STUDIES ON VALIDAMYCINS, NEW ANTIBIOTICS. IV

ISOLATION AND CHARACTERIZATIN OF VALIDAMYCINS A AND B

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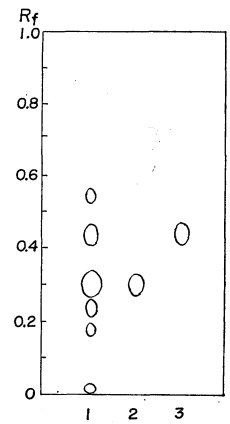
New antibiotics named validamycins A and B, active against the sheath blight of rice plants, were isolated from the culture filtrate of *Streptomyces hygroscopicus* var. *limoneus*. Validamycins A and B are water-soluble, glucose-containing basic antibiotics, and with molecular formulae of $C_{20}H_{33\sim37}NO_{13\sim14}$ and $C_{20}H_{33\sim37}NO_{14\sim15}$, respectively.

As described previously[1,2], antibiotics active in controlling the sheath blight of rice plants were produced by *Streptomyces hygroscopicus* var. *limoneus*. In the early separation studies KOSAKA's method[3], the reversed layer method[4] and color reactions for glycosides were used for the detection of the antibiotics. Two components were separated by chromatography on ion-exchange resin. From the physical, chemical and biological[2] properties, they were established to be new antibiotics and named validamycins A and B.

In this paper, the isolation and the characterization of validamycins A and B are described.

Fig. 1. Thin-layer chromatography of validamycins A and B
Silica gel G
Solvent system;
n-PrOH - AcOH - H_2O (4 : 1 : 1)
Detection by naphthoresorcin-H_2SO_4 reagent
(1) Crude powder of validamycin
(2) Validamycin A
(3) Validamycin B



Isolation

The culture broth of *Streptomyces hygroscopicus* var. *limoneus* was acidified with oxalic acid to pH 3~4 and the calcium ion in the broth was removed with the mycelium by filtration. To remove cationic and anionic impurities, the broth filtrate was first treated with Amberlite IR-120 (H) and IR-45 (OH) and then applied to a column of Dowex-50 X-2. The antibiotics were adsorbed on the resin and then eluted with aqueous ammonia. The eluate concentrate was chromatographed on Dowex-50X-2 with pyridine - acetic acid buffer at pH 6.5. Validamyin A was adsorbed on the column and validamycin B passed through. Validamycin A adsorbed on the resin was eluted with pyridine - acetic acid, pH 7.5, and the eluate was concentrated to give pure crystalline validamycin A.

The concentrate of the column effluent was dissolved in

water, applied to a column of Dowex-1 X-2 and developed with water to give pure validamycin B. The purity of each antibiotic was confirmed by thin-layer chromatography (Fig. 1) and their crystalline derivative.

