

EFFECT OF VALIDAMYCINS ON GLYCOHYDROLASES
OF *RHIZOCTONIA SOLANI*

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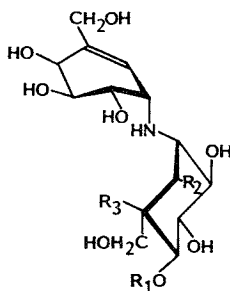
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The pseudo-oligosaccharides, validamycins, showed potent inhibitory activity against trehalase of *Rhizoctonia solani* while no significant inhibition was exhibited against cellulase, pectinase, chitinase, α -amylase, α - and β -glucosidases. In particular, validoxyamine A strongly inhibited trehalase in a competitive manner with a K_i value of 1.9×10^{-9} M. The uptake of the antibiotic into the cell and the amount of the intracellular trehalose were investigated by incubating the washed mycelia of *R. solani* with validamycins. It was found that validamycin A is transported into the cell and hydrolyzed therein by a β -glucosidase yielding validoxyamine A with greater inhibitory activity. Also validamycin A containing β -D-glucosyl residue is more favorably taken up into the cell than validamycin D containing α -D-glucosyl residue or their common aglycone, validoxyamine A. In addition, validamycin A suppressed the *in vivo* degradation of the intracellular trehalose at very low concentration of 0.1 μ g/ml.

The validamycin complex is produced by *Streptomyces hygroscopicus* subsp. *limoneus* and consists of validamycins A, B, C, D, E, F, G and validoxyamines A, B and G¹⁻³⁾. Validamycins A, C, D, E and F contain validoxyamine A as a common moiety, but differ from one another in the number, the site and/or the type of glucosidic attachment to validoxyamine A. Validamycins B and G contain validoxyamine B or validoxyamine G, respectively. Validamycin A is the major component and the most active one against sheath blight of rice plants caused by *Rhizoctonia solani*. Validamycin

Fig. 1. Structures of validamycins.



Validoxyamine A (VA-A)	$R_1 = R_2 = R_3 = H$
Validoxyamine B (VA-B)	$R_1 = R_3 = H$ $R_2 = OH$
Validoxyamine G (VA-G)	$R_1 = R_2 = H$ $R_3 = OH$
Validamycin A (VM-A)	$R_1 = \beta$ -D-Glucopyranosyl $R_2 = R_3 = H$
Validamycin B	β -D-Glucopyranosyl-VA-B
Validamycins C, E, F	α -D-Glucopyranosyl-VM-A
Validamycin D	α -D-Glucopyranosyl-VA-A
Validamycin G	$R_1 = \beta$ -D-Glucopyranosyl $R_2 = H$ $R_3 = OH$

A is regarded as a pseudo-trisaccharide which consists of two pseudo-aminosugars, valienamine and validamine, and D-glucopyranose.

In recent years, pseudo-oligosaccharides inhibiting α -glucosidase or α -amylase have been isolated from cultures of microorganisms⁴⁻⁷). They contain valienamine moiety in a core structure essential for inhibitory activity. We previously reported that the pseudo-aminosugars, which are microbial degradation products⁸) and the minor components from fermentation broth of *S. hygrosopicus* subsp. *limoneus*^{9,10}), exhibit inhibitory activity against α -glucosidases. The present paper describes the effect of validamycins on glycohydrolases of *R. solani* and the manner in which the antibiotic is taken up by cells of pathogenic fungi.

Materials and Methods

Organism and Culture Condition

A strain of *Rhizoctonia solani* TKF 44 (Culture type IA), maintained on potato - glucose agar, was inoculated into potato - sucrose liquid medium and incubated for 3 days at 27°C on a reciprocating shaker.

Preparation of Mycelial Suspension

After incubation, mycelia were harvested by filtration and washed with CZAPEK's salt solution (containing no carbon), pH 5.0. The resulting mycelial mat was removed and homogenized in a Waring Blender for 30 seconds with CZAPEK's salt solution. The homogenized mycelia were filtered and washed with the same solution. The washed mycelia, 20 g in wet weight, were suspended in 1 liter of CZAPEK's salt solution (pH 5.0).

