MICROBIAL DEGRADATION OF VALIDAMYCIN A BY *FLAVOBACTERIUM SACCHAROPHILUM*

ENZYMATIC CLEAVAGE OF C–N LINKAGE IN VALIDOXYLAMINE A

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The enzymatic cleavage of C–N linkage in the degradation of validamycin A by *Flavobacterium saccharophilum* was examined using *N-p*-nitrophenyl derivatives of validamine and valienamine as synthetic model substrates for validoxylamine A. Incubation of *N-p*-nitrophenylvalidamine with the membrane fraction from the organism led to formation of *N-p*-nitrophenyl-3-ketovalidamine, and succeeding cleavage of C–N linkage. As the products of the cleavage step, one was identified as *p*-nitroaniline and another keto compound could not be purified enough because of its instability. However, on the basis of its hydrogenation products, the structure of the keto compound could be established as 5D-(5/6)-5-*C*-(hydroxymethyl)-2,6-dihydroxy-2-cyclohexen-1-one. The same experiment was carried out with *N-p*-nitrophenylvalienamine. In this case, *N-p*-nitrophenyl-3-ketovalienamine could be isolated as an intermediate but the desired keto compound from the cleavage step could not be isolated because of its instability. The participation of two enzymes, that is, a dehydrogenase and a C–N lyase on the cleavage of C–N linkage was assured, and moreover, the analysis of its products, together with those of the previous studies allow us to propose a degradation pathway of validamycin A by *Flavobacterium saccharophilum*.

Validamycin A (Fig. 1), a main component of the validamycin complex produced by *Streptomyces hygroscopicus* var. *limoneus*[1,2], is extensively used as a curative fungicide for sheath blight of rice plants, soil-borne and other diseases caused by *Basidiomycetes*. In the viewpoint of microbial clearance of fungicides in fields, we have attempted to elucidate degradation processes of validamycins by soil bacteria for the past few years, and have published several reports on the subject in this journal[3~7].

Flavobacterium saccharophilum, which was isolated from the rice field of Kanazawa city, Japan, efficiently decomposes validamycin A. From previous results obtained by using resting cells, the outline of the degradation process is described as follows: (1) validamycin A is first hydrolyzed to validoxylamine A by a β-glucosidase, (2) the competitive cleavage of two C–N bonds in validoxylamine A leads to the formation of valienamine and validamine, (3) the deamination of validamine occurs to form 1-*epi*-3-ketovalidatol and 1-*epi*-4-ketovalidatol, and (4) the ketocyclitols may be mainly utilized, presumably after cleavage of the cyclitol ring, and in part can be reduced by its dehydrogenases to four epimers of validatol.

From these studies, we have become interested in the enzymatic cleavage of the C–N linkage in validoxylamine A and the paucity of reports on such enzymes prompted us to investigate the mechanism. To simplify the reaction mechanism of the C–N cleavage, we used synthetic model compounds *N-p*-nitrophenylvalidamine and *N-p*-nitrophenylvalienamine (Fig. 2), which are cleaved at one position of C–N linkage and are easy to detect because of their yellow color.

Fig. 1.

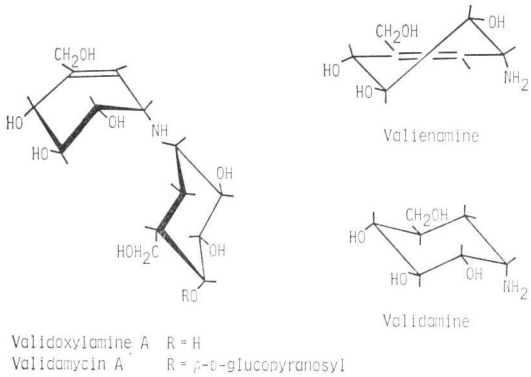
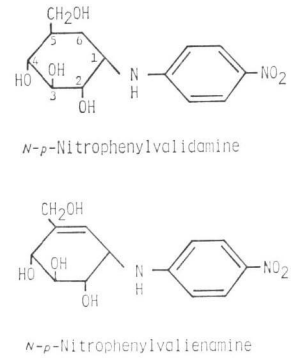


Fig. 2. Structure of model compounds.



