STUDIES ON VALIDAMYCINS, NEW ANTIBIOTICS. VIII

ISOLATION AND CHARACTERIZATION OF VALIDAMYCINS C, D, E AND F

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This paper describes the isolation and characterization of four components of validamycin complex, validamycins C, D, E and F, in addition to validamycins A and B reported in the previous papers[1~4]. Validamycins A, C, D, E and F are different from each other in the site and/or the type of glucosidic linkage to validoxylamine A[5]. The presence of validoxylamines A and B[5] in the culture filtrate was also demonstrated.

In the search for new antibiotic substances, it is usual to find a number of closely related compounds produced in fermentations. Validamycins A and B, antibiotics active against the sheath blight of rice plants, were reported in the previous papers[1~4]. The further chromatographic studies revealed in the fermentation broth of *Streptomyces hygroscopicus* var. *limoneus*[1], the presence of validoxylamines A and B[5], and four additional components designated validamycins C, D, E and F.

This report describes the chromatographic separation and physical and chemical properties of these components.

Experimental

Gas liquid chromatography:

Apparatus and chromatographic conditions: A Hitachi's Model 063 gas chromatograph equipped with flame ionization detector was used. The suitable chromatographic conditions should be chosen on the basis of the components to be examined.

The conditions for the chromatography of trimethylsilyl (TMS) validamycins A~F are:

Column: 1% silicone OV-1 on chromosorb W AW DMCS (glass column 2 m×3 mm I. D.)
Column temperature; 280°C, Injection temperature; 300°C,
Carrier gas (He): 60 ml/min.

The conditions for the separation of TMS-validoxylamines A and B are:

Column: 3% silicone OV-17 on chromosorb W AW DMCS (glass column 2 m×3 mm I. D.)
Column temperature; 250°C, Injection temperature; 300°C,
Carrier gas (He): 30 ml/min.

The conditions for the separation of TMS derivatives of the degradation products by hydrogenolysis are:

Column: 5% silicone OV-17 on chromosorb W AW DMCS (glass column 2 m×3 mm I. D.)
Column temperature: initial, 150°C; final, 280°C (program rate 10°C/min.); Injection temperature, 300°C.
Carrier gas (He): 45 ml/min.

Fig. 1. Gas-liquid chromatogram of TMS-validamycins A, B, C, D, E and F.

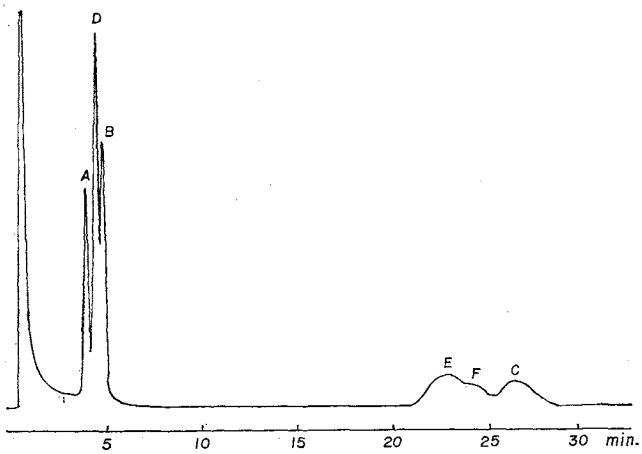
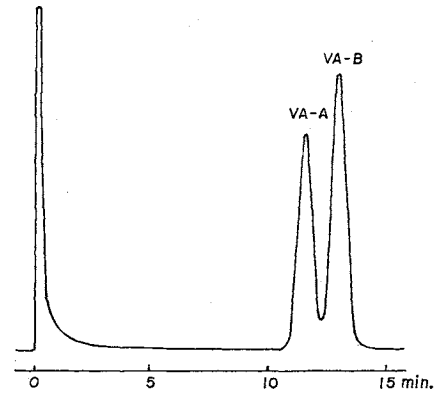


Fig. 2. Gas-liquid chromatogram of TMS-validoxylamines A (VA-A) and B (VA-B).



Typical chromatograms are shown in Figs. 1 and 2.

Silylation procedure: Approximately 1 mg of sample was weighed into a rubber-capped small tube and dissolved into 100 μl pyridine. Bis(trimethylsilyl) acetamide 100 μl and trimethylchlorosilane 50 μl were added. The tube was heated for 30 minutes at 70~80°C.

