods (paper disk of 8 mm in diameter). Amipurimycin was determined by a diffusion assay method with *Pyricularia oryzae* P-18 as the test organism on potato sucrose agar (pH 5). Before determining the antibiotic in culture fluid an unidentified byproduct* which inhibited the growth of *P. oryzae in vitro* was eliminated by extracting it with an equal volume of methyl isobutyl ketone.

Antiblast activity on rice plant

(1) Green house test. Twenty-five-day-old seedlings of rice plants (cultivar, Asahi-#4) were cultivated in pots of 9 cm diameter. They were inoculated with a spore suspension of *P. oryzae* P-18 ($6\sim8\times10^5$ spores/ml) which had been grown on rice-decoction agar for 10~14 days at 28°C. A sample of amipurimycin solution was sprayed on the rice plants one day after the inoculation. The controlling effect was estimated by measuring the percentage of disease area on the leaves.[6]

(2) Nursery bed test. This was conducted in a field at the Kyoto Herbal Garden of Takeda Chemical Industries, Ltd. Twenty-five-day-old seedlings of rice plants (Asahi #4) in each plot of 0.64 m^2, naturally infected with the blast disease were sprayed, twice at 3~6 days intervals, with 100 ml of an amipurimycin solution of various concentrations with 0.2% Dyne.** The percentage of lesion area was measured 10, 15 and 20 days after spraying.

(3) Effect on neck and panicle blast. Rice plants (Asahi #4) grown in a concrete box ($1\text{ m}\times1.5\text{ m}\times0.3\text{ m}$) were sprayed with 300 ml of an amipurimycin solution of a various concentration with 0.2% Dyne on Aug. 9, 1973, about 9 days after the beginning of heading, and on Aug. 12, 1973. Rice plants tested were subjected to natural infection of the blast fungus. The degree and rate of incidence were assessed on Oct. 10, 1973.

$$\text{Degree of incidence} = \frac{n_1+2n_2+3n_3}{3N}\times100\,(\%)$$

$$\text{Rate of incidence} = \frac{n_1+n_2+n_3}{N}\times100\,(\%)$$

N indicates the total number of heads examined, and n_1, n_2, n_3 indicate the number of heads showing index 1, 2, 3, respectively.

Index of incidence

0: Lesion area, none
1: Lesion area only at the neck
2: Lesion area expanding 1 to 2 cm above and below the neck
3: Lesion area expanding more than 2 cm above and below the neck

Sample of amipurimycin

The amipurimycin sample used in this study was purified amipurimycin (*ca.* 95% purity). Details of the purification procedure are described in the following paper.[1]

Results and Discussion

1. Taxonomic Characteristics of Strain T-36496

Strain T-36496 formed abundant aerial mycelium on oatmeal agar, yeast extract-malt extract agar, CZAPEK's agar, glucose asparagine agar, glycerol asparagine agar, inorganic salts-starch agar, and tyrosine agar. Aerial hyphae showed monopodial branching which terminated

* As methyl isobutyl ketone extract of the filtered broth was ineffective against rice blast in the green house tests, a further examination on this byproduct has not been done.

** The surfactant sold by Takeda Chemical Industries, Ltd. containing 20% polyoxy ethylenealkyl arylether and 12% calcium ligninsulfonate.

in spirals of 4~7 turns (Fig. 1). Clusters were sometimes observed in aerial mycelium. Spore chains contained more than 10 spores, the shape of which was oval or sometimes oblong, $0.6\sim1.3\mu\times0.7\sim1.8\mu$ in size. The surface of the spore was usually smooth, but occasionally it showed some irregularity (Fig. 2). Sporangia and sclerotia were not observed on media commonly used in taxonomic studies of *Actinomycetes*.