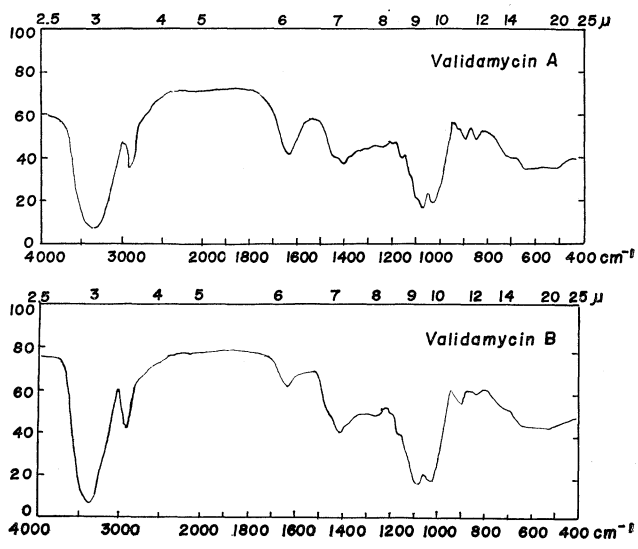
Characterization

Validamycin A was obtained as colorless hydrophilic powder and did not show sharp melting point; softened about 100°C and decomposed about 135°C, $[\alpha]_D^{24}$ 110°± 15° (*c* 1, H_2O), 110°±15° (*c* 1, pyridine), 92°±10° (*c* 1, dimethylformamide), pKa 6.0. The molecular weight was found to be 519±30 by the titration method. The elemental analysis and the molecular weight of validamycin A supported the molecular formula of $C_{20}H_{33\sim37}NO_{13\sim14}$. It is soluble in water, methanol, dimethylformamide and dimethylsulfoxide, sparingly soluble in ethanol and acetone and insoluble in ethylacetate and diethylether. It shows a green color with the anthrone reagent, reddish brown color with phenol-sulfuric acid, reddish brown color with orcin sulfuric acid, blue-violet color with GREIG-LEABACK's reagent[5] and positive with benzidine-periodate, but is negative to SAKAGUCHI's and ELSON-MORGAN's reagents. The ultraviolet spectrum of validamycin A in aqueous solution shows only end absorption, and the infrared spectrum indicates the presence of hydroxyl and ether linkage (1000~1100 cm^{-1}) (Fig. 2). The NMR spectrum (in D_2O) of validamycin A indicates the presence of an anomeric proton (δ 6.3 ppm) and many methine protons which are adjacent to hydroxyls (-$\overset{|}{\underset{H}{C}}$-OH, δ 3~5 ppm) (Fig. 3).

Validamycin A monohydrochloride is a colorless crystalline powder; m.p. 95°C (decomp.), $[\alpha]_D^{22}$ +49° ±10° (*c* 1, H_2O), soluble in water, methanol and ethanol, and insoluble in acetone and diethylether.

Validamycin A acetate was obtained by acetylation of validamycin A with acetic anhydride in pyridine as colorless needles, m.p. 100°C (decomp.). The molecular weight determined in ethyl acetate by vapor pressure osmometry was 1,007±100. In the mass spectrum of validamycin A acetate the highest mass peak was found at m/e 959. These facts and the elemental analysis of validamycin A acetate supported the molecular formula of $C_{42\sim44}H_{55\sim61}NO_{24\sim26}$.

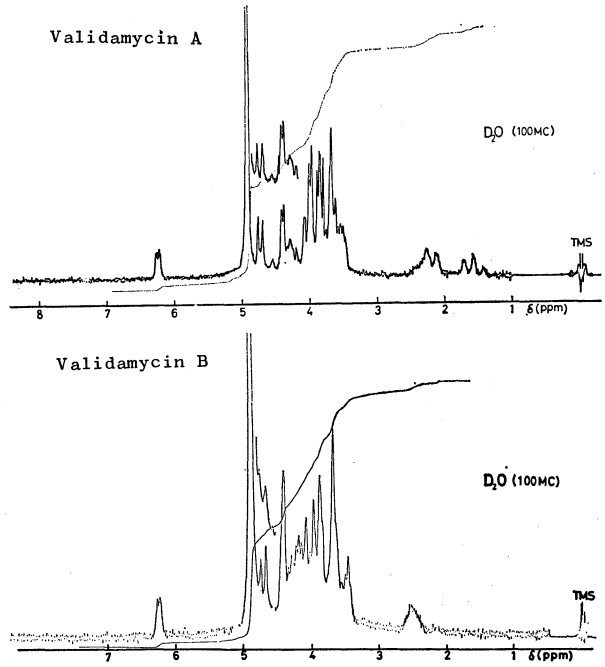
Fig. 2. Infrared spectra of validamycins A and B (KBr)



Validamycin B was obtained as basic hydrophilic substance, colorless powder; $[\alpha]_D^{24}$ 102°±10° (*c* 1.0, H_2O), pKa 5.0. The molecular weight was found to be 530±30 by the titration method.

The elemental analysis and the molecular weight of validamycin B indicate the molecular formula of $C_{20}H_{33\sim37}NO_{14\sim15}$. The solubility and the color reaction of validamycin B were similar to those of validamycin A, and the ultraviolet (in H_2O) and infrared (KBr) spectra of validamycin B were also similar to those of validamycin A. However, the NMR spectrum (in D_2O) of validamycin B was different from that of validamycin A (Fig. 3). Validamycin B also formed a salt, validamycin B monohydrochloride.

Fig. 3. NMR spectra of validamycins A and B. (100 MC in D_2O, TMS as reference)



Validamycin B acetate was obtained by acetylation of validamycin B with acetic anhydride in pyridine as colorless needles, m.p. 155°C (decomp.). The molecular weight determined in ethyl acetate by vapor pressure osmometry was 1,040±100. The molecular weight and the elemental analysis of validamycin B acetate suggested the molecular formula of $C_{44\sim46}H_{57\sim63}NO_{25\sim27}$.