Automatic liquid chromatography:

The Jeol JLC-3BC$_2$ amino acid analyzer (Japan Electron Optics Laboratory Co.) was used as the chromatographic system. In the detection of validamycins A~F, a wave length of 440 mμ was used to detect orcinol-sulfuric acid reaction products, using a visible ray detector. The samples were analyzed with the following two column chromatographic systems.

System I: The column (15 cm×8 mm I.D.) was packed with Jeol Resin LC-R-3® (strongly basic ion-exchange resin, 8% cross linkage; borate form) and

Fig. 3. Automatic liquid chromatogram of validamycins A, B, D and E.
Conditions: system II.

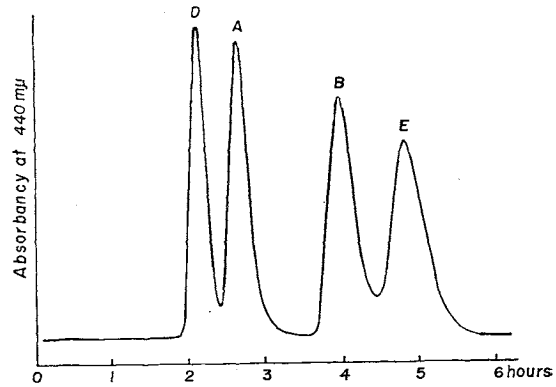


Fig. 4. Automatic liquid chromatogram of validamycins E and F.
Conditions: system II.

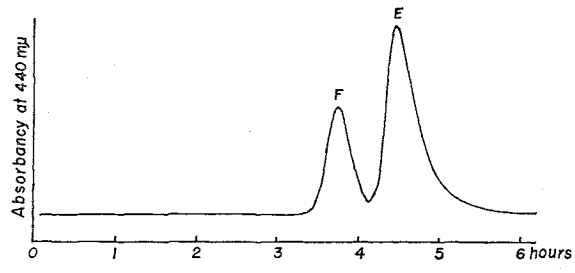
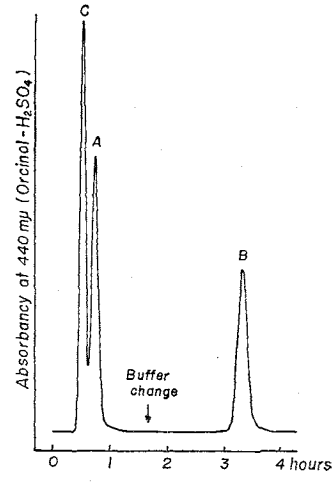


Fig. 5. Automatic liquid chromatogram of validamycins A, B and C.
Conditions: system I.



was jacketed to maintain the temperature at 50°C. The column was developed with 0.11 M borate buffer, pH 7.5 (first buffer) and 0.25 M borate buffer, pH 9.0 (second buffer; buffer change time 100 minutes), at a flow rate of 0.49 ml/min.

System II: The column (70 cm×8 mm I. D.) was packed with Bio-Rad resin AG 1×2® (200~400 mesh, OH form) and developed with water at a flow rate of 0.37 ml/min. Some typical chromatograms are shown in Figs. 3, 4 and 5.

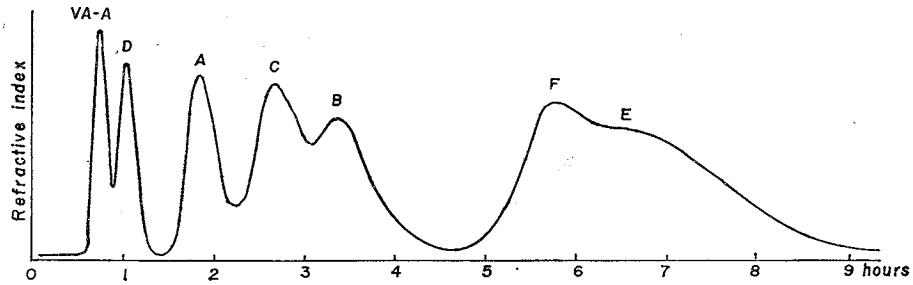
On a gravity flow, the differential refractometer R403 (Water Associates Inc.) is suitable for the detection of validoxylamines A and B, which are negative to orcinol-sulfuric acid reaction, as well as the detection of validamycins A~F.

