TREHALASE INHIBITORS, VALIDOXYLAMINE A AND RELATED COMPOUNDS AS INSECTICIDES

NAOKI ASANO, MASAYOSHI TAKEUCHI, YUKIHIKO KAMEDA, KATSUHIKO MATSUI and YOSHIAKI KONO[†,††]

School of Pharmacy, Hokuriku University, Kanazawa 920-11, Japan
[†]Agro Division, Takeda Chemical Industries, Ltd., Tokyo 103, Japan

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Validoxylamine A showed a potent inhibitory activity against insect trehalase in a competitive manner with a *Ki* value of 4.3×10^{-10} M. The other validoxylamines and validamycins also exhibited the activity *in vitro*. Injection of these compounds to young last instar larvae of the tobacco cutworm, *Spodoptera litura*, elicited the morphological abnormality followed by death after the cessation of feeding. Validoxylamine A showed 100% mortality at a dose of 10 μg/ last instar larva.

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a nonreducing disaccharide and has been isolated from algae, bacteria, fungi, insects, invertebrates, and yeasts, as well as from some lower vascular plants and a few flowering plants[1]. This sugar is of importance to some microorganisms as means of sugar transport and as a readily available supply of energy. Trehalose is now recognized as a characteristic blood sugar of insects. It has been found in all insect species examined. The metabolic use of trehalose requires its cleavage, presumably to an equimolar mixture of α-D-glucose and β-D-glucose[2,3] by the enzyme trehalase (α,α-trehalose glucohydrolase, E.C. 3.2.1.28). Trehalase appears to play a primary metabolic role in organisms that store trehalose as a reserve carbohydrate. From a practical viewpoint, the specific inhibitors for trehalases may find applications in the regulation of the metabolism of this energy reserve.

Validamycin A is an antibiotic to control rice sheath blight caused by a phytopathogenic fungus, *Rhizoctonia solani*[4]. We have reported that validamycin A, and validoxylamine A formed by intracellular hydrolysis of the β-glucosidic bond of validamycin A, are potent trehalase inhibitors both *in vitro* and *in vivo*[5]. In particular, validoxylamine A exhibits a very strong competitive inhibitory activity against trehalases in all organisms examined[5,6]. In the present paper, inhibition of validoxylamine A and its related compounds against insect trehalase and their insecticidal activity were investigated in the tobacco cutworm, *Spodoptera litura*.

Materials and Methods

Validamycin Complex

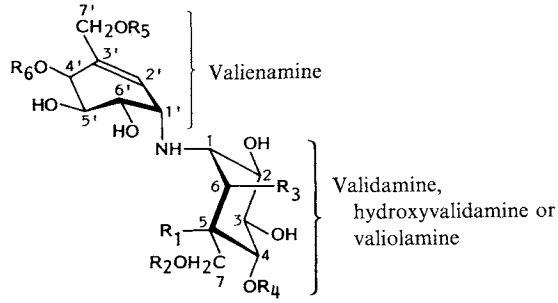
Validamycins A, B, C, D, E, F, G and validoxylamines A, B and G were purified as previously described[4,7,8].

Assay of Trehalase Inhibitory Activity

Trehalase was prepared from the last instar larvae of *S. litura*, by the method of KALF and RIEDER[9].

[††] Present address: Department of Medical Entomology, National Institute of Health, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan.

Fig. 1. Structure of the validamycin complex.



Validoxylamine A	R_1=H	R_2=H	R_3=H	R_4=H	R_5=H	R_6=H
Validoxylamine B	R_1=H	R_2=H	R_3=OH	R_4=H	R_5=H	R_6=H
Validoxylamine G	R_1=OH	R_2=H	R_3=H	R_4=H	R_5=H	R_6=H
Validamycin A	R_1=H	R_2=H	R_3=H	R_4=β-D-Glc	R_5=H	R_6=H
Validamycin B	R_1=H	R_2=H	R_3=OH	R_4=β-D-Glc	R_5=H	R_6=H
Validamycin C	R_1=H	R_2=H	R_3=H	R_4=β-D-Glc	R_5=α-D-Glc	R_6=H
Validamycin D	R_1=H	R_2=α-D-Glc	R_3=H	R_4=H	R_5=H	R_6=H
Validamycin E	R_1=H	R_2=H	R_3=H	R_4=α-D-Glc(1-4)-β-D-Glc	R_5=H	R_6=H
Validamycin F	R_1=H	R_2=H	R_3=H	R_4=β-D-Glc	R_5=H	R_6=α-D-Glc
Validamycin G	R_1=OH	R_2=H	R_3=H	R_4=β-D-Glc	R_5=H	R_6=H

Glc: Glucopyranosyl.

