STUDIES ON VALIDAMYCINS, NEW ANTIBIOTICS. II

PRODUCTION AND BIOLOGICAL PROPERTIES OF VALIDAMYCINS A AND B

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The fermentation conditions for the production of validamycin by *Streptomyces hygroscopicus* var. *limoneus* No. T-7545 were studied. Validamycins A and B showed no antimicrobial activity on agar media against bacteria, yeasts and fungi, but caused an abnormal branching at the tips of the hyphae of *Pellicularia sasakii*. Although they were inactive *in vitro*, validamycins A and B controlled the sheath blight of rice plants and the damping off of cucumber seedlings in green house tests. Validamycin A was effective against the sheath blight at a concentration of 30 ppm. Both antibiotics showed low toxicity to plants, fishes and mice.

Validamycins A and B[1] are weakly basic, water soluble antibiotics produced by *Streptomyces hygroscopicus* var. *limoneus*[2]. Although they showed good activity against the sheath blight of rice plants and the damping off of cucumber seedlings caused by *Pellicularia sasakii* and *Rhizoctonia solani*, respectively, the growth of bacteria and fungi including *P. sasakii* and *P. solani* were not inhibited by validamycins A and B on agar media in ordinary *in vitro* tests. However, it was observed that under certain conditions the hyphae of *P. sasakii* and *R. solani* were subject to an abnormal branching at the tips and further development was repressed. Using this phenomenon, new assay methods[3] were devised: a dilution assay designated the "dendroid-test method", and a diffusion method called the "reversed layer method". Culture conditions for the production of validamycins and the biological properties of validamycins are reported in the present paper.

Materials and Methods

1. Strain: *S. hygroscopicus* var. *limoneus* No. T-7545 was incubated on glucose asparagine agar slant for 7 days at 27°C, and then held at 10°C until used.

2. Cultivation

Seed preparation: A loopful of the stock culture was added to 20 ml of the liquid medium in a 100 ml Erlenmeyer flask. This was incubated for 48 hours at 27°C on the rotary shaker (5.0 cm radius) at 220 rpm. The medium for the seed culture consisted of 3.0 % glucose, 2.2 % soy bean flour, 0.3 % peptone, and 0.4 % calcium carbonate added after adjustment to pH 7.

Production: Unless otherwise stated a 100 ml Erlenmeyer flask containing 20 ml of

medium was inoculated with 1 ml of the seed culture and incubated at 27°C for 5~7 days on the rotary shaker. Validamycin potency is expressed in terms of validamycin A standard.

3. Assay method: The details of the two assay methods are described in another paper[3].

With the dendroid-test method, the maximum dilution causing abnormal branching of hyphae of the test organism is measured. In the reversed layer method, the diameter of an inhibition zone is measured and the quantity of validamycin calculated from a standard curve.

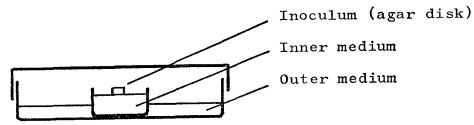
4. Investigation on the biological properties of validamycins

Antimicrobial activity *in vitro*: Antimicrobial activities of purified validamycins A and B on agar media were examined by an agar dilution method.

The preparation of plant juice: Ten grams of 3-week old rice plants without roots were cut, homogenized in 100 ml of water, and filtered. The plant juice of broad beans was prepared in the same manner.

Effect on the development of the hyphae of *P. sasakii*: A modified dish technique[4] was used. At the center of a 9-cm Petri dish, a small dish, 1.6 cm diameter and 1 cm height, was placed, and into which 2 ml of a certain agar medium was poured. Twenty ml of a certain agar medium with or without crude validamycin (2,000 units/mg) at a concentration of 100 μg/ml was poured into the outer part (Fig. 1). *P. sasakii* was inoculated at the center of the inner medium. The appearance of the hyphae grown on the outer media was observed after incubation at 27°C.

Fig. 1. Modified dish technique.



Green house test: Rice plants were planted in a 1/5,000-are WAGNER pot, 3 stocks per pot. Three pots per group, in the ear-bearing stage, were used for the test. Thirty milliliters of a sample solution containing 0.05 % Dyne* was sprayed evenly over the foliage with a spray-gun. An agar disk, 15 mm in diameter, of *P. sasakii* cut from a culture on potato sucrose agar incubated at 30°C for 48 hours was inserted into the sheath near the soil immediately after spraying with antibiotic, and inoculated pots were kept at 25~32°C in 70~100 % relative humidity. The lengths of lesions were measured at 7, 14 and 21 days after application of drugs, and the expanding rate** was calculated.