Isolation procedure:

The preparation of crude validamycins have been described in previous papers of this series[2,4]. In a typical column chromatogram for preparative purposes, the crude validamycins (15 g) were chromatographed on Dowex 1×2 column (OH form, 100~200 mesh, 870 ml), and the column was developed with water to give the eight components: validoxylamines A and B, and validamycins D, A, C, B, F and E, in order of elution from the column.

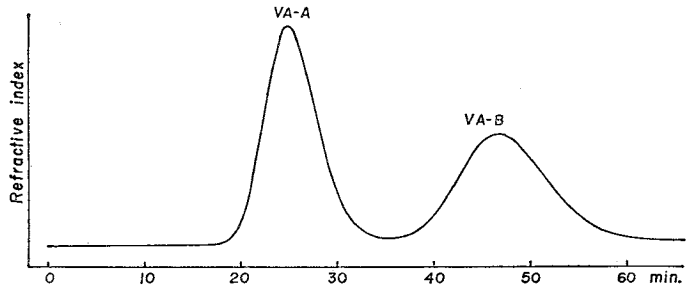
The peak of validamycin C appeared just before validamycin B, and these two components were slightly overlapped. On the other hand, validamycin D was eluted just before validamycin A. Validamycin C-rich fractions and fractions mainly containing

Fig. 6. Chromatogram of authentic mixture of validamycins A, B, C, D, E, F and validoxylamine A (VA-A).
Column: Dowex 1×2 (OH form, 100~200 mesh, 27 cm×15 mm I. D.)
Carrier: Water (gravity flow rate; 1.1 ml/min.)



validamycin D were rechromatographed on a silica gel column (elution with *n*-PrOH·AcOH·H_2O 4:1:1) and Dowex 50W×2 column (elution with pyridine-acetic acid buffer, pH 6.0).

Fig. 7. Chromatogram of authentic mixture of validoxylamines A (VA-A) and B (VA-B).
Column: Dowex 1×2 (OH form, 100~200 mesh, 23.5 cm×12 mm I. D.)
Carrier: Water (gravity flow rate; 2.4 ml/min.)



Validamycins E and F were strongly retarded on Dowex 1×2 column owing to their great affinity for the resin, these showed a rather diffuse elution pattern on a gravity flow. The complete resolution of validamycins E and F was not accomplished on Dowex 1×2 column. Validamycin E-rich fractions and validamycin F-rich fractions obtained by Dowex 1×2 chromatography were further chromatographed on Dowex 50W×2 column (elution with pyridine-acetic acid buffer, pH 6.0). In this chromatography, early fractions contained validamycin F. Thereafter, validamycin E was eluted. These chromatographic

purifications were repeated once more, if necessary. Finally, chromatographically homogeneous validamycins C, D, E and F were obtained by rechromatography on Dowex 1×2 (OH form, developed with water).

Validoxylamine A was eluted just before validamycin D on Dowex 1×2 column, and crystallization from water-ethanol gave the pure validoxylamine A. Although validoxylamine B and validamycin D were almost overlapped on Dowex 1×2 chromatography, these components were easily separated on Dowex 50W×2 column (elution with pyridine-acetic acid buffer, pH 6.0). These compounds were identical with validoxylamines A and B obtained by the hydrolysis of validamycins A and B, respectively.

The chromatograms on Dowex 1×2 columns of these components are shown in Fig. 6 (validamycins A, B, C, D, E, F and validoxylamine A) and Fig. 7 (validoxylamines A and B).

Acid hydrolysis:

Each component (validamycins A, C, D, E and F) was heated on a steam bath with 1 N sulfuric acid for 10 hours to give validoxylamine A and D-glucose. The milder acid hydrolysis (0.5 N sulfuric acid, for 3 hours) of validamycins C, E and F gave validamycin A as a partial hydrolysis product, but no validamycin A could be isolated from the hydrolyzate of validamycin D.

After removal of sulfate ions with barium hydroxide, the hydrolyzate was chromatographed on Dowex 50W×2 (H form). D-Glucose passed through the column, whereas validoxylamine A and validamycin A were adsorbed on the resin and then eluted with 1 N hydrochloric acid. Each compound was further purified by Dowex 1×2 (OH form) chromatography.