The reaction mixture consisting of 50 μl of 0.2 M phosphate buffer (pH 6.0), 80 μl of inhibitor solution or distilled water and 50 μl of enzyme solution was preincubated at 37°C for 3 minutes. The reaction was started by adding 20 μl of 0.25 M trehalose solution and incubated at 37°C for 15 minutes. The released D-glucose was measured by the glucose oxidase method using the commercially available Glucose B-Test Wako Kit (Wako Pure Chemical Industries, Ltd.). Protein was determined according to LOWRY *et al.*[10] using bovine serum albumin as the standard.

Assay Method of Insecticidal Activity

The stock culture of *S. litura* was maintained on artificial diet under 16 hour light-8 hour dark photoperiod at 25°C. The last instar larvae of *S. litura* were collected 1 day after the final larval molt from the stock culture and subjected to the assay. One μl per larva of aqueous solution containing 10 μg test chemicals was injected into the body cavity of the last instar larvae anesthetized with CO_2 using microsyringe. Treated larvae were fed with the artificial diet individually in polyethylene cup and observed their subsequent developmental abnormality until the adult eclosion.

Results

Trehalase Inhibitory Activity

Enzyme inhibitory activity of validoxylamine A and its related compounds against insect trehalase is shown in Table 1 and Fig. 2. All validoxylamines and validamycins, except validamycin C, showed a potent inhibitory activity against insect trehalase. Kinetic analysis with trehalose as the substrate indicated that all validoxylamines and validamycins were competitive inhibitors. Validoxylamine A was found to be the most potent inhibitor against the insect trehalase (*Ki* value: 4.3×10^{-10} M) in all its relatives tested. The attachment of a hydroxyl group in C-5 or C-6 position of validoxylamine A (validoxylamine G or B) seemed to weaken the trehalase inhibitory activity. The introduction of D-glucose into C-4 or C-7 position of validoxylamine A (validamycin A or D), to the contrary, did not reduce the inhibitory activity so prominently, comparing with the results obtained with the trehalase in *R. solani*[5]. Furthermore, the

Table 1. Inhibitory effect of validoxylamine A and related compounds on insect trehalase.

($Km = 1.4 \times 10^{-3}$ M)

Compound	IC_{50}(M)[a]	Ki (M)[b]
Validamycin A	3.7×10^{-7}	4.7×10^{-8}
Validamycin B	1.8×10^{-6}	1.9×10^{-7}
Validamycin C	1.3×10^{-4}	2.8×10^{-6}
Validamycin D	2.6×10^{-7}	3.2×10^{-9}
Validamycin E	1.2×10^{-6}	1.4×10^{-7}
Validamycin F	5.8×10^{-6}	3.3×10^{-7}
Validamycin G	7.9×10^{-7}	1.9×10^{-7}
Validoxylamine A	4.8×10^{-8}	4.3×10^{-10}
Validoxylamine B	6.6×10^{-6}	5.5×10^{-8}
Validoxylamine G	5.9×10^{-6}	1.2×10^{-7}

[a] Molar concentration required to give 50% inhibition.
[b] Inhibition constant.

Fig. 2. Lineweaver-Burk plot of *Spodoptera litura* trehalase with validoxylamine A or validamycin A.

● Validoxylamine A (8×10^{-9} M), ▲ validamycin A (2×10^{-7} M), ■ none: $Km = 1.4 \times 10^{-3}$ M.

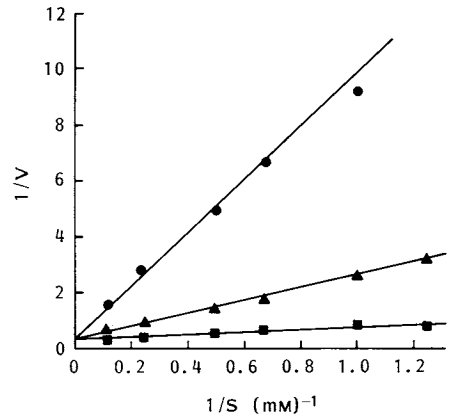
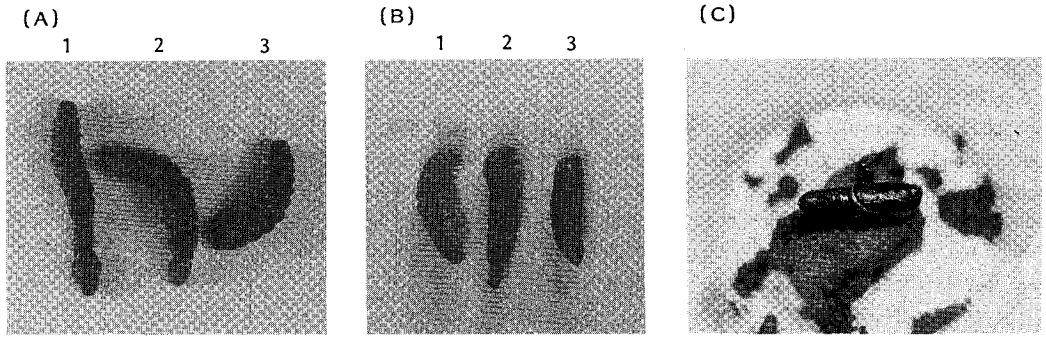


Fig. 3. Effect of validoxylamine A injection (50 μg) upon the last instar larvae of *Spodoptera litura*.