The present paper describes the preparation of crude enzymes responsible for the cleavage, the characterization of the products and their relation to the degradation processes.

Materials and Methods

Organism and Culture Condition

A strain of *Flavobacterium saccharophilum* IFO 13984, maintained on nutrient agar, was inoculated into a 1-liter Erlenmeyer flask containing 100 ml of sterilized nutrient broth. The flask was incubated at 27°C on a reciprocating shaker (120 rpm) for 24 hours. A 1.0% (v/v) transfer of this culture growth was used to inoculate nine 5-liter Erlenmeyer flasks containing 1 liter of the same medium. The fermentation was continued for 24 hours at 27°C on the reciprocating shaker.

Preparation of Membrane Fraction

The cells from 8 liters of the culture were harvested by centrifugation at 20,000×*g* for 15 minutes, washed once with 400 ml of 0.05 M phosphate buffer (pH 7.0) containing 0.8% NaCl and suspended in 200 ml of the buffer. Batches of 100 ml of the suspension were disrupted for 6 minutes with a sonic oscilator (TOMY Model UR 2000P). Combined disrupted extracts were then centrifuged at 20,000×*g* for 15 minutes to remove cell debris. The supernatant was recentrifuged at 120,000×*g* for 60 minutes and the precipitate of membrane fraction was suspended in 200 ml of the same buffer.

Determination of the Products of the Enzymatic Degradation

Ten ml of a 2 mM *N-p*-nitrophenylvalidamine solution in 0.2 M phosphate buffer (pH 7.0) or a 2 mM *N-p*-nitrophenylvalienamine solution was added to 10-ml of the membrane suspension and the mixture was incubated at 37°C on a rotary shaker. A sample (1 ml) was withdrawn at suitable intervals and applied to a short column of Sep-pak C_{18}. The column was washed with water (2 ml) and then eluted with 80% acetonitrile (2 ml). The eluate was analyzed for the products by high performance liquid chromatography (HPLC). The HPLC was carried out using a reverse phase C_{18} μBondapak column (Waters Associates Ltd.) and 25% acetonitrile in water as a mobile phase. The substrates and products were detected by their UV absorption at 254 nm using a spectrometer.

Synthesis of *N-p*-Nitrophenylvalidamine and *N-p*-Nitrophenylvalienamine

Validamine (1.5 g) was dissolved in 50 ml of *N,N*-dimethylformamide and triethylamine (2 ml) and *p*-nitrofluorobenzene (3 ml) was added. The solution was stirred overnight at 75°C. The solvent was evaporated under reduced pressure. The residue was suspended in 200 ml of water, acidified to pH 2.0 with 2 N HCl and washed with 100 ml of toluene. The aqueous layer was extracted four times with 100 ml of 1-butanol at pH 10.0. The extract was evaporated and the residue was recrystallized from water to afford *N-p*-nitrophenylvalidamine as yellow needles (1.2 g); mp 185~188°C; $[\alpha]_D^{20}$ +95.1° (*c* 1, MeOH); *Anal* Calcd for $C_{13}H_{18}N_2O_6$: C 52.35, H 6.08, N 9.39. Found: C 52.18, H 6.21, N 9.29.

In the same manner, *N-p*-nitrophenylvalienamine was obtained as yellow needles (1.4 g); mp 188~191°C; $[\alpha]_D^{20}$ +136.0° (*c* 1, MeOH); *Anal* Calcd for $C_{13}H_{16}N_2O_6$: C 52.70, H 5.44, N 9.45. Found: C 52.63, H 5.38, N 9.19.

General

The ^1H and ^{13}C NMR spectra were obtained on a Jeol FX-100. HPLC was carried out using Waters ALC/GPC 206 equipped with an ultraviolet detector (254 nm).

