A NOVEL MACROLIDE ANTIBIOTIC, NOTONESOMYCIN A

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Fermentation of *Streptomyces aminophilus* subsp. *notonesogenes* 647-AV$_1$ produced a mixture of antifungal antibiotics. Notonesomycin A, the main component has been characterized as a new macrolide antibiotic containing a sulfate group, a malonate moiety, two deoxysugars and a 1,3,4-trisubstituted benzene system in the molecule. It was active against some fungi and Gram-positive bacteria *in vitro* and effective against the sheath blight disease of rice plant in a green house test.

In the course of our screening program for antibiotics effective against the sheath blight disease of rice plant, a new antifungal antibiotic was isolated from the mycelium of *Streptomyces aminophilus* subsp. *notonesogenes* 647-AV$_1$ and designated notonesomycin A (Fig. 1). This report describes the taxonomy of the producing organism, fermentation and isolation procedures as well as physico-chemical and biological properties of notonesomycin A.

Taxonomy of the Producing Strains

Notonesomycin A was obtained by the fermentation of the strains 647-AV$_1$ and 652-MC$_1$. The cultures are identical except that the 647-AV$_1$ showed slight, or almost no, aerial mycelium formation for taxonomical studies. Therefore, 652-MC$_1$ was used as a type strain. However, 647-AV$_1$ was utilized for the production of the antibiotic because of its high activity. The notonesomycin A-producing strains 647-AV$_1$ and 652-MC$_1$ were isolated from soil samples collected from a kitchen garden in Tamagusuku-son and a broad-leaved forest in Ozato-son, respectively, both located in Shimajiri-gun, Okinawa Prefecture. The type strain of *S. aminophilus* KCC S-0619 (ISP 5186) obtained from Dr. A. SEINO of Kaken Chemical Co., Ltd., Tokyo, was used for the reference culture. Characterization of the strains was performed in accordance with the methods of the International *Streptomyces* Project (ISP)[1] and additionally by the methods of WAKSMAN,[2] and analysis of whole cell hydrolysate was carried out by the method of BECKER *et al.*[3]

The strains were in perfect agreement with regard to morphological characteristics observed by light and electron microscopes. Mature spore chains generally contained 10 to 20, or more, spores which formed irregular spirals of two to four turns often with hooks at the ends of short sporophores branched monopodially on the aerial hyphae (Fig. 2). Spores were 0.3 to 0.4 μm in diameter, 0.6 to 0.7 μm in length, elliptical to oblong with smooth surface, and non-motile (Fig. 3). No sporangia, sclerotia or other structures were observed. Both strains were assigned to cell wall type I, because whole cell hydrolysates of the strains contained LL-diaminopimelic acid. Cultural and physiological characteristics are summarized in Tables 1 and 2, respectively.

On the basis of the comparative morphological, cultural and physiological characteristics mentioned

Fig. 1. Structure of notonesomycin A free acid.

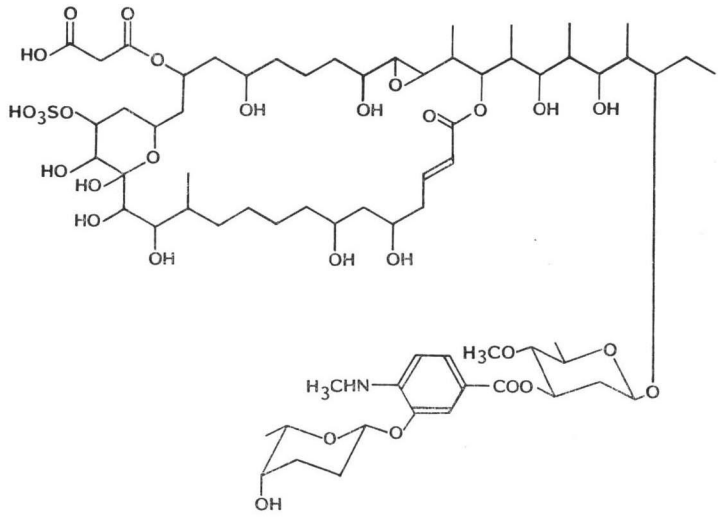


Fig. 2. Aerial mycelium of strain 652-MC_1 on sucrose - nitrate agar, 21-day.