(A): 1 and 2, extrusion of hind- and mid-gut outside of the body in prepupae; 3, normal prepupa; (B): 1 and 2, unsuccessful metamorphosis from larvae stage to prepupae stage and to pupae stage; 3, normal pupa; (C): partial separation between wingpads and abdomen in pupa.

insect trehalase appeared to be very sensitive to validamycins E and F, while such pseudo-tetrasaccharides as validamycins C, E and F had no inhibitory effect on the trehalase of *R. solani*[5].

Insecticidal Activity of Validoxylamine A and Related Compounds

Effect of validoxylamine A injection (50 μg) upon the last instar larvae of *S. litura* is shown in Fig. 3. The characteristic of abnormality in prepupae was the extrusion of hind- and mid-gut outside of the body after the cessation of feeding (Fig. 3A) and death followed. Fig. 3B shows the unsuccessful metamorphosis from larvae stage to prepupae stage or to pupae stage. The pupae succeeded in the pupal ecdysis showed a morphological abnormality characterized by the partial separation between wingpads and abdomen (Fig. 3C), and no adult emerged from such abnormal pupae. Effect of injection of validoxylamine A relatives (10 μg/larva) on the morphogenesis of *S. litura* is shown in Table 2. Validoxylamine A is the most potent insecticidal compound among the validamycin complex. Validamycins A and D provided over 50% mortality, but validamycin A showed a lower adult ecdysis rate from normal pupae than validamycin D, although they had the same extent of inhibitory activity against trehalase *in vitro*.

Table 2. Effect of trehalase inhibitors injection (10 μg) to the last instar larvae on the later development in *Spodoptera litura*.

Compound	Test larvae	Death in prepupae	Abnormal pupae[a]	Normal pupae	Adult ecdysis/ normal pupae
Validamycin A	20	5	6	9	1/9
Validamycin B	20	0	0	20	17/20
Validamycin C	20	3	5	12	7/12
Validamycin D	20	9	4	7	5/7
Validamycin E	20	5	7	8	3/8
Validoxylamine A	20	14	6	0	
Validoxylamine B	20	0	0	20	17/20
Validoxylamine G	20	2	2	16	14/16
None	20	2	0	18	16/18

[a] No adult emerged from abnormal pupae.

Validamycin C which showed less activity ($IC_{50} = 1.3 \times 10^{-4}$ M) *in vitro* exerted a fairly good activity *in vivo*. This seems to be due to the conversion of validamycin C into the potent insecticidal compounds in the insect body. The further attachment of the hydroxyl group to C-5 or C-6 position in validoxylamine A as seen in validoxylamines B, G and validamycins B and G had a marked effect on the efficacy of insecticidal activity.

Discussion

We have previously reported that validamycin A and its aglycone, validoxylamine A, strongly inhibit the trehalase of phytopathogenic fungus, *R. solani* both *in vitro* and *in vivo*[5]. In the present paper, we demonstrated that validoxylamine A and its relatives showed inhibitory activity against insect trehalase and insecticidal activity in *S. litura*. The further attachment of the hydroxyl group to C-5 or C-6 position in validoxylamine A provided weak insecticidal activity as well as weak trehalase inhibition, while validamycin C exhibited a fairly good insecticidal activity despite its weak trehalase inhibition. The insecticidal activity of validamycin C seems to depend on its hydrolysis in the insect body to active metabolites such as 7′-*O*-α-D-glucopyranosylvalidoxylamine A, 4-*O*-β-D-glucopyranosylvalidoxylamine A (validamycin A) or validoxylamine A.

Taking the essential role of trehalose and its hydrolyzing enzyme, trehalase in insect sugar metabolisms, lethal activity was easily expected from the inhibition of trehalase. The effect of trehalase inhibitors, however, appeared as morphological abnormalities at the metamorphosis, several days after the dosing. According to the experimental results in the silkworm pupa, *Bombyx mori*[11], validoxylamine A lost its activity in 2 days after the injection into body cavity. Therefore, it can be considered that the inhibition of trehalase at the younger age of last instar larva causes the morphological abnormalities. Detailed mechanism of trehalase inhibitor action is to be elucidated.

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