Preparation of Cellulase, Pectinase and Chitinase

To induce enzyme production, either 0.5% carboxymethyl cellulose (Brown Company) for cellulase, 0.5% pectic acid (Nakarai Chemicals, Ltd.) for pectinase, or 1% chitin (Seikagaku Kogyo Co.) for chitinase was added to mycelial suspension. For suppression of bacteria, 10 μ g/ml of dibekacin (Meiji Seika Kaisha, Ltd.) was added to the medium. The mycelial suspension was incubated for 3~5 days at 27°C on a reciprocating shaker and centrifuged at 20,000 \times g for 10 minutes. The resulting supernatant was used as the enzyme solution.

Preparation of α - and β -Glucosidases, α -Amylase and Trehalase

The washed mycelia (120 g in wet weight) were suspended in 400 ml of 10 mM phosphate buffer (pH 6.0) and homogenized in a Waring Blender for 2 minutes. This suspension was further disrupted for 20 minutes with a sonic oscillator (TOMY Model UR 2000p) under cooling and then centrifuged at 3,000 \times g for 20 minutes. Solid ammonium sulfate was added to the supernatant to 40% saturation. The resulting precipitate was removed by centrifugation at 16,000 \times g for 20 minutes and the supernatant solution was brought to 60% saturation by further addition of ammonium sulfate. The resulting precipitate was collected by centrifugation at 16,000 \times g for 20 minutes and dissolved in a small volume of 1 mM phosphate buffer (pH 6.0). This solution was then dialyzed against 1 mM phosphate buffer (pH 6.0) at 4°C overnight. The dialyzed solution served as the enzyme source of α - and β -glucosidases, and α -amylase. The pellet was suspended in distilled water and homogenized by means of a motor driven teflon pestle. This suspension was centrifuged at 3,000 \times g for 20 minutes, and the precipitate was dialyzed against water at 4°C overnight and served as the source of trehalase.

Assay of Enzymes and Enzyme Inhibitory Activity

(a) Cellulase, pectinase and chitinase activities were measured by following the decrease in viscosity of the reaction mixture with an Ostwald viscometer at 30°C according to the method of LISKER *et al.*¹¹⁻¹³). The reaction mixture contained 2 ml of enzyme solution, 4 ml of substrate (2.5% sodium carboxymethyl cellulose (Wako Pure Chemical Ind.) for cellulase, 3.0% sodium polypectate (Sigma Chemical Company) for pectinase or 0.5% ethylene glycol chitin (Seikagaku Kogyo Co.)

for chitinase in 50 mM citrate buffer at pH 5.0), and 4 ml of inhibitor solution in 50 mM citrate buffer or 4 ml of the same buffer.

(b) The assay of α - and β -glucosidases and trehalase was based on the colorimetric determination of D-glucose released from the substrate. The reaction mixture consisted of 125 μ l of enzyme solution, 50 μ l of 0.4 M substrate (maltose for α -glucosidase, cellobiose for β -glucosidase or trehalose for trehalase), 200 μ l of inhibitor solution or distilled water, and 125 μ l of 0.2 M phosphate buffer (pH 6.0). After incubation for 15 minutes at 37°C, the released D-glucose was measured by the glucose oxidase method¹⁴.

(c) α -Amylase was assayed by measuring the amount of reducing sugar released from the substrate with dinitrosalicylic acid reagent¹⁵. The reaction mixture consisted of 200 μ l of enzyme solution, 50 μ l of 1% soluble starch, 50 μ l of 0.2 M phosphate buffer (pH 6.9), and 200 μ l of inhibitor solution or distilled water. After incubation for 5 minutes at 37°C, 200 μ l of this reaction mixture and 200 μ l of the reagent were heated for 5 minutes in boiling water and then cooled. After addition of 2 ml of distilled water, the optical density (540 nm) of this solution was determined photometrically.

