ALL EIGHT POSSIBLE MONO-β-D-GLUCOSIDES OF VALIDOXYLAMINE A

II. BIOLOGICAL ACTIVITIES

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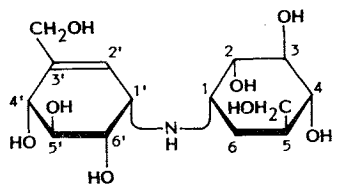
The biological activities of all eight possible mono-β-D-glucosides of validoxylamine A against *Rhizoctonia solani* were studied. The attachment of the D-glucosyl residue to validoxylamine A generally diminished the inhibitory activity against trehalase. The introduction of the D-glucosyl residue at the C-3 position did not cause serious loss in activity, while substitution at the C-6′ position caused complete loss in trehalase inhibitory activity. Of the eight β-D-glucosides, 4-*O*-β-D-glucopyranosylvalidoxylamine A (4-*O*-β-Glc-VA), 3-*O*-β-Glc-VA and 5′-*O*-β-Glc-VA exhibited very strong activity against *R. solani* in the "dendroid-test method". The antagonistic activity of sugars (1 mM) against validoxylamine A and 4-*O*-β-Glc-VA was examined using the "dendroid-test method". The inhibitory effect of validoxylamine A on hyphal extension was not antagonized by any sugars tested, whereas that of 4-*O*-β-Glc-VA was antagonized by β-1,3- and β-1,4-glucooligosaccharides. Of 2-*O*-, 3-*O*-, 4-*O*- and 7-*O*-β-Glc-VAs, 7-*O*-β-Glc-VA exhibiting the lowest activity was not antagonized by any β-glucooligosaccharides tested. The inhibitory effect of 3-*O*- and 4-*O*-β-Glc-VAs was antagonized by most β-glucooligosaccharides. The uptake of 4-*O*-β-Glc-VA into the mycelia was inhibited by laminaribiose and cellobiose but not by maltose.

Validamycin A (4-*O*-β-D-glucopyranosylvalidoxylamine A; 4-*O*-β-Glc-VA) has been used to control some diseases caused by *Rhizoctonia solani*, particularly sheath blight of rice plants. It is known that validamycin A decreases the maximum rate of hyphal extension and increases hyphal branching without affecting the organism's specific growth rate[1~4]. WAKAE and MATSUURA reported that *meso*-inositol antagonized the effect of validamycin A on the pathogenicity of *R. solani in vivo* because of its structural similarity to one or more component moieties of the antibiotic[5]. However, addition of inositol to culture medium failed to reverse the effect of the antibiotic on the morphology of *R. solani* or *Rhizoctonia cerealis*[3,6]. The inhibitory effect of validamycin A (0.2 μM) on *R. cerealis* was antagonized by 20 mM glucose[3]. TRINCI suggested that the antagonism observed between validamycin A and glucose may either result from catabolite repression or from competition for a common uptake mechanism although the latter explanation would seem unlikely in view of the structure of these

Fig. 1. Eight theoretically possible mono-β-D-glucosides of validoxylamine A.



Validoxylamine A (**1**)

2-*O*-β-D-Glucopyranosylvalidoxylamine A (2-*O*-β-Glc-VA)
3-*O*-β-D-Glucopyranosylvalidoxylamine A (3-*O*-β-Glc-VA)
4-*O*-β-D-Glucopyranosylvalidoxylamine A (4-*O*-β-Glc-VA: Validamycin A)
7-*O*-β-D-Glucopyranosylvalidoxylamine A (7-*O*-β-Glc-VA)
4′-*O*-β-D-Glucopyranosylvalidoxylamine A (4′-*O*-β-Glc-VA)
5′-*O*-β-D-Glucopyranosylvalidoxylamine A (5′-*O*-β-Glc-VA)
6′-*O*-β-D-Glucopyranosylvalidoxylamine A (6′-*O*-β-Glc-VA)
7′-*O*-β-D-Glucopyranosylvalidoxylamine A (7′-*O*-β-Glc-VA)

two compounds[2,3]. We previously reported that validoxylamine A powerfully inhibits the trehalase of *R. solani* in a competitive manner with a *Ki* value of 1.9×10^{-9} M, and that addition of validamycin A (0.1 μg/ml) to the mycelial suspension suppresses the degradation of intracellular trehalose[7]. Also it has been found that validamycin A is more readily taken up into the mycelia than validoxylamine A and validamycin D (7-*O*-α-Glc-VA). *R. solani* may have an oligosaccharide uptake system which is capable of transporting validamycin A.