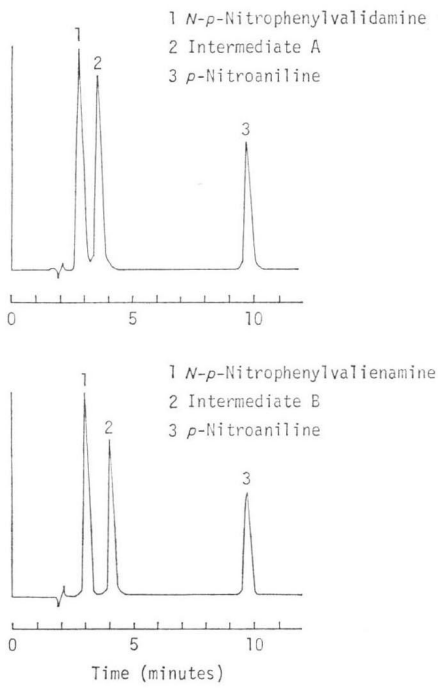
Results

Incubation of *N-p*-Nitrophenylvalidamine and *N-p*-Nitrophenylvalienamine with the Membrane Fraction of *F. saccharophilum*

When the membrane fraction was incubated with *N-p*-nitrophenylvalidamine, in the analysis of reaction mixture by HPLC, three UV absorbing peaks were observed as shown in Fig. 3. The compound of the first peak (retention time 2.9 minutes) and of the third (9.7 minutes) were identified to be the substrate (*N-p*-nitrophenylvalidamine) and *p*-nitroaniline, respectively. As shown in Fig. 4, the substrate decreased with time as *p*-nitroaniline increased. However the second peak increased at first and then decreased. From these findings, it was assumed that the second peak is an intermediate in the enzymatic cleavage. Furthermore, the abundant accumulation of the intermediate was observed when the reaction was carried out at pH 5.0, depressing the C–N cleavage in Fig. 4.

In the case of *N-p*-nitrophenylvalienamine as the substrate, similar results were obtained agreeing approximately with those expected, and the existence of an intermediate was also detected.

Fig. 3. HPLC of enzymatic reaction mixture of model compounds.
Column: μBondapak C_{18}
Mobile phase: CH_3CN - H_2O (25: 75)
Flow rate: 1 ml/minute
Detecter: UV 254 nm



Isolation and Structural Elucidation of the Intermediates

A mixture of *N-p*-nitrophenylvalidamine (250 mg) and the membrane fraction obtained

Fig. 4. Time course of enzymatic degradation reaction of *N-p*-nitrophenylvalidamine.

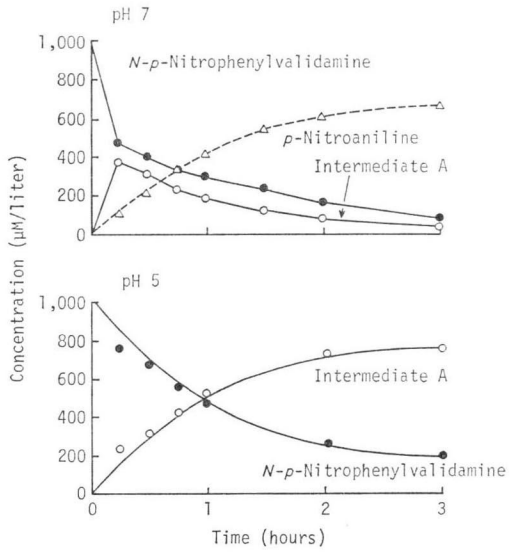


Table 1. ^{13}C NMR spectral data of intermediates A and B.