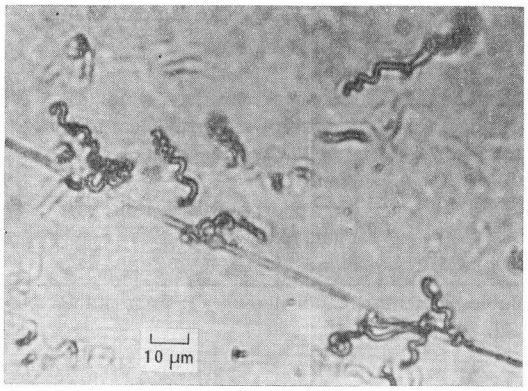
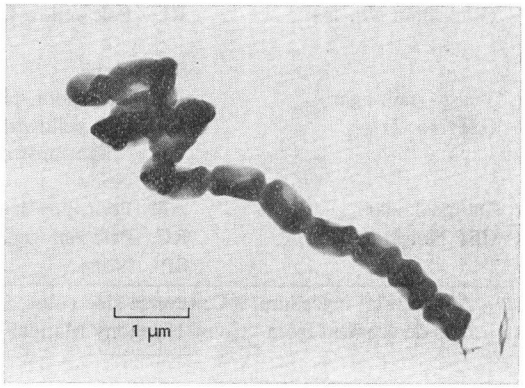


Fig. 3. Electron micrograph of spore chain of strain 652-MC_1 from 21-day culture on inorganic salts-starch agar.



above, both strains were concluded to belong to the same taxon, and are placed into the genus *Streptomyces*. Both strains are characterized as follows; 1) the spore chain morphology is spiral, 2) the spore surface is smooth, 3) the aerial mass color belongs to the yellow color-series, 4) the reverse side of the colony does not contain any distinctive pigments (pale yellow to pale yellowish brown or light brownish gray) but becomes pink or pale orange to light reddish orange on some media, 5) melanoid pigments and other soluble pigments are not formed, and 6) sucrose, *i*-inositol and raffinose are not utilized for growth.

On the basis of these characteristics, species fitting both strains were searched for among validly published species of the genus *Streptomyces*,[4,5] consulting with the existing keys[6,7] and additionally our key. As a result, it was found that both strains most resembled *S. aminophilus* Wooldridge.[6,8] *S. aminophilus* ISP 5186 (type strain) and the strains 647-AV_1 and 652-MC_1 were compared for characteristics under the same experimental conditions (Table 3). The type strain of *S. aminophilus* differed from both producing strains in that aerial mycelia on various media were relatively more abun-

Table 1. Cultural properties of the notonesomycin-producing strains.

		647-AV_1	652-MC_1
Sucrose - nitrate agar	AM	None	Very poor, powdery, white (a)
(Waksman No. 1)	RC	Yellowish-brownish tint (3ba)	Yellowish-brownish tint (3ba)
	SP	None	None
Glucose - asparagine agar	AM	None	Poor, powdery, white (a)
(Waksman No. 2)	RC	Light yellow (2db) to brownish white (3ca)	Light yellow (2db) to pale yellowish brown (2ic)
	SP	None	None
Glycerol - asparagine agar	AM	None	Poor, powdery, yellow or red color-series (3ba)
(ISP No. 5)	RC	Brownish white (4ca) to light reddish orange (6ga)	Pale yellow (2ca) to light reddish orange (6ga)
	SP	Trace brownish tinge	None
Inorganic salts - starch agar	AM	Very poor, white (a)	Poor, powdery, yellow color-series (1ba)
(ISP No. 4)	RC	Light yellow (2fb) to pale yellowish brown (3gc)	Light brownish gray (3ec) to light orange (5ia)
	SP	None	None
Tyrosine agar	AM	None	Poor, powdery, white (a)
(ISP No. 7)	RC	Pale yellow (2ca) to pink (6ea)	Pale yellow (2ca) to brownish white (3ca)
	SP	None	None
Peptone - beef agar	AM	None	Poor, powdery, white (a)
(Waksman No. 14)	RC	Pale yellow (2ca)	Light yellow (2ga) to pale orange (5ba)
	SP	None	None
Yeast - malt agar	AM	Very poor, powdery, white (a)	Very poor, powdery, white (a)
(ISP No. 2)	RC	Pale yellowish brown (3gc) to light brownish gray (4ec)	Light brownish gray (3ec) to dark yellowish orange (3ne)
	SP	None	None
Oatmeal agar	AM	Poor, powdery, white (a)	Very poor, powdery, white (a)
(ISP No. 3)	RC	Pale yellow (2ca)	Light yellow (2db)
	SP	None	None