Extraction and Measurement of Validamycins and Trehalose in Mycelia

Mycelial suspension with or without the antibiotic was incubated at 27°C with shaking. The incubation mixture was filtered through a glass filter, washed with water, and dried with acetone and ether. Validamycins and trehalose were extracted from dried mycelia with 10% TCA at room temp with stirring for 24 hours. The precipitate was removed by centrifugation, and the supernatant was extracted with ethyl ether to remove TCA. This aqueous solution was designated as the "TCA soluble fraction". This fraction was deionized with Amberlite MB-3 resin and used for the determination of trehalose. On the other hand, the TCA soluble fraction was applied to a short column of Dowex 50W-X8 (H⁺ form), and eluted with 0.5 N aq ammonia. The eluate concentrate was desalted by a short column of Dowex 1-X2 (OH⁻ form) and used for the determination of validamycins. Trehalose and validamycins were determined by gas-liquid chromatography².

Results

As shown in Table 1, some validamycins showed potent inhibitory activity against trehalase of *R. solani*, whereas no significant activity was exhibited against cellulase, pectinase, chitinase, α -amylase,

Table 1. Effects of validamycins against trehalase of *Rhizoctonia solani* and on *R. solani* in "dendroid-test method".

Compound	Trehalase inhibitory activity; IC ₅₀ (M) ^a	Dendroid-test method (μ g/ml) ^b
Validamycin A	7.2×10^{-5}	0.01
Validamycin B	3.5×10^{-5}	0.50
Validamycin C	—	10
Validamycin D	1.5×10^{-5}	25
Validamycin E	—	0.01
Validamycin F	—	0.01
Validamycin G	5.2×10^{-6}	0.50
Validoxylamine A	1.4×10^{-7}	1.00
Validoxylamine B	1.6×10^{-5}	50
Validoxylamine G	7.4×10^{-6}	2.50

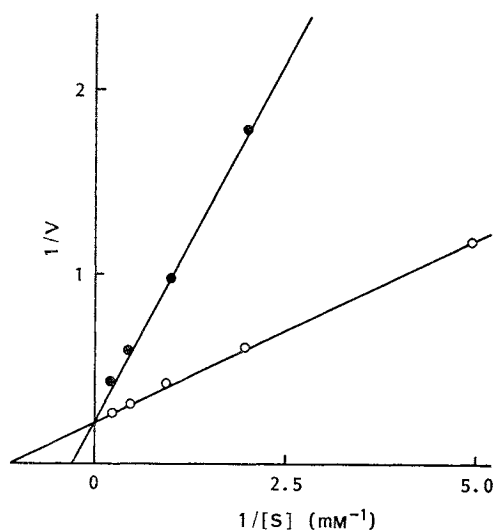
^a Molar concentration required to give 50% inhibition.

^b Minimum concentration causing abnormal branching.

—: No inhibition ($>10^{-2}$ M).

Fig. 2. Lineweaver-Burk plots for the inhibition on trehalase of *Rhizoctonia solani* by validoxylamine A.

● $K_i = 1.9 \times 10^{-9}$ M, ○ $K_m = 9.1 \times 10^{-4}$ M.



α - and β -glucosidases. Validoxylamine A exhibited the greatest inhibitory activity against trehalase. Validoxylamine A inhibited trehalase in a competitive manner with a K_i value of 1.9×10^{-9} M (Fig. 2). The K_i value was about 10^{-6} times smaller than the K_m value (9.1×10^{-4} M) for trehalose. The attachment of the D-glucosyl residue to validoxylamine A diminished the inhibitory activity against trehalase. The pseudo-tetrasaccharides, such as validamycins C, E and F, had no inhibitory effect on trehalase.

Fig. 3 records the time-course of validamycin A uptake into the mycelia. Interestingly, the formation of validoxylamine A as a hydrolysis product was shown. Only validoxylamine A was detected until the initial 2 hours and since then large amount of validamycin A was accumulated in the mycelia. It seems likely that the uptake of validamycin A becomes faster as the time proceeds and the rate becomes much greater than that of hydrolysis of validamycin A.