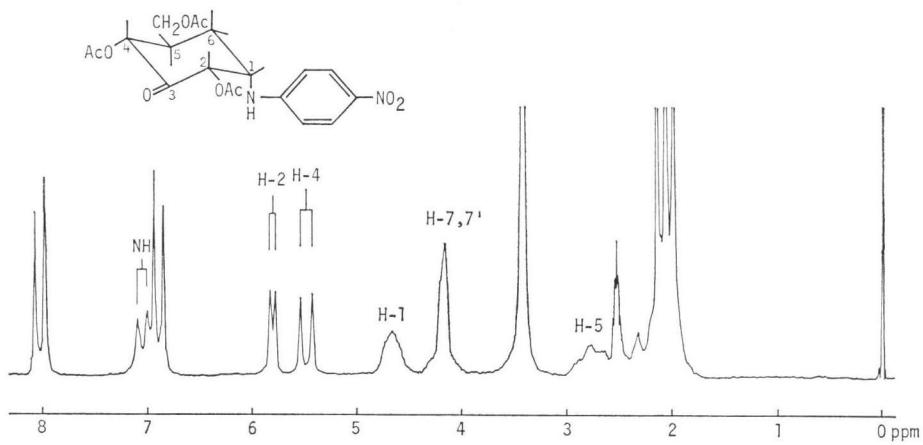
Carbon No.	Chemical shifts δ (ppm)*	
	Intermediate A	Intermediate B
C-1	56.0 (d)	55.4 (d)
C-2	73.8 (d)	71.4 (d)
C-3	209.8 (s)	207.6 (s)
C-4	75.0 (d)	74.5 (d)
C-5	42.8 (d)	144.2 (s)
C-6	27.7 (t)	118.6 (d)
C-7	61.5 (t)	60.5 (t)
C-1′	154.9 (s)	155.1 (s)
C-2′, 6′	112.0 (d)	112.0 (d)
C-3′, 5′	125.8 (d)	125.6 (d)
C-4′	136.1 (s)	136.0 (s)

* Chemical shifts are downfield from internal TMS in DMSO-d_6.

Table 2. ^1H NMR spectral data of intermediate A triacetate (100 MHz, in DMSO-d_6).

Proton	Chemical shift δ (ppm)	Coupling constant (Hz)
H-1	4.62 (1H, m)	
H-2	5.76 (1H, d)	$J_{1,2}=4.9$
H-4	5.44 (1H, d)	$J_{4,5}=11.2$
H-5	2.66 (1H, m)	
H-6	1.70~2.40 (2H)	
H-7	4.12 (2H, br s)	
H-2′, 6′	6.84 (2H, d)	$J=9.3$
H-3′, 5′	7.97 (2H, d)	$J=9.3$
NH	7.00 (1H, d)	$J=9.0$
$-COCH_3$	1.97 (3H, s)	
	2.03 (3H, s)	
	2.12 (3H, s)	

Fig. 5. ^1H NMR spectrum of intermediate A triacetate (100 MHz, in DMSO-d_6).



from 40 g wet cells in 0.05 M acetate buffer (pH 5.5, 100 ml) was incubated at 37°C for 8 hours. The reaction mixture was dialyzed against water (1 liter) at 4°C. The outer solution was applied to a column of Amberlite XAD-4 (50 ml), washed with water and eluted with MeOH (150 ml). The concentrate of the MeOH eluate was subjected to Sephadex LH-20 column chromatography (200 ml) using CH_2Cl_2 - MeOH (5: 1) to afford intermediate A as yellow needles (147 mg); mp 150~154°C; $[\alpha]_D^{20}$ −11.2° (*c* 0.5, MeOH). *Anal* Calcd for $C_{13}H_{16}N_2O_6$: C 52.70, H 5.44, N 9.45. Found: C 52.55, H 5.24, N 9.18. Intermediate A forms a triacetate; mp 160~162°C; $[\alpha]_D^{20}$ +55.0° (*c* 0.5, $CHCl_3$); MS *m/z* 422 (M^+). *Anal* Calcd for $C_{19}H_{22}N_2O_9$: C 54.03, H 5.25, N 6.63. Found: C 53.98, H 5.24, N 6.44. The IR spectrum (in KBr) of intermediate A showed the presence of a carbonyl function (1740 cm^{-1}). The ^{13}C NMR spectrum (Table 1) showed the presence of four methines, two methylenes and a carbonyl group as well as four resonances derived from the *p*-nitrophenyl group. The ^1H NMR spectrum (Fig. 5, Table 2) of the triacetate showed two characteristic doublets at δ 5.44 ($J_{4,5}=11.2$ Hz) and δ 5.76 ($J_{1,2}=4.9$ Hz), which were assigned to H-4 and H-2, respectively. These results indicate the presence of a carbonyl group at C-3