Toxicity:

Toxicity to killifish: Ten killifishes were tested in 1 liter of water containing a fixed concentration of validamycin A. After 48 hours the LD_{50} was calculated.

Acute toxicity: Four week-old IRC-JCL/T male mice and 5 week-old SD/Ta female rats were treated with validamycins A and B intravenously, subcutaneously or orally. After 7-day observation the LD_{50} was calculated.

Results and Discussion

I. Production of Validamycin

1. Temperature of Submerged Culture

As the optimum temperature for the growth of the strain No. T-7545 was found to be fairly high, it was cultivated at 37°C. Since the fermented broth thus obtained was turbid and filtration was very difficult, fermentation at 27°C was compared with that at 37°C. As shown in Table 1, the validamycin titer of the filtered broth

* A spreader manufactured by Takeda Chemical Industries, Ltd. containing 20 % polyoxyethylene-alkylaryl ether and 12 % calcium lignin sulfonate.

** Expanding rate $= \frac{\text{average length of lesion per stem treated with drugs}}{\text{average length of lesion per stem untreated}} \times 100\ (\%)$

obtained in 37°C incubation was a little higher than that at 27°C, but filtration was more difficult, and essentially equivalent results were obtained in a green house test. So the lower temperature was used to prepare the antibiotic.

2. Relationship between Volume of Medium and Production of Validamycin

Erlenmeyer flasks of 200 ml capacity containing 20 ml or 60 ml of the fermentation medium* were compared. As shown in Table 2, 60 ml of the medium gave superior production.

3. Investigations on the Composition of Production Medium

Table 1. Effect of temperature on validamycin production

Incubation period (days)	27°C		37°C	
	Validamycin titer* (unit/ml)	Broth**	Validamycin titer (unit/ml)	Broth
4	20,000	Clear, easy filtration	35,000	Turbid, difficult filtration
6	20,000	do	35,000	do

* Assayed by "dendroid-test method".
** The medium used in this experiment consists of 5.0 % of glucose, 3.6 % of soy bean flour, 0.5 % of peptone and 0.6 % of $CaCO_3$, pH adjusted to 7. Sixty ml of the medium in each Erlenmeyer flask of 200 ml capacity was inoculated and cultivated on the rotary shaker (5.0 cm radius) at 220 rpm.

Table 2. Effect of the volume of the medium on validamycin production

Incubation period (hrs)	Volume of the medium**			
	20 ml		60 ml	
	pH	Validamycin titer* (unit/ml)	pH	Validamycin titer (unit/ml)
66	8.0	10,000	7.4	15,000
90	8.0	15,000	7.8	20,000
114	8.2	20,000	8.0	50,000
138	8.6	20,000	8.2	50,000

* Assayed by "dendroid-test method".
** Composition of the medium used in this experiment is the same as in Table 1.

(1) Selection of carbon and nitrogen sources

Table 3. Production of validamycin by *S. hygroscopicus* var. *limoneus* No. T-7545 and its growth on various media

		Medium											
		1	2	3	4	5	6	7	8	9	10	11	12
Component (%)	Glucose	5			5			5			5		
	Glycerol		5			5			5			5	
	Starch			5			5			5			5
	Peptone	1	1	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Beef extract	0.5	0.5	0.5									
	C. S. L.				3	3	3	1	1	1	1	1	1
	S. B. F.	0.5	0.5	0.5	1	1	1				3	3	3
	C. G. M.							3	3	3			
	NaCl	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	$CaCO_3$	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Growth**		⫫	⫫	+～⫫	⫫	⫫	⫫～⫫	⫫	⫫	⫫	⫫	⫫	+～⫫
pH		7.5	7.25	8.0	8.2	7.3	8.3	7.75	7.15	8.0	7.55	8.1	8.6
Validamycin titer* (μg/ml)		64	12.5	12.5	140	12.5	74	103	12.5	38	64	28	12.5

* Five-day culture was assayed by "reversed layer method".
** ⫫ : Very good growth ⫩ : Good growth + : Fair growth

* The medium employed in this experiment had the following composition: 5.0 % glucose, 3.6 % soy bean flour, 0.5 % peptone, 0.6 % $CaCO_3$ (pH 7).