In this report we describe the trehalase inhibitory activity and the activity in the "dendroid-test method" of all eight possible mono-β-D-glucosides of validoxylamine A (Fig. 1) described in our preceding paper[8], the effect of sugars on the activity of the antibiotics and the effect of disaccharides on 4-*O*-β-Glc-VA uptake into the mycelia.

Materials and Methods

General

Maltooligosaccharides, isomaltooligosaccharides, laminarioligosaccharides, cellooligosaccharides and gentiobiose were purchased from Seikagaku Kogyo Co., Ltd. Sophorose was prepared from pods of *Sophora japonica* according to the method of CLANCY[9]. Preparation of trehalase and assay of the enzyme were carried out according to the previously reported procedure[7].

Determination of Antagonistic Effect

An agar disk of *R. solani* (inoculum) was put on a glass disk (7-mm i.d.), previously placed on a water agar plate containing an antibiotic in the presence or absence of 1 mM sugar (antagonist), followed by incubation at 27°C for 48 hours. The length of hyphal extension or the occurrence of abnormal branching was compared with that in a control culture (without antibiotic and sugar).

Measurement of Validamycin A Taken up into Mycelia

A mycelial suspension[7] (50 ml) containing validamycin A (2.5 mg) in the presence or absence of 1 mM disaccharide was incubated at 27°C with shaking. The mycelia were filtered through a glass filter, washed with water, and dried with acetone and ether. The dried mycelia (100 mg) were extracted with 50% methanol (10 ml) under reflux for 1 hour and after centrifugation the resulting extract was concentrated *in vacuo* to remove methanol. The concentrated extract was applied to a short column of Dowex 50W-X2 (H^+, 1 ml), and washed with water. The resin adsorbed antibiotic was heated at 100°C for 4 hours. After cooling the resin was filtered through a glass filter, washed with water, and eluted with 0.5 N aq ammonia. The eluate was concentrated *in vacuo* to dryness and dissolved in water. The amount of validoxylamine A in this solution was determined on the basis of trehalase inhibition[7].

Results

Inhibitory Activity against Trehalase

As shown in Table 1, the attachment of the D-glucosyl residue to validoxylamine A generally diminished the inhibitory activity against trehalase of *R. solani*. Of the eight β-glucosides of validoxylamine A, 3-*O*-β-Glc-VA exhibited the most potent inhibitory activity ($IC_{50} = 8.4 \times 10^{-7}$ M), while 6′-*O*-β-Glc-VA had no effect on trehalase. The introduction of a D-glucosyl residue to the C-3 or C-5′ position, which corresponds to the C-3 or C-3′ position of trehalose, did not cause serious loss in trehalase inhibitory activity. However, substitution at the C-2 or C-6′ position, which corresponds to the C-2 or C-2′ position of trehalose, caused serious loss in the activity.

Inhibitory Activity by the "Dendroid-test Method"

As shown in Table 2, 4-*O*-β-Glc-VA, 3-*O*-β-Glc-VA and 5′-*O*-β-Glc-VA exhibited very strong activity

Table 1. Effect of β-glucopyranosylvalidoxylamines A against trehalase of *Rhizoctonia solani*.

Compound	Trehalase inhibitory activity; IC_{50} (M)
Validoxylamine A	2.0×10^{-7}
2-*O*-β-D-Glucopyranosylvalidoxylamine A	5.0×10^{-4}
3-*O*-β-D-Glucopyranosylvalidoxylamine A	8.4×10^{-7}
4-*O*-β-D-Glucopyranosylvalidoxylamine A	5.0×10^{-4}
7-*O*-β-D-Glucopyranosylvalidoxylamine A	4.0×10^{-5}
4′-*O*-β-D-Glucopyranosylvalidoxylamine A	3.2×10^{-4}
5′-*O*-β-D-Glucopyranosylvalidoxylamine A	5.0×10^{-6}
6′-*O*-β-D-Glucopyranosylvalidoxylamine A	—
7′-*O*-β-D-Glucopyranosylvalidoxylamine A	8.0×10^{-5}

—: No inhibition ($> 10^{-2}$ M).

Table 2. Effect of β-glucopyranosylvalidoxylamines A on *Rhizoctonia solani* in the "dendroid-test method".