Fig. 6. Enzymatic degradation of model compounds.

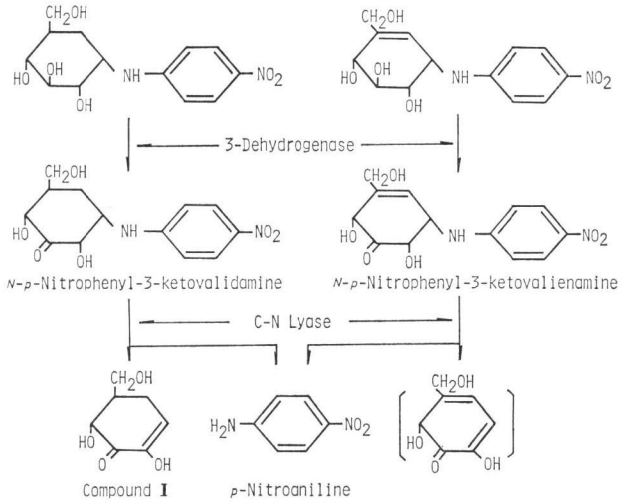
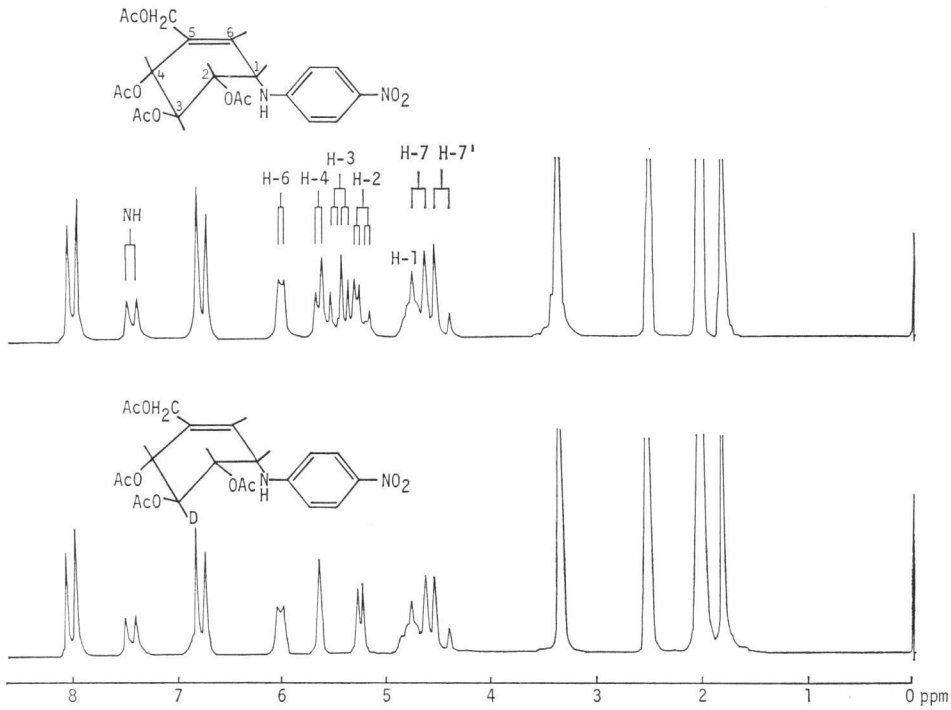


Fig. 7. ^{1}H NMR spectra of *N-p*-nitrophenylvalienamine and *N-p*-nitrophenyl[3-^{2}H]valienamine (100 MHz, in DMSO-d_6).



position. These data established the structure of intermediate A as *N-p*-nitrophenyl-3-ketovalidamine (Fig. 6).