The hydrolyzates were also qualitatively and quantitatively analyzed by gas liquid chromatography and automatic liquid chromatography.

Hydrogenolysis:

The hydrogenation of each component (validamycins A~F) was carried out in water using a platinum catalyst and resulted in the uptake of about two mole-equivalents of hydrogen to yield a mixture of several degradation products. These were separated by chromatography on Dowex 1×2 (OH form, developed with water), Dowex 50W×2 (elution with pyridine-acetic acid buffer) and silica gel column (elution with *n*-PrOH - AcOH - H_2O, 4 : 1 : 1).

Results and Discussion

Validamycins C, D, E and F are all white amorphous solids which have no definite melting point. A variety of chromatographic procedures have indicated that they are distinguishable from each other and are homogeneous.

All are soluble in water, dimethyl formamide and dimethyl sulfoxide, sparingly

Table 1. Properties of validamycins A~F and validoxylamine A, B (1)

	Formula	Elemental analysis					
		Found			Calcd.		
		C %	H %	N %	C %	H %	N %
Validamycin A	$C_{20}H_{35}NO_{13}\cdot H_2O$	46.64	7.15	2.95	46.60	7.24	2.72
Validamycin B	$C_{20}H_{35}NO_{14}\cdot H_2O$	45.58	6.92	2.71	45.20	7.02	2.64
Validamycin C	$C_{26}H_{45}NO_{18}\cdot H_2O$	46.48	7.22	2.15	46.08	6.99	2.07
Validamycin D	$C_{20}H_{35}NO_{13}\cdot H_2O$	46.97	7.23	2.58	46.60	7.24	2.72
Validamycin E	$C_{26}H_{45}NO_{18}\cdot H_2O$	46.22	7.19	1.94	46.08	6.99	2.07
Validamycin F	$C_{26}H_{45}NO_{18}\cdot H_2O$	46.00	6.90	1.95	46.08	6.99	2.07
Validoxylamine A	$C_{14}H_{25}NO_8\cdot H_2O$	47.86	7.86	3.89	47.58	7.70	3.96
Validoxylamine B	$C_{14}H_{25}NO_9\cdot H_2O$	46.01	7.28	3.59	45.52	7.37	3.79

Table 2. Properties of validamycins A~F and validoxylamines A, B (2)

	$[\alpha]_D$ (H_2O)	pKa' value* (neut. equiv.)	tlc** Rf value	nmr (D_2O)*** anomeric proton	
Validamycin A	+112.5°	6.0±0.2 (520±30)	0.24	δ 4.75 (J=7.5 Hz)	
Validamycin B	+102.3°	5.0±0.2 (530±30)	0.30	δ 4.75 (J=7.5 Hz)	
Validamycin C	+132.9°	6.0±0.2 (680±30)	0.15	δ 4.73 (J=7.3 Hz)	δ 5.12 (J=3.3 Hz)
Validamycin D	+169.3°	6.0±0.2 (520±30)	0.24	δ 5.12 (J=3.3 Hz)	
Validamycin E	+148.2°	6.1±0.2 (680±30)	0.15	δ 4.75 (J=7.3 Hz)	δ 5.59 (J=3.3 Hz)
Validamycin F	+130.7°	6.1±0.2 (680±30)	0.15	δ 4.75 (J=7.3 Hz)	δ 5.61 (J=3.3 Hz)
Validoxylamine A	+170.0°	6.2±0.2 (355±30)	0.34	——	
Validoxylamine B	+130.7°	5.0±0.2 (370±30)	0.42	——	

* In water. ** Solvent: *n*-propanol-acetic acid-water (4:1:1), Silica gel G.
*** At 100 MHz with TMS standard.

soluble or insoluble in ethanol, acetone, ethyl ether, benzene, chloroform and ethyl acetate. In methanol, validamycin D is easily soluble, but validamycins C, E and F are less soluble. All are weakly basic substances giving positive reaction with anthrone reagent, orcinol-sulfuric acid, GREIG-LEABACK's reagent and benzidine periodate, but negative reaction with SAKAGUCHI's and ELSON-MORGAN's reagents.