AM: Aerial mycelium, RC: reverse side color, SP: soluble pigments. Color name ( ): Code of the color tabs determined from "Color Harmony Manual" 4th Ed.

dantly sporulated and stronger in yellow color, and that pink or pale orange to light reddish brown pigments on the reverse side of the colony were not observed on some media. The results mentioned above lead us to conclude that both notonesomycin A-producing strains should be assigned to a new subspecies of *S. aminophilus*. We hereby propose the name *Streptomyces aminophilus* Wooldridge subsp. *notonesogenes* Furihata et Shimazu subsp. nov. (noto-neso-genes, Gr. *notos* south+Gr. *nesos* island+Gr. adj *genes* origin, referring to the place where the soil samples were obtained from which both organisms were isolated) with strain 652-MC_1 as the type strain of this taxon.

Fermentation

A well-grown agar slant of the strain 647-AV_1 was inoculated into 20 ml of a seed culture medium consisting of starch 1% and soybean meal 3% (pH 7.0) in a 100-ml Erlenmeyer flask. The inoculated flask was shaken on a rotary shaker (220 rpm) at 28°C for 5 days. Seed flasks (500-ml Erlenmeyer) containing 100 ml of the same medium were inoculated with 5 ml of the first seed and then shaken at 28°C for 43 hours. A 30-liter jar fermentor with 20 liters of medium consisting of glucose 1.5%,

Table 2. Physiological properties of the notonesomycin-producing strains.

	647-AV$_1$	652-MC$_1$
Growth range of temperature (°C)	20~30	20~30
Formation of melanoid pigments		
Tyrosine agar	None	None
Peptone - yeast - iron agar	None	None
Tryptone - yeast broth	None	None
Hydrolysis of starch	Good	Good
Liquefaction of gelatin	Slight	Slight
Peptonization of milk	Slight	Slight
Coagulation of milk	Slight	Slight
Utilization of carbon		
L-Arabinose	++	++
D-Xylose	++	++
D-Glucose	++	++
D-Fructose	+	+
Sucrose	−	−
i-Inositol	−	−
Rhamnose	++	++
Raffinose	−	−
D-Mannitol	++	++

++ Good growth, + fair growth, − no growth.

Table 3. Comparison of taxonomical properties of the notonesomycin-producing strains and the type strain of *S. aminophilus*.

	647-AV$_1$	652-MC$_1$	*S. aminophilus* ISP 5186
Spore chains	Spirals (hooks)	Spirals (hooks)	Spirals (hooks)
Spore surface	Smooth	Smooth	Smooth
Formation of AM	None or very poor	Very poor to poor	Poor to moderate
Color of colony	ID	ID or yellow	Yellow
Reverse side of colony	NDP or pink to light reddish brown	NDP or pale orange to light reddish brown	NDP
Melanoid pigments	None	None	None
Other soluble pigments	Trace or none	None	None
Utilization of			
L-Arabinose	+	+	+
D-Xylose	+	+	+
D-Fructose	+	+	+
D-Mannitol	+	+	+
Rhamnose	+	+	+
Sucrose	−	−	±
i-Inositol	−	−	±
Raffinose	−	−	±

AM: Aerial mycelium, ID: inadequate for determination of aerial mass color, NDP: no distinctive pigments (pale yellow to pale yellowish brown).
+ Growth, ± trace of growth, − no growth.

glycerol 1.0%, meat extract 1.0%, Polypeptone 1.0% and $CaCO_3$ 0.4%, (pH 7.2) was inoculated with 3% of the mature seed broth. The fermentation was incubated at 28°C for 76 hours with aeration at 20 liters/minute and agitation at 250 rpm. The progress of the fermentation was monitored by the antifungal activity against *Rhizoctonia solani* as reported previously.[9]

Fig. 4. Purification procedure for notonesomycin A.