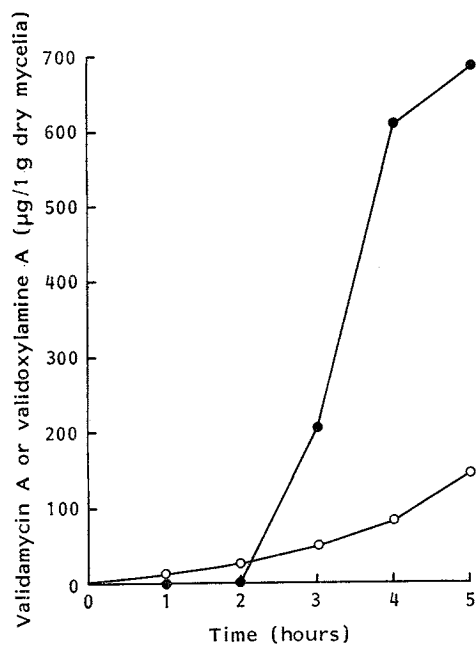
The difference of activity among the validamycins was expected to be responsible for the membrane transport of the antibiotic. Therefore, the uptake of validoxylamine A, validamycin A (β -D-glucopyranosylvalidoxylamine A) and validamycin D (α -D-glucopyranosylvalidoxylamine A) was examined. Based on the fact that within 2 hours only validoxylamine A was detected in the mycelia and validamycin D was also hydrolyzed to validoxylamine A by the intracellular α -glucosidase of *R. solani* (data not shown), the amount of validoxylamine A was determined on the basis of trehalase inhibition. The enzyme was prepared from larvae of moth, *Spodoptera litura*, according to the method of KALF and RIEDER¹⁰. A linear relationship was observed between the logarithm of validoxylamine

A concentration (0.001 ~ 0.1 μ g/ml) and the inhibition rate (Fig. 4).

Fig. 3. Time course of validamycin A uptake into mycelia.

Mycelial suspension with validamycin A (100 μ g/ml) was incubated at 27°C with shaking.

● Validamycin A, ○ validoxylamine A (hydrolysis product).



A concentration (0.001 ~ 0.1 μ g/ml) and the inhibition rate (Fig. 4). The determination of the uptake of these compounds into the mycelia is summarized in Fig. 5. It was found that validamycin A was more readily taken up into the cell than its aglycone, validoxylamine A. As to validamycin D, only trace amounts of validoxylamine A were detected from the mycelium within 30 minutes.

In order to investigate whether validamycin A inhibits a trehalase of *R. solani in vivo*, the

Fig. 4. The relationship between the concentration of validoxylamine A and the inhibition rate against insect trehalase.

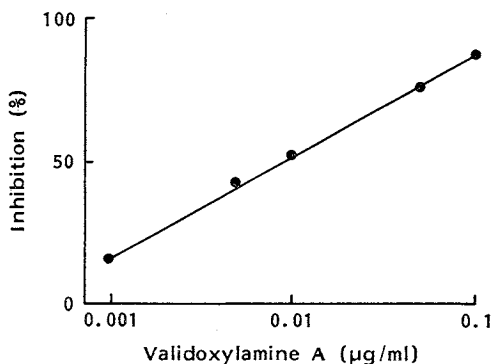


Fig. 5. Time course of validamycins uptake into mycelia.

Mycelia suspension with validamycins (each 100 $\mu\text{g/ml}$) was incubated at 27°C with shaking.

Δ Validamycin A, \circ validamycin D, \bullet validoxylamine A.

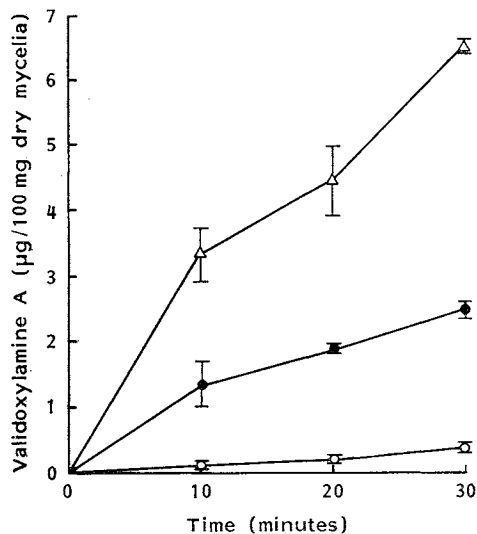
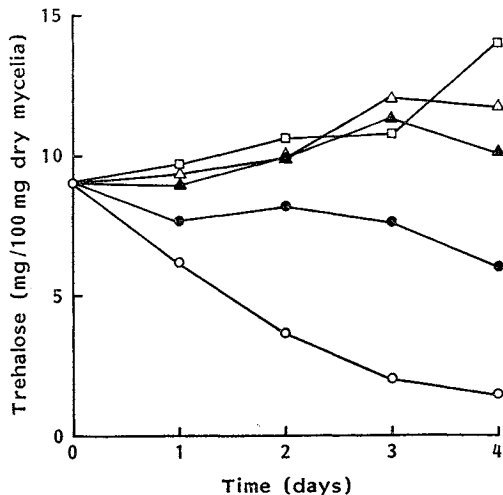


Fig. 6. Suppression of degradation of intracellular trehalose by validamycin A.