A variety of media containing glucose, glycerol or starch as a carbon source and various nitrogen sources such as peptone, beef extract, corn steep liquor (C.S.L.), soy bean flour (S.B.F.) and corn gluten meal (C.G.M.) were compared by the reversed layer method. The results are shown in Table 3. Good growth occurred in the media containing glucose or glycerol as the carbon source regardless of nitrogen sources.

As for the yield of validamycin, glucose was notably superior to other carbon sources, and when it was used, no remarkable differences were found among the nitrogen sources. As a result of repeated examinations C.S.L. and C.G.M. were judged relatively better than S.B.F. and beef extract.

When starch was adopted as the carbon source, considerable validamycin production was obtained, although slowly, in the medium containing C.S.L. and C.G.M. as the nitrogen sources.

(2) Effects of inorganic salts

Effects of $FeSO_4$, $MnSO_4$, $ZnSO_4$, NaCl, KCl and NH_4Cl on the production of validamycin were investigated in the following two kinds of media; (a) 5.0 % glucose, 3.0 % S.B.F., 1.0 % C.S.L., 0.5 % $CaCO_3$, (b) 4.0 % glucose, 2.0 % corn starch, 2.0 % C.S.L., 3.0 % C.G.M., 1.5 % $CaCO_3$. Little effect of these inorganic salts was found in the first medium, but in the latter medium remarkable effects of NaCl and NH_4Cl were found as shown in Table 4.

Table 4. Effects of NH_4Cl and NaCl on the production of validamycin

Salts added (%)	Growth	pH	Validamycin titer* (μg/ml)
NH_4Cl 0.5	+++	7.8	345
NaCl 1.5	+++	7.45	300
NH_4Cl 0.5 + NaCl 1.5	+++	7.1	380
No addition	+++	8.4	75

* Seven-day culture was assayed by "reversed layer method".

(3) Selection of medium for high yield of validamycin

From these and other results, a medium composed of 2 % glucose, 4 % corn starch, 2 % C.S.L., 4 % C.G.M., 0.5 % NH_4Cl, 1.5 % NaCl and 1.5 % $CaCO_3$ was selected, yielding about 620 μg/ml of validamycin.

II. Biological Properties of Validamycin

1. Effect on the Growth of Bacteria and Fungi

(1) Antimicrobial activity on agar plates

Validamycins A and B, at 10,000 μg/ml, did not inhibit the growths of bacteria and fungi including *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, *Escherichia coli*, *Proteus vulgaris*, *Xanthomonas oryzae*, *Mycobacterium avium*, *Mycobacterium* ATCC 607, *Pellicularia sasakii*, *Rhizoctonia solani*, *Pyricularia oryzae*, *Colletotrichum lagenarium*, *Alternaria kikuchiana*, *Aspergillus niger*, *Penicillium chrysogenum*, *Saccharomyces cerevisiae*, *Candida albicans* and *Trichophyton mentagrophytes*. In addition, 2,177 strains of fungi and 762 strains of bacteria were not inhibited by the antibiotic under various assay conditions.

(2) Effects of other antibiotic substances and of plant juice on the activity of validamycin

No interactions[5] between validamycin A and griseofulvin, blasticidin S, aristeromycin, polyoxin, kasugamycin, mikamycin, dihydrostreptomycin, chromomycin A_3,

streptonigrin, lateriomycin, and 6-mercaptopurine at concentrations of 100~2,000 μg/ml was found. No interactions was found between validamycin A and triphenyl tetrazolium chloride, fuchsin, methylene blue, methyl orange, methyl red, litmus, bromocresol green, aniline blue, rose bengal and gentian violet at concentrations of 0.02~0.04 %.

The inhibitory effect of validamycin A against the growth of *P. sasakii* was examined on agar media containing the plant juice of broad beans or rice plants. The growth of the fungus was found to be somewhat repressed, but not notably so.

2. Effect on the Development of the Hyphae of *P. sasakii*

Despite the lack of inhibitory effect against *P. sasakii* on agar media in ordinary assay methods, validamycin inhibited remarkably the extension of the disease, and development of the hyphae on the rice plants seemed to be thoroughly inhibited. In view of similar results reported by ISHIZAKI[6] on methyl arsonate, the *in vitro* activity of validamycin against *P. sasakii* was investigated by a modified dish technique. Modified PFEFFER's agar was poured into the inner part and various media with or without validamycin were used in the outer one. An agar disk of *P. sasakii* was placed in the center of the inner part, incubated at 27°C, and after 24, 40 and 64 hours incubation, the diameter of the mycelium which developed on the medium in the outer part was measured.