The ultraviolet absorption spectra of validamycins C, D, E and F all show only end absorption. The infrared absorption spectra of these are very similar to those of validamycins A and B, and are suggestive of polyhydroxy compounds. Other characterization data on validamycins A~F are presented in Tables 1 and 2.

The biological assays[3] for validamycins are rather time-consuming and incapable of composition analyses. The gas liquid chromatography and the automatic liquid chromatography are rapid and convenient methods for analyzing the complex mixtures and the residues of validamycins.

The gas chromatographic method is especially of value in monitoring validoxylamines A and B. The presence of validoxylamines A and B in the fermentation broth was first demonstrated by this analytical method, because of their low biological activity by the "dendroid-test method"[3] and negative orcinol-sulfuric acid reaction. It was also demonstrated that the validamycins were not degraded to validoxylamines during the procedure of this gas chromatographic analysis.

Validamycins C, E and F gave one mole of validoxylamine A and two moles of D-glucose by acid hydrolysis, and differed from validamycins A and D which gave each one mole of validoxylamine A and D-glucose. This evidence and elemental analyses suggest that validamycins C, E and F have the tentative molecular formula $C_{26}H_{45}NO_{18}$, and validamycin D has the tentative molecular formula $C_{20}H_{35}NO_{13}$.

The nmr anomeric signals of validamycins C, E and F showed that they had two (α- and β-) types of glycosidic linkages as shown in Table 2. The optical rotation data also confirmed these anomeric configurational assignments.

Hydrogenolysis of validamycins C and F, respectively, afforded α-D-glucopyranosyl-validatol* and β-D-glucopyranosyl-validamine* (nmr: δ 4.77, d, J=7.5 Hz). However, α-D-glucopyranosyl-validatol (nmr: δ 5.09, d, J=3.3 Hz) derived from validamycin C was distinguished from the validatol derivative (nmr: δ 5.38, d, J=

* The structural studies of validamine, (1*S*)-(1,2,4/3,5)-1-amino-5-hydroxymethyl-2,3,4-cyclohexanetriol and validatol, (1,2,4/3)-1-hydroxymethyl-2,3,4-cyclohexanetriol, were reported in detail[6,7].

3.3 Hz) derived from validamycin F, by nmr spectrum and gas liquid chromatography, and these differences may be due to the point of attachment of the glycosidic linkage to the validatol. On the other hand, validamycin E gave α-D-glucopyranosyl-(β-D-glucopyranosylvalidamine) (nmr: δ 4.74, d, J=7.5 Hz and δ 5.61, d, J=3.3 Hz) and validatol by hydrogenolysis.

Validamycin D is very similar to validamycin A in their pKa' values, analytical data and thin-layer chromatographic mobilities. However, validamycin D gave α-D-glucopyranosyl-validamine (nmr: δ 5.10, d, J=3.3 Hz) by hydrogenolysis and could be distinguished from validamycin A by the different anomeric configurations of their glycosidic linkages (β type for validamycin A and α type for validamycin D).

Table 3. The minimum concentration causing the abnormal branching at the tops of the hyphae of *Pellicularia sasakii*

Compound	Minimum concentration (mcg/ml)
Validamycin A	0.01
Validamycin B	0.5
Validamycin C	10
Validamycin D	100
Validamycin E	0.013
Validamycin F	0.013
Validoxylamine A	10
Validoxylamine B	>100

Validamycins E and F are highly active against *Pellicularia sasakii* by the "dendroid-test method" and the greenhouse test. Although validamycins C and D, as well as validoxylamines A and B showed very low activity by the "dendroid-test method" as shown in Table 3*, they showed considerable activity by the greenhouse test. These results have provided an interesting evidence for structure-activity relationship in the validamycin group. The results of the greenhouse test will be reported in a separate paper by O. WAKAE and his coworkers of the Phytopathology Department.

The structure of validamycin A has been tentatively proposed to be *N*-[3-hydroxymethyl-(4,6/5)-4,5,6-trihydroxy-2-cyclohexenyl][*O*-β-D-glucopyranosyl-(1→3)-(1*S*)-(1,2,4/3,5)-2,3,4-trihydroxy-5-hydroxymethylcyclohexyl]amine, and further details will be also reported elsewhere. Further work is now in progress on the chemistry of validamycins A~F and the isolation of other minor components.

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