Fig. 1. Aerial hyphae of *Streptomyces novoguineensis* on inorganic salts starch agar, 14 days (×300)

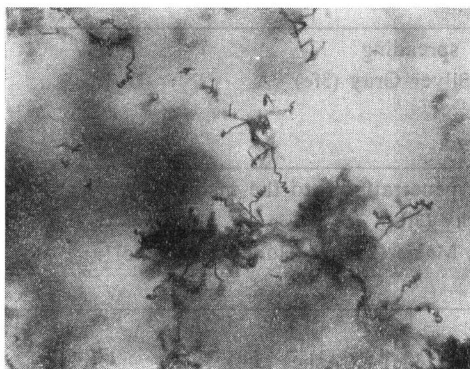
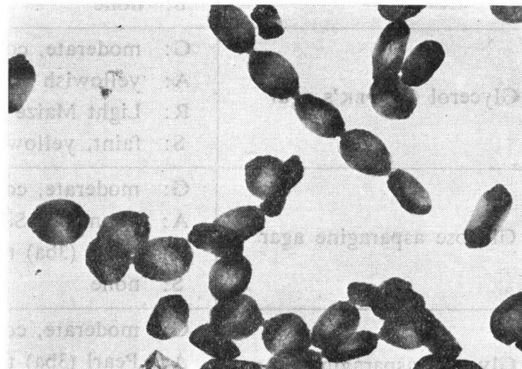


Fig. 2. Smooth spores of *Streptomyces novoguineensis*; electron micrograph from 14-day culture on inorganic salts starch agar. (×6,700)



Cultural characteristics of strain T-36496 are shown in Table 1. In synthetic media, it formed light brownish gray aerial mycelium on colorless to yellowish gray vegetative mycelium. No or, if any, a faint yellowish brown soluble pigment was produced. In complex media, the culture formed yellowish white to light gray aerial mycelium on yellowish white to light yellowish brown to pinkish brown vegetative mycelium. Brown soluble pigment was produced.

Physiological properties of strain T-36496 are shown in Table 2. It grew at 15~43°C and the optimum temperature range was 34~37°C. It showed a positive reaction in starch hydrolysis and peptonization of milk, a negative reaction in nitrate reduction to nitrite, liquefaction of gelatin and coagulated horse serum, cellulose decomposition and coagulation of skimmed milk. Formation of melanoid pigment was observed in peptone-yeast extract iron agar (ISP No. 6) and tryptone-yeast extract broth (ISP No. 1) and in ARAI's melanine formation test,[7] but negative in tyrosine agar (ISP No. 7). This strain, therefore, was placed in the so-called chromogenic type of *Streptomyces*.

As shown in Table 3, inositol, D-mannitol, D-xylose, L-arabinose, D-galactose, D-glucose, D-fructose, melibiose, maltose, lactose, trehalose, D-mannose, starch, glycerol, sodium acetate, sodium succinate and sodium citrate were utilized well but erythritol, adonitol, D-sorbitol, dulcitol, L-sorbose, rhamnose, sucrose and raffinose were either not used or only slightly used for growth.

The morphological, cultural and physiological characteristics of strain T-36496 were compared with known species reported previously.[3,8~13] It was found that the strain resembled to *Streptomyces miharaensis*,[14] *Streptomyces anandii*[15] and *Streptomyces phaeofaciens*.[16] The difference between *Streptomyces miharaensis* and strain T-36496 is as follows:

S. miharaensis does not form aerial mycelium on CZAPEK's agar, glycerol asparagine agar and most agar media commonly used in taxonomic studies. It forms aerial mycelium of fawn color with white patches on starch-yeast extract agar containing M/15 phosphate buffer (pH 7).