Compound	Dendroid-test method (μg/ml)[a]
Validoxylamine A	1.00
2-*O*-β-D-Glucopyranosylvalidoxylamine A	0.50
3-*O*-β-D-Glucopyranosylvalidoxylamine A	0.0125
4-*O*-β-D-Glucopyranosylvalidoxylamine A	0.00625
7-*O*-β-D-Glucopyranosylvalidoxylamine A	1.25
4′-*O*-β-D-Glucopyranosylvalidoxylamine A	0.50
5′-*O*-β-D-Glucopyranosylvalidoxylamine A	0.025
6′-*O*-β-D-Glucopyranosylvalidoxylamine A	5.00
7′-*O*-β-D-Glucopyranosylvalidoxylamine A	0.50

[a] Minimum concentration causing abnormal branching.

Table 3. Effect of sugars on the activity of validoxylamine A and 4-*O*-β-D-glucopyranosylvalidoxylamine A in the "dendroid-test method".

Sugar (1 mM)	Glucosidic linkage	Validoxylamine A (2 μg/ml)		4-*O*-β-Glc-VA (0.1 μg/ml)	
		Colony diameter (mm)	Abnormal branching	Colony diameter (mm)	Abnormal branching
None		30	+	34	+
D-Glucose		31	+	39	+
Trehalose	α (1→1)	29	+	34	+
Maltose	α (1→4)	28	+	35	+
Maltotriose	α (1→4)	29	+	34	+
Isomaltose	α (1→6)	31	+	34	+
Isomaltotriose	α (1→6)	30	+	35	+
Sopholose	β (1→2)	28	+	44	+
Laminaribiose	β (1→3)	27	+	65	−
Laminaritriose	β (1→3)	27	+	65	−
Laminaritetraose	β (1→3)	27	+	60	−
Cellobiose	β (1→4)	28	+	65	−
Cellotriose	β (1→4)	28	+	58	−
Cellotetraose	β (1→4)	28	+	40	+
Gentiobiose	β (1→6)	28	+	50	+

Control without antibiotic exhibited the colony diameter of 72 mm in the "dendroid-test method".

against *R. solani* in the "dendroid-test method". In contrast, the activity of 6′-*O*-β-Glc-VA and 7-*O*-β-Glc-VA were lower than that of the corresponding aglycone, validoxylamine A. In general, the introduction of a D-glucosyl residue to the valienamine moiety tends to weaken the inhibitory activity, compared with that of the validamine moiety.

Antagonistic Activity of Sugars against Antibiotics

Various sugars were added at a concentration of 1 mM to the medium to investigate the effect of sugars on the inhibition of hyphal extension and abnormal branching by antibiotics as described in Materials and Methods section. As shown in Table 3, although the activity of validoxylamine A was not antagonized by any sugars tested, the activity of 4-*O*-β-Glc-VA was strongly antagonized by laminarioligosaccharides and cellooligosaccharides, except for cellotetraose. Sophorose and gentiobiose partially reversed the inhibition of hyphal extension by the antibiotic.

Table 4. Effect of β-oligosaccharides on the activity of β-glucopyranosylvalidoxylamines A in the "dendroid-test method".

Sugar (1 mM)	2-*O*-β-Glc-VA[a] (2 μg/ml)		3-*O*-β-Glc-VA (0.1 μg/ml)		4-*O*-β-Glc-VA (0.1 μg/ml)		7-*O*-β-Glc-VA (5 μg/ml)	
	Colony diameter (mm)	Abnormal branching	Colony diameter (mm)	Abnormal branching	Colony diameter (mm)	Abnormal branching	Colony diameter (mm)	Abnormal branching
None	32	+	45	+	38	+	35	+
Sophorose	60	−	65	−	55	+	41	+
Laminaribiose	75	−	75	−	75	−	48	+
Laminaritriose	75	−	70	−	75	−	43	+
Laminaritetraose	62	−	75	−	70	−	42	+
Cellobiose	43	+	65	−	70	−	35	+
Cellotriose	47	+	65	−	65	−	33	+
Cellotetraose	40	+	65	−	45	+	35	+
Gentiobiose	60	−	65	−	55	+	40	+

Control without antibiotic exhibited the colony diameter of 80 mm in the "dendroid-test method".
[a] 2-*O*-β-D-Glucopyranosylvalidoxylamine A.