As shown in Table 5 when the medium in the outer part was water agar, the mycelium of *P. sasakii* on validamycin containing medium developed to some extent but an abnormal branching occurred at the tips of the hyphae, and further development was inhibited. Microscopic observation of this part revealed that branching at nearly right angle to main axis occurs at the tips of the hyphae and something like a fine drop of water was also observed among the branching hyphae (Fig. 2). This phenomenon was also observed, though weakly, when the medium in the outer part was modified PFEFFER's agar without

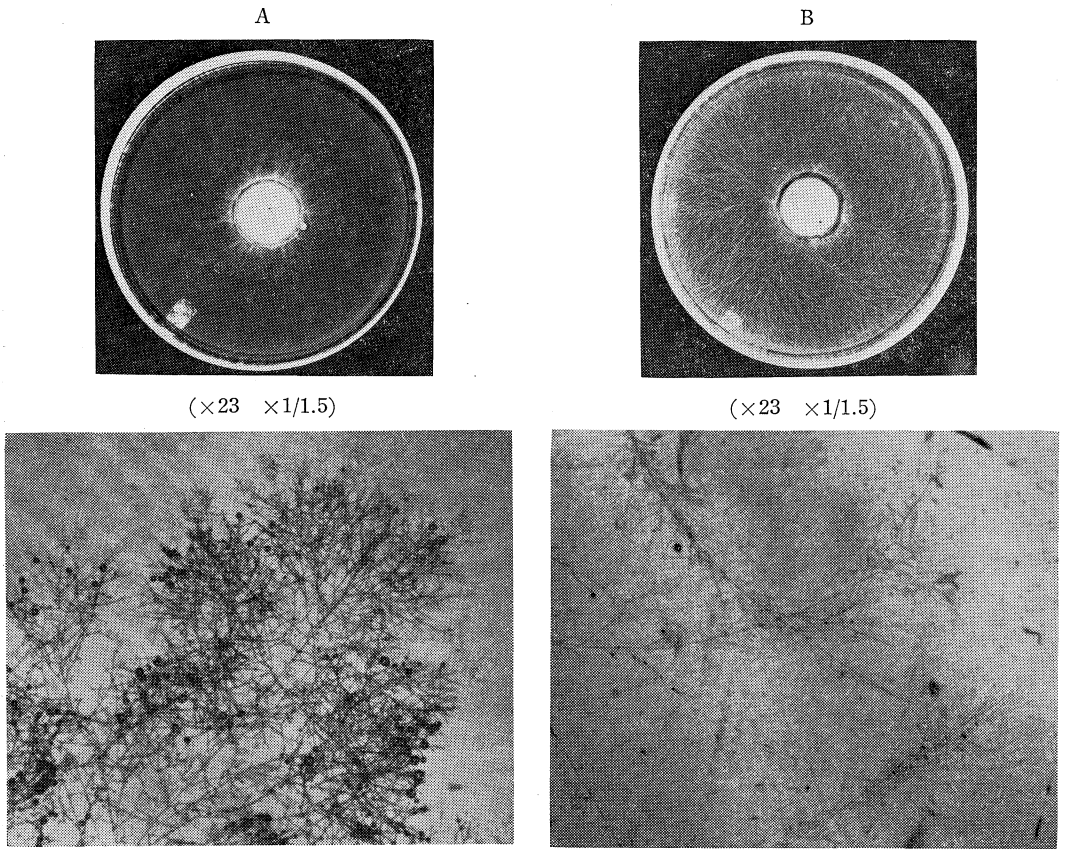
Table 5. Effect of validamycin (VM) on the growth of *Pellicularia sasakii* in modified dish technique

Outer medium		Diameter of mycelium (mm)		
		24 hrs.	40 hrs.	64 hrs.
Modified PFEFFER's agar	VM added	57	76	90
	no addition	78	90	90
Modified PFEFFER's agar without carbon source	VM added	51	61	66
	no addition	73	90	90
Modified PFEFFER's agar without nitrogen source	VM added	40	44	52
	no addition	80	90	90
Water agar	VM added	32	35	36
	no addition	70	90	90