Table 1. Cultural characteristics of strain T-36496

Medium	Characteristics
CZAPEK's agar	*G: moderate, colorless to Light Maize (2ea) A: moderate, Silver Gray (3fe) partially Sand (3cb) R: Natural (3dc) to Light Maize (2ea) S: none
Glucose CZAPEK's agar	G: moderate, colorless A: yellowish white to Silver Gray (3fe) R: Light Ivory (2ca) S: none
Glycerol CZAPEK's agar	G: moderate, colorless, spreading A: yellowish white to Silver Gray (3fe) R: Light Maize (2ea) S: faint, yellowish brown
Glucose asparagine agar	G: moderate, colorless, penetrating into the medium A: abundant, Silver Gray (3fe) R: Pearl (3ba) to Light Maize (2ea) to Honey Gold (2ic) S: none
Glycerol asparagine agar (**I.S.P. No. 5)	G: moderate, colorless A: Pearl (3ba) to Natural (3dc) to Ashes (5fe) R: Light Maize (2ea) partially Beige Brown (3ig) S: faint, yellowish brown
Ca-malate agar	G: moderate, colorless A: poor, thin, Light Ivory (2ca) R: Maize (2hb) S: faint, yellow
Inorganic salts-starch agar (I.S.P. No. 4)	G: moderate, colorless, spreading, also penetrating into the medium A: abundant, Silver Gray (3fe) R: Light Ivory (2ca) to Light Maize (2ea) S: none
Tyrosine agar (I.S.P. No. 7)	G: colorless A: abundant, Silver Gray (3fe) to Sand (3cb) R: Natural (3dc) to Light Melon Yellow (3ea) to Light Amber (3ic) S: faint, yellowish brown
Oatmeal agar (I.S.P. No. 3)	G: Light Ivory (2ca) A: Natural (3dc) to Ashes (5fe), periphery of the colony Light Ivory (2ca) R: Light Beige (3ec) to Maple (4le) S: faint brown
Nutrient agar (37°C)	G: moderate, colorless A: poor, Pearl (3ba) R: Light Ivory (2ca) S: Light Brown (4ng)
Glucose nutrient agar (37°C)	G: moderate, colorless, wrinkled A: moderate, Pearl (3ba) to Sand (3cb) R: Light Ivory (2ca) S: Light Brown (4ng)

(continued)

Nutrient broth (37°C)	G: surface, colorless, also flocculent growth in the bottom A: none S: Oak Brown (4pi)
Glucose nutrient broth (37°C)	G: surface, pellicle, Light Ivory (2ca) A: Pearl (3ba) to Sand (3cb) S: Oak Brown (4pi)
Yeast extract-malt extract agar (I.S.P. No. 2)	G: moderate, colorless, folded A: moderate, Beige (3ge) partially Pearl (3ba) R: Light Melon Yellow (3ea) to Honey Gold (2ic) to Tan (3ie) S: faint brown
Peptone yeast extract iron agar (I.S.P. No. 6)	G: colorless, glossy, wrinkled A: none R: Chamois (2gc) S: Ebony Brown (8pn)
Gelatin (24°C)	G: colorless A: poor, yellowish white S: Russet Brown (5pi) slow liquefaction
Milk (37°C)	G: surface ring, Light Ivory (2ca) A: none S: Pastel Orange (4ic) to Light Brown (4ng), pH 8.0 to 8.3 peptonization without coagulation
Potato plug	G: moderate, colorless A: abundant, Pearl (3ba) to Natural (3dc) plug turns to brownish gray
Carrot plug	G: moderate, colorless A: abundant, Natural (3dc) to Silver Gray (3fe) plug turns slightly brownish
LÖFFLER's medium	G: colorless A: none S: Deep Brown (4pi) only near the colony
Cellulose	G: poor, colorless A: poor, Natural (3dc) S: none

* G: Growth A: Aerial mycelium R: Reverse S: Soluble pigment.
** Medium employed by International Streptomyces Project.

S. miharaensis reduces nitrate to nitrite, utilizes rhamnose, raffinose and D-sorbitol and shows positive tyrosinase reaction. It produces antibiotics miharamycins which are different from amipurimycin.

Streptomyces anandii forms poor, white aerial mycelium on glucose asparagine agar. The color of aerial mycelium on glycerol asparagine agar is white. *S. anandii* reduces nitrate to nitrite, utilizes D-sorbitol, sucrose and raffinose but does not assimilate sodium acetate, and shows a positive tyrosinase reaction. It produces a pentaene antibiotic which is not found among the metabolites of strain T-36496.