Cultured broth (15 liters)
↓
Mycelial cake
↓ 50% Me_2CO
Extract
↓ concd
↓ BuOH
BuOH layer
↓ concd
↓ added EtOAc
Crude powder
↓ silica gel column chromatography
↓ EtOAc - MeOH - H_2O, 65: 25: 4
Active fraction
↓ HPLC S-343 ODS-C_{18}
↓ CH_3CN - 0.01 M CH_3COONH_4, 2: 3
Notonesomycin A fraction
↓ Toyopearl HW-40 column chromatography
↓ MeOH - H_2O, 7: 3
Active fraction
↓ lyophilized
Notonesomycin A (150 mg)

Table 4. Physico-chemical properties of notonesomycin A.

MP	194~195°C
$[\alpha]_D^{24}$	−29.7° (*c* 1.0, $CHCl_3$ – MeOH, 1: 1)
Molecular formula	$C_{83}H_{109}NO_{30}SNa_2$
Anal (%)	Found C 54.21, H 7.85, N 1.33, S 2.07 Calcd C 54.51, H 7.28, N 0.94, S 2.14
FAB-MS (*m/z*)	Positive 1,498 $(M+H)^+$; Negative 1,452 $(M-2Na+H)^-$
UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$)	310 nm (65)
IR (KBr) cm^{-1}	3400, 1720, 1700, 1600, 1280, 1060
Color reaction	Positive; $KMnO_4$, vanillin-sulfuric acid Negative; ninhydrin, Sakaguchi

Isolation

The isolation and purification procedures for notonesomycin A are summarized in Fig. 4. The mycelial cake was separated by filtration from cultured broth (15 liters) and extracted twice with 10 liters of 50% aqueous acetone. The extract was concentrated to an aqueous solution (5 liters) and then extracted twice with 3 liters of butanol. The combined solvent fraction was evaporated *in vacuo* to give a brown syrup which was dissolved in a small amount of methanol. An active principle was precipitated by the addition of ethyl acetate. The precipitate (10 g) was dissolved in a solvent chloroform - methanol (1: 1), mixed with an equal weight of silica gel and evaporated *in vacuo*. The dried silica gel was placed on the top of a silica gel column (7×20 cm) packed with ethyl acetate. After washing with ethyl acetate, the column was developed with a mixture of ethyl acetate - methanol - water (65: 25: 4). Active fractions were collected and concentrated to dryness *in vacuo* to give a crude powder (1.2 g). Separation of notonesomycin A from its minor components was achieved by preparative HPLC on S-343 ODS-C_{18} (Yamamura Chemical Lab. Co., Ltd.) with acetoni-

Fig. 5. IR spectrum of notonesomycin A in KBr.

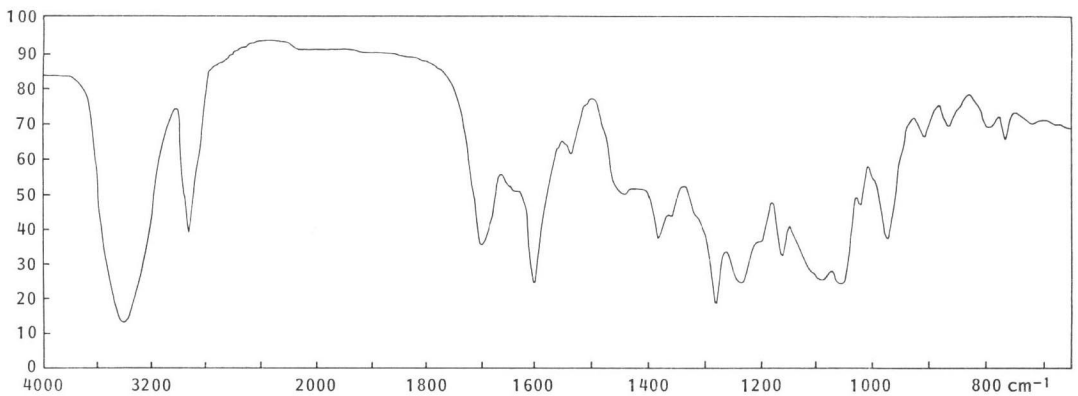


Fig. 6. ^{13}C NMR spectrum of notonesomycin A (100 MHz in Me_2CO-d_6 - D_2O, 4: 1).