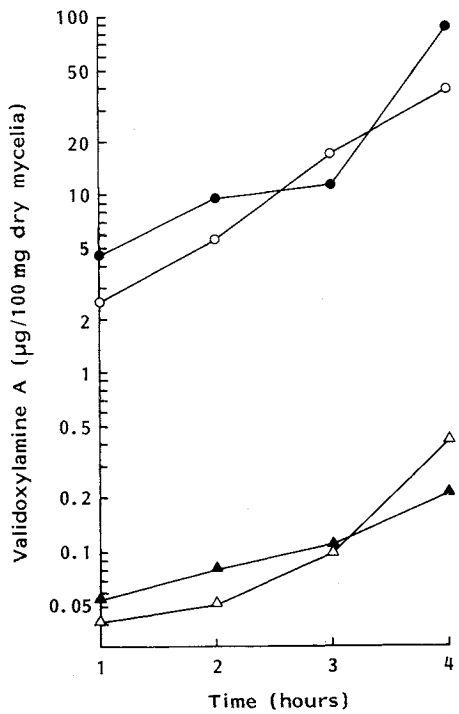
Of 2-*O*-β-Glc-VA, 3-*O*-β-Glc-VA, 4-*O*-β-Glc-VA and 7-*O*-β-Glc-VA, the abnormal branching caused by 7-*O*-β-Glc-VA, which exhibited the lowest activity (Table 2), was not antagonized by any β-oligosaccharides tested (Table 4). Laminaribiose partially reversed the inhibition of hyphal extension by 7-*O*-β-Glc-VA, but other β-oligosaccharides did not show any significant antagonistic activity. In contrast, the inhibition of hyphal extension and abnormal branching by 3-*O*-β-Glc-VA was antagonized by all β-oligosaccharides tested.

Effect of Disaccharides on 4-*O*-β-Glc-VA Uptake into Mycelia

The washed mycelia of *R. solani* were incubated with 4-*O*-β-Glc-VA in the presence or absence of disaccharides. The antibiotic taken up was extracted from the mycelia and adsorbed on Dowex 50W-X2. The antibiotic adsorbed on the resin was heated and hydrolyzed to the powerful trehalase inhibitor, validoxylamine A. The amount of validoxylamine A was determined on the basis of trehalase inhibition as previously reported[7]. As shown in Fig. 2, maltose, which did not antagonize the inhibitory activity of 4-*O*-β-Glc-VA, gave no effect on its uptake into the mycelia. However, by addition of

Fig. 2. Time course of 4-*O*-β-D-glucopyranosylvalidoxylamine A uptake into mycelia in the presence or absence of 1 mM disaccharides.

○ No addition of sugar, ● maltose, △ laminaribiose, ▲ cellobiose.



Mycelial suspension with the antibiotic (50 μg/ml) was incubated at 27°C with shaking.

1 mM laminaribiose or cellobiose which strongly antagonized the activity of the antibiotic, the antibiotic uptake into the fungal cell was markedly depressed. The uptake of the antibiotic in the presence of laminaribiose or cellobiose was over one hundred times lower than that in the absence of sugar or that in the presence of maltose.

Discussion

ROBSON *et al.*[4] reported that only a limited range of fungal pathogens, *Basidiomycotina*, were sensitive to validamycin A and this sensitivity may be due to a common factor shared by *Basidiomycotina*, possibly a transport system which is necessary for the uptake of validamycin A.

We have previously reported that validamycin A (4-*O*-β-Glc-VA) and its aglycone, validoxylamine A, strongly inhibit the trehalase of *R. solani* both *in vitro* and *in vivo*[7]. In addition, we recovered validamycin A from the mycelium of *R. solani* incubated in the presence of the antibiotic and showed that it is hydrolyzed intracellularly to validoxylamine A and D-glucose. It should be also noted that validamycin A is more readily taken up into the cell than validoxylamine A and validamycin D (7-*O*-α-Glc-VA). These observations suggest that *R. solani* has an uptake system capable of transporting validamycin A. In our present paper, 4-*O*-β-Glc-VA and 3-*O*-β-Glc-VA exhibited powerful activity in the "dendroid-test method" and their activity was strongly antagonized by laminarioligosaccharides and cellooligosaccharides. This antagonism appears to result from competition for a common uptake system, since the uptake of 4-*O*-β-Glc-VA was markedly depressed by laminaribiose and cellobiose. It seems likely that highly active β-glucosides of validoxylamine A such as 4-*O*-β-Glc-VA, 3-*O*-β-Glc-VA and 5′-*O*-β-Glc-VA are readily transported into the cell by the common uptake system with laminarioligosaccharides and cellooligosaccharides and intracellularly converted into the potent trehalase inhibitor, validoxylamine A by β-glucosidases.

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