Streptomyces phaeofaciens forms poor aerial mycelia with white to gray color on glucose

Table 2. Physiological properties of strain T-36496

Temperature and pH ranges*	growth occurs at 15~43°C, better growth and aerial mycelium formation at 34~37°C, no growth at 10°C and 50°C, growth occurs at pH 5~10, no growth at pH 4, optimum range pH 7~8
Gelatin liquefaction	very slow
Starch hydrolysis	positive diameter of hydrolysed area/diameter of colony=27/12
Melanine production	positive in peptone yeast extract iron agar (I.S.P. No. 6) and tryptone yeast extract broth (I.S.P. No. 1) negative in tyrosine agar (I.S.P. No. 7)
Nitrate reduction	negative in peptone solution doubtful in CZAPEK's solution
Skimmed milk	peptonization without coagulation
Liquefaction of serum	negative
Cellulose decomposition	negative
Product	amipurimycin

* On glucose asparagine agar.

Table 3. Carbon source utilization of strain T-36496

Carbon source	Growth	Carbon source	Growth
Erythritol	±	Sucrose	±
Adonitol	±	Lactose	++
D-Sorbitol	±	Raffinose	± +
Inositol	++	Trehalose	++
D-Mannitol	++	Salicin	+
Dulcitol	±	Esculin	+
D-Xylose	++	Inulin	+
L-Arabinose	++	D-Mannose	++
L-Sorbose	±	Starch	++
D-Galactose	++	Glycerol	++
D-Glucose	++	Na-Acetate	++
D-Fructose	++	Na-Succinate	++
Rhamnose	±	Na-Citrate	++
Melibiose	++	Carbon-free control	±
Maltose	++		

++: Good growth +: Fair growth
±: No or very poor growth

Table 4. Time course of amipurimycin production

Incubation time (hr)	pH	Amipurimycin (μg/ml)
30	5.7	0
66	7.3	46
90	7.3	54
114	7.3	73

asparagine agar. The reverse color on yeast malt agar is greenish yellow to light olive brown. *S. phaeofaciens* does not hydrolyze or only slightly hydrolyzes starch, reduces nitrate to nitrite, utilizes rhamnose but does not assimilate sodium citrate and sodium succinate. It produces an antibiotic phaeofacin which is different from amipurimycin.

From the comparison mentioned above, strain T-36496 was considered to be a new species of *Streptomyces*, and the name, *Streptomyces novoguineensis* sp. nov is proposed. The specific epithet "novoguineensis" is the modern Latin adjective meaning New Guinea where the soil sample of this study was collected.

Strain T-36496 is designated the type strain of the species and has been deposited in the Institute for Fermentation Osaka and assigned accession number IFO-13572.

2. Production of Amipurimycin

A typical time course of amipurimycin production is shown in Table 4. Amipurimycin was detected in culture fluid at 66 hours, reached a maximum at 102~114 hours, and showed

Table 5. Antimicrobial spectrum of amipurimycin

Test organisms	Medium*	Temp. (°C)	Time (hr)	Minimum inhibitory concentration (μg/ml)
Pyricularia oryzae IFO-4874	A	28	64	5
P. setariae IFO-6694	A	28	64	10
Alternaria kikuchiana IFO-7515	A	28	64	10~20
Helminthosporium sigmoideum var. *irregulare* IFO-5273	A	28	64	5~10
Pythium aphanidermatum IFO-7030	A	28	64	5
Cochliobolus miyabeanus IFO-5277	A	28	64	10~20
Sclerotinia sclerotiorum IFO-9395	A	28	88	20
Monilinia fructicola IFO-9068	A	28	64	20~50
Elsinoe fawcettii IFO-8417	A	28	88	>100
Aspergillus niger IFO-4066	A	28	40	>100
Penicillium chrysogenum IFO-4626	A	28	40	>100
Trichophyton mentagrophytes IFO-5466	D	28	40	10
Candida albicans IFO-0538	A	28	40	>100
Saccharomyces cerevisiae IFO-0209	A	28	40	>100
Bacillus subtilis IFO-3513	B	37	20	>100
Staphylococcus aureus IFO-3061	B	37	20	>100
Escherichia coli IFO-12734	B	37	20	>100
Proteus vulgaris IFO-3045	B	37	20	>100
Pseudomonas aeruginosa IFO-12045	B	37	20	>100
Mycobacterium phlei IFO-3158	C	37	40	>100