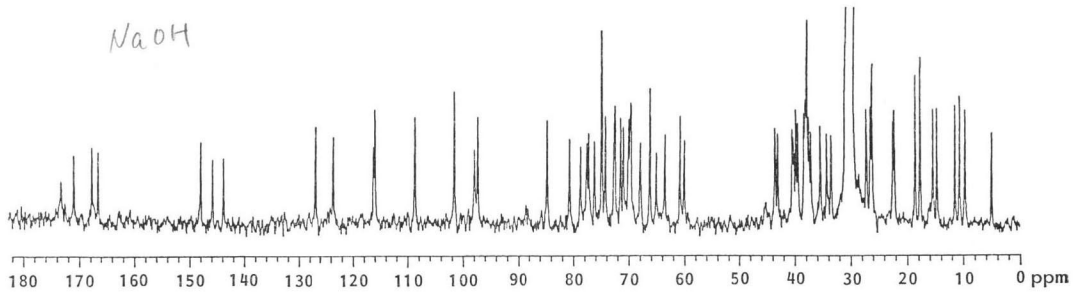
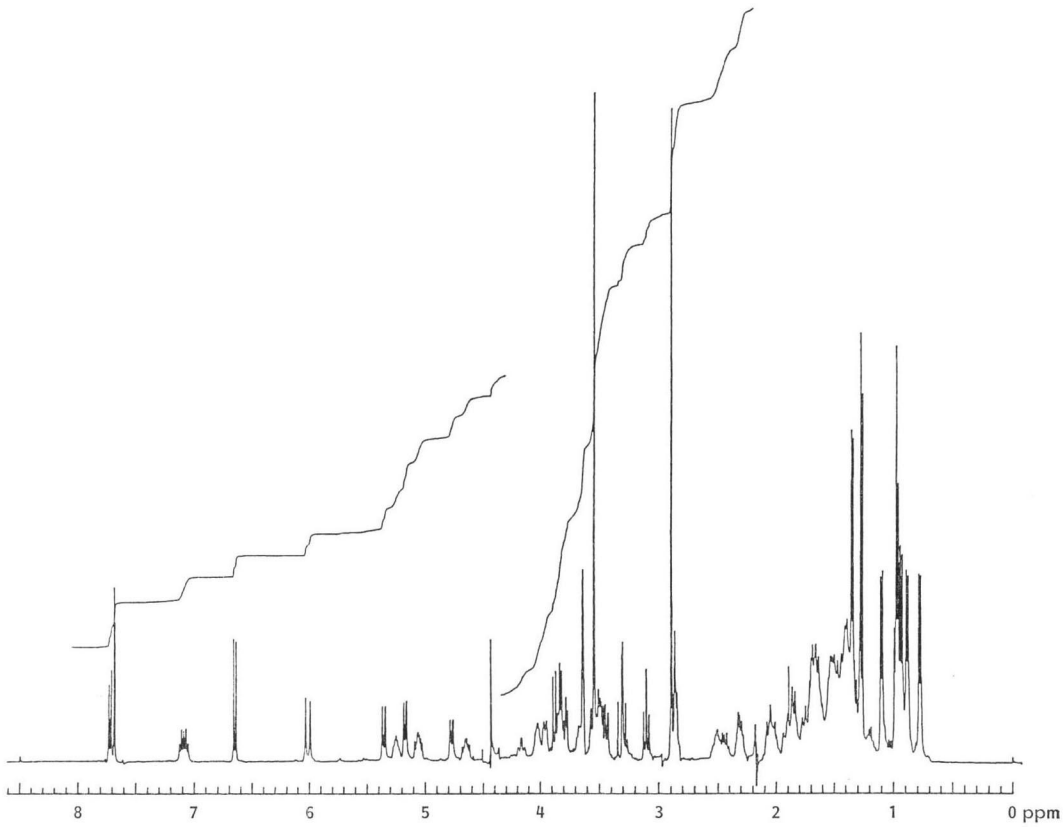


Fig. 7. ^1H NMR spectrum of notonesomycin A (400 MHz in Me_2CO-d_6 - D_2O, 4: 1).



trile - 0.01 M aqueous ammonium acetate (2: 3). Fractions containing notonesomycin A were collected and concentrated under reduced pressure. The concentrate was desalted by Toyopearl HW-40 column chromatography with methanol - water (7: 3). The combined active fraction was lyophilized to give an amorphous powder of notonesomycin A (150 mg).