Table 6. Effects of validamycins A and B on some phytopathogenic fungi

Test organisms	Minimum concentration causing abnormal branching (μg/ml)	
	Validamycin A	Validamycin B
Pellicularia sasakii	0.01	0.5
Rhizoctonia solani	0.02	50
Pellicularia praticola	5	—
Corticium rolfsii	>100	—
Stereum fasciatum	>100	—
Glomerella cingulata	>100	>100
Alternaria kikuchiana	>100	>100
Fusarium oxysporum f. *niveum*	>100	>100
Sclerotinia scrlotiorum	>100	>100
Botrytis cinerea	>100	—
Phytophthora infestans	>100	—

Fig. 2. Hyphae of *P. sasakii* in modified dish technique.
A: Validamycin in the outer part (0.2 μg/ml).
B: No validamycin.



nitrogen source, but not observed in case of modified PFEFFER's agar or that without carbon source.

With nutrient-deficient medium in the inner part and nutrient-rich medium containing validamycin in the outer part, abnormal branching did not occur. When the medium containing plant constituents such as rice straw infusion agar was used, the phenomenon was observed only with nutrient-deficient medium with validamycin in the outer part. Thus, when *P. sasakii* growing from nutrient-rich medium develops onto nutritionally very poor medium containing validamycin, the hyphae on the latter undergo abnormal branching at the tips, and subsequent growth is inhibited.

The occurrence of this phenomenon in other phytopathogenic fungi was examined by the modified dish technique. The minimum concentration causing abnormal branching is shown in Table 6. *P. sasakii*, *Rhizoctonia solani* and *Pellicularia praticola* were sensitive to validamycin. This sensitivity to validamycin seems to be limited to *P. sasakii* and related species.

3. Activity on Sheath Blight of Rice Plants in Green House Tests

As shown in Table 7, validamycin A at a concentration of 30 ppm was as effective as a commercial organoarsenical (Monkit dust), with no injury to the rice plant.

Table 7. Effects of validamycins A and B against the sheath blight of rice plants in green house test

	Concentration (ppm)	Expanding rate* (%)			
		7 days	14 days	21 days	28 days
Untreated		100	100	100	100
Validamycin A	15	4	5	47	76
	30	0	0	0.1	0.2
	60	0	0	0	0
Validamycin B	40	27	63	115	109
	80	3	4	65	105
	160	3	1	33	72
Monkit dust**		9	4	0.1	0.3

* Expanding rate $= \frac{\text{Average length of lesion per stem treated with drugs}}{\text{Average length of lesion per stem untreated}} \times 100$

** Containing 0.4% of iron ammonium-methane arsonic acid.

4. Toxicity to Fish

The LD_{50} for the killifish measured after 48 hours was found to be greater than 1,000 μg/ml.

5. Acute Toxicity

In oral administration of validamycins A and B at the dose of 10 g/kg to mice and rats, or in subcutaneous and intravenous administration at the dose of 2 g/kg to mice, all animals examined survived without any change for 7 days. Also no irritative effects on the skin and on the cornea in the rabbit were observed.

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References

1) IWASA, T.; Y. KAMEDA, M. ASAI, S. HORII & K. MIZUNO: Studies on validamycins, new antibiotics. IV. Isolation and properties of validamycins A and B. J. Antibiotics 24 : 119~123, 1971
2) IWASA, T.; H. YAMAMOTO & M. SHIBATA: Studies on validamycins, new antibiotics. I. *Streptomyces hygroscopicus* var. *limoneus* nov. var., validamycin-producing organism. J. Antibiotics 23 : 595~602, 1970
3) IWASA, T.; E. HIGASHIDE & M. SHIBATA: Studies on validamycins, new antibiotics. III. Bioassay methods for the determination of validamycin. J. Antibiotics 24 : 114~118, 1971
4) SCHÜTTE, K. H.: Translocation in the fungi. New Phytologist 55 : 164~182, 1955
5) MACCACARO, G. A.: The assessment of the interaction between antibacterial drugs. Progr. Indust. Microbiol. 3 : 175~210, 1961
6) ISHIZAKI, H.: On a mechanism of action of organo-arsenic (As^{5+}) compound. Ann. Phytopath. Soc. Japan 32 : 102, 1966