* A: Modified PFEFFER's agar (Synthetic agar medium)[18] (pH 7).
B: Bouillon agar (pH 7).
C: Glycerol bouillon agar (pH 7).
D: 1.0% glucose, 0.4% $(NH_4)_2HPO_4$, 0.07% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, 0.1% NaCl, 0.003% $FeSO_4 \cdot 7H_2O$, 1.5% agar (pH 7).

no marked drop thereafter. The fermentation was stopped at 114 hours and the broth was harvested.

3. Antimicrobial Activity *in vitro*

The antimicrobial activities of amipurimycin against phytopathogenic fungi, yeasts, Gram-positive and Gram-negative bacteria are shown in Table 5 and Table 6. Amipurimycin inhibited the growth of some phytopathogenic fungi such as *Pyricularia oryzae*, *P. setariae*, *Alternaria kikuchiana*, *Helminthosporium sigmoideum* var. *irregulare*, *Pythium aphanidermatum*, *Cochliobolus miyabeanus* and *Sclerotinia sclerotiorum* at concentrations of 5~50 μg/ml. Partial inhibition was observed in *P. oryzae*, *P. setariae*, *A. kikuchiana*, *H. sigmoideum* var. *irregulare*, *S. sclerotiorum* and *Monilinia fructicola* at concentrations of 1/5~1/10 of each minimum inhibitory concentration shown in Table 5. It also inhibited the growth of *Trichophyton mentagrophytes*, a dermatophyte, at a concentration of 10 μg/ml. It showed no antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, yeasts and saprophytic fungi tested. Phytopathogenic fungi such as *Botrytis cinerea*, *Colletotrichum lagenarium*, *Fusarium oxysporum*, *Verticillium albo-atrum*, *Venturia pirina*, *Ceratocystis fimbriata*, *Diaporthe citri*, *Gibberella zeae*, *Ustilago zeae*, *Pellicularia sasakii* and yeasts such as *Rhodotorula glutinis* and *Hansenula anomala*

were not inhibited by the presence of 100 μg/ml of amipurimycin. The strong antiblast activity *in vivo* and physicochemical properties of amipurimycin suggested a similarity to miharamycin. Table 6 compares both antibiotics. Miharamycin is characterized by dominant activity against *Pyricularia oryzae* and *Pseudomonads*[14] whereas amipurimycin showed no activity against the 26 strains of *Pseudomonas aeruginosa* and 3 strains of *P. fluorescens* tested.

4. Effect of Amipurimycin on the Incidence of Rice Blast

As shown in Table 7 amipurimycin showed a strong curative effect against blast disease of

Table 6. Comparison of amipurimycin with miharamycins A and B in their antimicrobial spectra

Test organisms	Medium**[17]	Temp. (°C)	Time (hr)	Diameter of inhibition zone (mm)				
				Miharamycin A[17]		Miharamycin B[17]		Amipuri-mycin
				1,000 μg/ml	500 μg/ml	1,000 μg/ml	500 μg/ml	1,000 μg/ml
Pseudomonas tabaci	GPA	28	20	26.7	26.0	27.3	25.5	0
P. aeruginosa	GPA	37	20	27.3	25.4	19.7	17.2	0
P. fluorescens	GPA	28	20	16.7	12.6	15.3	11.3	0
P. trifolii	GPA	28	20	12.3	+	13.6	+	0
Pyricularia oryzae	YSA	28	72	—	69.5	64.0	61.5	45
Colletotrichum lagenarium	YSSA	28	72	40.0	45.0	40.0	30.0	0
Aspergillus niger	YSSA	28	72	30.0	15.0	20.0	0	(60)*
Glomerella cingulata	YSSA	28	72	25.0	20.0	18.0	15.0	0
Fusarium oxysporum	YSSA	28	72	20.0	15.0	0	0	0
Rhizopus nigricans	SA	28	72	26.8	26.5	—	—	0
Penicillium expansum	YSA	28	72	15.0	15.0	12.0	10.0	0
Guignardia laricina	YSA	28	72	60.0	50.0	40.0	30.0	63

* Quite obscure inhibition zone.