Physico-chemical Properties

Physico-chemical properties of notonesomycin A are summarized in Table 4. Notonesomycin A was obtained as a colorless amorphous powder, easily soluble in dimethyl sulfoxide, but not easily soluble in water, methanol, chloroform and acetone. However, it was soluble in a mixture of meth-

Table 5. Antimicrobiol activity of notonesomycin A.

Test organisms	MIC (μg/ml)
Xanthomonas campestris pv. *oryzae*	>100
X. campestris pv. *citri*	>100
Pseudomonas syringae pv. *tabaci*	>100
P. syringae pv. *lachrymans*	>100
Erwinia carotovora subsp. *carotovora*	>100
Corynebacterium michiganense pv. *michiganense*	25
Pyricularia oryzae	25
Diaporthe citri	100
Colletotricum lagenarium	25
Alternaria kikuchiana	25
Gromerella cingulata	25
Botrytis cinerea	25
Fusarium oxysporum f. sp. *lycoperisici*	>100
Gibberella fujikuroi	100
Cochliobolus miyabeanus	25
Rhizoctonia solani	50

The MICs were determined on potato semisynthetic agar (*X. oryzae*) and potato - sucrose agar (the other organisms) by the agar dilution method.

Table 6. Effect of notonesomycin A on rice sheath blight in a green house test.

Concentration (μg/ml)	Protective value (%)	
	Validamycin	Notonesomycin A
100	100	100
50	96	95
25	64	80
12.5	49	58

anol - water (7: 3), acetone - water (4 : 1) or chloroform - methanol (1 : 1). The antibiotic was stable at alkaline conditions but very unstable at acidic conditions. The molecular formula was established to be $C_{68}H_{109}NO_{30}SNa_2$ based on elemental analysis, mass and ^{13}C NMR spectral analysis. The fast atom bombardment mass spectrum (FAB-MS, positive mode) of notonesomycin A showed a quasimolecular ion peak at *m/z* 1,498. This result is in agreement with the ion peak at *m/z* 1,452 $(M-2Na+H)^-$ in the negative FAB-MS. Elemental analysis showed that a sulfur atom is a constituent f notonesomycin A.

A fragment ion peak (*m/z* 1,395, M – SO_3Na) in FAB-MS and the strong abosrptions (1280 cm^{-1} and 1060 cm^{-1}) in the IR spectrum (Fig. 5) indicated the presence of a sulfate ester in notonesomycin A.

The ^{13}C NMR spectrum of notonesomycin A (Fig. 6) showed 68 carbon signals. They are classified to the following groups according to the chemical shift trends and INEPT experimental results; four ester carbonyl (δ_C 173.0, 171.0, 167.7 and 166.6), eight olefinic acrbons (147.9~108.8), one hemiketal carbon (98.2), two hemicacetal carbons (101.6, 97.4), 21 oxymethine carbons (84.7~60.1), five methine carbons (39.8~37.6), 17 methylene carbons (45.6~22.2), one methoxy carbon (60.8), one *N*-methyl carbon (29.9) and 8 *C*-methyl carbons (18.5~4.9). The 400 MHz ^1H NMR spectrum of notonesomycin A is shown in Fig. 7. Two olefinic proton signals [δ_H 6.02 (d, J=16 Nz), 7.08 (dt, J=7, 16 Hz)] indicate the presence of an α,β-unsaturated ester group. The aromatic protons [δ_H 7.72 (dd, J=8.3, 1.6 Hz), 7.68 (d, J=1.6 Hz) and 6.66 (d, J=8.3 Hz)] were assigned to a 1,2,4-tri-substituted benzene system. The isolated methylene protons [δ_H 3.30, s, δ_C 45.6 in DMSO-d_6] were gradually exchanged by deuterium upon addition of D_2O. The above result strongly suggests that malonate ester is a part of notonesomycin A.

Extensive ^1H and ^{13}C NMR analysis of notonesomycin A and chemical degradation revealed the gross structure of notonesomycin A as shown in Fig. 1. Details of the structure determination of notonesomycin A will be reported in due course.[10]

Biological Properties

The minimum inhibitory concentrations (MIC) of notonesomycin A against phytopathogenic bacteria and fungi were determined by the agar dilution method, and the results are given in Table 5. The antibiotic was as effective as validamycin[11] against the sheath blight of rice plant caused by *R.*

solani in a green house test (Table 6). The acute toxicity (LD_{50} in mice) of notonesomycin A was 0.25 to 0.50 mg/kg when administered intraperitoneally.

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