Thin-layer chromatography of validamycins A and B was carried out on a plate of silica gel G by developing with a mixture of *n*-propanol - acetic acid - water (4 : 1 : 1), and detecting with the above-mentioned color reactions (Fig. 1).

Hydrolyses of validamycins A and B: Validamycins A and B were refluxed with Amberlite IR-120 (H) in water to give D-glucose which was identified with the authentic sample.

Validamycins A and B seem to be related to trehalosamine[6], mannosyl-glucosaminide[7] and hygromycin B[8] in some chemical properties. However, from the data of the above-mentioned physicochemical properties and the biological characteristics[2], they are recognized to be new antibiotics.

Experimental

Separation. The culture broth of *Streptomycces hygroscopicus* var. *limoneus* (180 liters, 100 μg/ml[4]) was acidified with oxalic acid to pH 3~4, and filtered with the aid of Hyflo-supercel. The filtrate was passed through columns of Amberlite IR-120 (H, 6 liters) and IR-45 (OH, 7 liters) successively and the effluent was applied to a column of Dowex-50 WX-2 (H, 7.2 liters). The antibiotics adsorbed on Dewex-50 WX-2 were eluted with 0.5 N aqueous ammonia and the active eluate (35 liters, 550 μg/ml) was concentrated to give crude validamycin (21 g, 650 μg/mg).

Validamycin A. The crude validamycin (21 g) obtained by the above-described procedure was dissolved in 0.1 N pyridine - acetic acid buffer solution (11.5 liters, pH 6.5), and

then applied to a column of Dowex-50 WX-2 (50~100 mesh, 35 liters), previously treated with the same solution. The column was washed with the same buffer (50 liters) until validamycin B passed through completely. Validamycin A adsorbed on the resin was eluted with 0.1 N pyridine - acetic acid buffer solution (pH 7.5). The active eluate was concentrated, and the residue was added with acetone to give a colorless powder of pure validamaycin A (15 g, 987 μg/mg).

Anal. Found: C 46.64, H 7.15, N 2.95 %.

Validamycin B. The effluent from the buffered Dowex-50 WX-2 column as mentioned above was concentrated to give a residue. The residue was dissolved in water and applied to a column of Dowex-1 X-2 (OH, 200 ml), then washed with water. The aliquot of anthrone positive effluent was concentrated, and the concentrate was treated with acetone to give pure validamycin B as a coloress powder (3 g).

Anal. Found: C 45.58, H 6.92, N 2.71 %.

Validamycin A acetate. Validamycin A (450 mg) was acetylated with acetic anhydride (1 ml) in pyridine (3.5 ml) for 40 hours. The reaction mixture was poured into ice-water to yield a precipitate (600 mg). The dried precipitate was dissolved in a mixture of benzene - ethyl acetate (1 : 1), applied on a preparative thin-layer plate of silica gel (Merck), and developed with a mixture of benzene - ethyl acetate (1 : 1). Validamycin A acetate, detected with aqueous 1 % $KMnO_4$ solution (near Rf 0.7), was extracted with ethyl acetate, and the extract was concentrated. Crystallization of the concentrate from a mixture of ethyl acetate - diethyl ether gave colorless needles.

Anal. Found: C 52.55, H 6.16, N 1.60 %.

Validamycin B acetate. Validamycin B (500 mg) was acetylated with acetic anhydride (1 ml) in pyridine (5 ml) for 24 hours and the reaction mixture was evaporated. The resultant residue was washed with water and purified by column chromatography on silica gel, using a mixture of chloroform - ethyl acetate (1 : 1) as a deleloping solvent. The effluent was concentrated, and the residue was crystallized from aqueous ethanol to give colorless needles of validamycin B acetate.

Anal. Found: C 51.33, H 5.83, N 1.44 %.

Hydrolysis of validamycins A and B. Validamycin A or B (2 g) in water was refluxed with Amberlite IR-120 (H, 20 ml) for 8 hours. After filtration, the reaction mixture was treated with active carbon to remove colored impurities, and then concentrated to give a white powder. It was identified with authentic D-gulucose by paper chromatography[9] using two solvent systems of 80 % phenol and *n*-butanol - acetic acid - water (4 : 1 : 5).

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