** GPA: Glucose peptone agar YSA: Yeast starch agar YSSA: Yeast saccharose agar SA: SABOURAUD's agar

Table 7. Controlling effect of amipurimycin on blast of rice plant in the green house test

Antibiotics	Concentration (ppm)	Curative effect percent lesion area (%)	Phytotoxicity*
Amipurimycin	20	0.05	++
	10	1.6	+
	5	5.9	±
	2.5	9.9	±
	1.25	23.6	—
Blasticidin S	20	0.3	+
	10	2.9	+
	5	9.2	—
	2.5	16.7	—
	1.25	39.7	—
Untreated check		90.0	

* The strength of the phytotoxicity is expressed as ++, + and ± in the order of the intensity estimated by visual inspection.

Table 8. Control of blast of rice plant with amipurimycin in nursery bed test in field

Antibiotics	Concentration (ppm)	Percent lesion area (%) Days after spray			Phytotoxicity*
		10 days	15 days	20 days	
Amipurimycin	20	2.0	3.4	5.1	±～+
	16	2.7	5.9	6.2	±
	12.8	4.4	12.5	13.0	±
	10.2	4.1	10.2	14.2	
	8.1	5.9	15.8	20.9	
	6.4	4.9	14.2	27.5	
	5.1	9.7	22.7	37.9	
Blasticidin S	20	2.0	4.0	5.3	±
	16	5.0	8.5	8.0	
	12.8	5.2	12.4	13.2	
	10.2	6.5	16.4	18.9	
	8.1	8.9	18.5	20.9	
	6.4	7.6	18.0	30.3	
	5.1	9.4	28.0	37.3	
Untreated check		9.7	22.7	37.9	

* See the footnote of Table 7.

Table 9. Controlling effect of amipurimycin on neck and panicle blast of rice plant

Antibiotics	Concentration (ppm)	Degree of incidence (%)	Rate of incidence (%)	Phytotoxicity*
Amipurimycin	40	9.1	17.1	±～+
	20	9.1	14.9	±
	10	14.6	26.5	
Blasticidin S	40	7.0	12.9	chlorosis
	20	10.1	15.2	do
	10	13.8	25.7	
Untreated check		36.7	64.3	

* See the footnote of Table 7.

rice plants in the green house test, even at a concentration of 2.5 ppm. Phytotoxicity was observed as small, brown spots which are different from the chlorosis caused by blasticidin S. The controlling effect of amipurimycin was equal to or a little stronger than that of blasticidin S.

In field tests, amipurimycin showed sufficient controlling effect against blast disease at a concentration of 16 ppm (Table 8). Phytotoxicity seems to be less serious than that observed in the green house tests probably due to less humid conditions in field tests.

The efficacy of amipurimycin against neck and panicle blast which causes poor harvest of rice was studied in the field. As shown in Table 9 amipurimycin showed a strong curative effect.

Thus, 10～20 ppm solutions of amipurimycin showed an excellent curative effect against blast of rice plants not only in the green house tests but also in the field tests. The type and

strength of activity of amipurimycin against blast disease resembled those of blasticidin S.

5. Toxicity

Toxicity of amipurimycin to the killifish was not observed at 10 ppm after 2 days, but all fish tested died after 3 days.

LD_{50} values of amipurimycin by intravenous administration in mice and rats were 1~5 mg/kg, and by oral administration were 10~20 and 20~30 mg/kg, respectively. In the rabbit, no irritation was observed on the cornea at 500 ppm of amipurimycin, but strong irritation was observed on the skin by daily application of 200 ppm for 10 days.

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