Validamycin A: \square 100 $\mu\text{g/ml}$, Δ 10 $\mu\text{g/ml}$, \blacktriangle 1 $\mu\text{g/ml}$, \bullet 0.1 $\mu\text{g/ml}$, \circ no addition.



amount of intracellular trehalose was measured when the mycelial suspension was incubated with validamycin A. Validamycin A significantly suppressed the degradation of intracellular trehalose at the concentration exceeding 0.1 $\mu\text{g/ml}$ (Fig. 6).

Discussion

Validamycin A does not significantly suppress the growth of *R. solani* on a nutritionally rich medium, but specifically it causes an abnormal branching of hyphae and the cessation of colony development on a water-agar¹³. NIOH and MIZUSHIMA¹⁷) concluded that validamycin does not inhibit the growth of fungal mass, causes no change in the amount of protein, nucleic acid, and cell wall components, but alters the morphology of the fungus. Validamycin significantly inhibits the extension of the main hyphae but not of the primary and secondary branches^{17,18}). WAKAE and MATSUURA¹⁹) suggested that *meso*-inositol antagonizes the inhibitory effect of validamycin A on *R. solani* because of its structural similarity to one or more component moieties of the antibiotic. However, TRINCI²⁰) reported that the inhibitory effect of validamycin A on the radial growth rate of colonies of *Rhizoctonia cerealis* was not antagonized by 400 μM *meso*-inositol. Recently, UEDA *et al.*²¹) reported that the production of laminarinase and glucan synthetase in *R. solani* was apparently affected by validamycin, whereas validamycin had no effect on the production of glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, glucose-phosphate isomerase, glucosyltransferase or glucoamylase.

In the present paper, we investigated the inhibitory effect of validamycins on glycohydrolases of *R. solani*. As shown in Table 1, it was seen that some validamycins show potent inhibitory activity against trehalase of *R. solani*. In particular, validoxylamine A powerfully inhibited trehalase in a competitive manner with K_i value of 1.9×10^{-9} M. It was also found that validamycin A is efficiently transported into the mycelia and hydrolyzed therein by a β -glucosidase yielding validoxylamine A with greater inhibitory activity. Furthermore, addition of validamycin A suppressed degradation of intracellular trehalose at very low concentration of 0.1 $\mu\text{g/ml}$. It was observed that *R. solani* contains a very high concentration of trehalose, about 10% of the dry cell. Trehalose is well-known as a storage carbohydrate and trehalase plays the essential role in the transport of D-glucose in insects

and fungi²²⁻²⁴). *R. solani* is one of the most typical examples of a fungus that grows rapidly by transporting nutrients from the basal part to the tip part through long stretches of hyphae²⁵). NIJH and MIZUSHIMA¹⁷) suggest that the supply of nutrient to the tip of hyphae is related to the action of validamycin. This idea is supported by the fact that the effect of validamycin was significantly less noticeable on a nutritionally rich medium where nutrient is always supplied at the elongation tip^{17,20}). Validamycin A is more readily taken up into the cell than validoxylamine A and validamycin D (Fig. 5). Namely, β -D-glucoside (validamycin A) is the more favorable form for uptake than the aglycone or the α -D-glucoside (validamycin D).

Pseudo-tetrasaccharides, validamycins E and F, which exhibit no inhibitory activity against trehalase but are as active in "dendroid-test method"²⁶) as validamycin A (Table 1), may be regarded as inactive prodrugs that are converted to active form by cellular enzymes. However, to explain the difference of biological activity among validamycins, further investigation of the relationship between structure and the permeability of validamycins will be required.

Acknowledgments

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