The same experiment was carried out with *p*-nitrophenylvalienamine (250 mg) as the substrate and afforded unstable intermediate B (56 mg). The IR spectrum (in KBr) showed the presence of carbonyl function (1730 cm^{-1}). The ^{13}C NMR spectrum (Table 1) showed the presence of four methines (con-

Table 3. ^{1}H NMR spectral data of *N-p*-nitrophenylvalienamine and ^{2}H-derivatives tetraacetates (100 MHz, in DMSO-d_6).

Proton	*N-p*-Nitrophenylvalienamine tetraacetate		*N-p*-Nitrophenyl[3-^{2}H]-valienamine tetraacetate		*N-p*-Nitrophenyl[3-^{2}H]-3-*epi*-valienamine tetraacetate	
	Chemical shift (ppm)	Coupling constant (Hz)	Chemical shift (ppm)	Coupling constant (Hz)	Chemical shift (ppm)	Coupling constant (Hz)
H-1	4.74 (1H, m)		4.74 (1H, m)		4.84 (1H, m)	
H-2	5.22 (1H, dd)	$J_{1,2}$=4.5	5.22 (1H, d)	$J_{1,2}$=4.5	5.46 (1H, d)	$J_{1,2}$=4.5
H-3	5.43 (1H, dd)	$J_{2,3}$=10.2				
H-4	5.63 (1H, d)	$J_{3,4}$=6.6	5.62 (1H, s)		5.60 (1H, s)	
H-6	5.99 (1H, d)	$J_{1,6}$=4.6	5.99 (1H, d)	$J_{1,6}$=4.6	5.97 (1H, br s)	
H-7	4.44 (1H, d)	$J_{7,7'}$=13.0	4.44 (1H, d)	$J_{7,7'}$=13.0	4.58 (2H, br s)	
	4.67 (1H, d)		4.67 (1H, d)			
H-2′, 6′	6.75 (2H, d)	J=9.3	6.75 (2H, d)	J=9.3	6.83 (2H, d)	J=9.3
H-3′, 5′	7.99 (2H, d)	J=9.3	7.99 (2H, d)	J=9.3	8.00 (2H, d)	J=9.3
NH	7.42 (1H, d)	J=9.3	7.42 (1H, d)	J=9.3	7.26 (1H, d)	J=9.0
$-COCH_3$	1.79 (3H, s)		1.79 (3H, s)		1.95 (3H, s)	
	1.98 (3H, s)		1.97 (3H, s)		1.99 (3H, s)	
	2.01 (6H, s)		2.01 (6H, s)		2.03 (6H, s)	

taining a olefinic methine), a methylene, a quarternary carbon and a carbonyl group derived from the cyclitol moiety. The structure of intermediate B was confirmed by reduction with sodium borodeuteride which gave *N-p*-nitrophenyl[3-^{2}H]valienamine and -3-*epi*-valienamine in the ratio 1: 2. The ^{2}H-derivatives were subjected to Sephadex LH-20 column chromatography (170 ml) using CH_2Cl_2 - MeOH (6: 1). *N-p*-Nitrophenyl[3-^{2}H]-3-*epi*-valienamine was eluted first, followed by *N-p*-nitrophenyl[3-^{2}H]valienamine. Each ^{2}H-derivative forms a tetraacetate.

N-p-Nitrophenyl[3-^{2}H]valienamine tetraacetate: mp 70~72°C; $[\alpha]_D^{25}$ +70.0° (*c* 0.4, $CHCl_3$); MS *m/z* 465 (M^+). *Anal* Calcd for $C_{21}H_{23}N_2DO_{10}$: C 54.19, H 4.98, N 6.01. Found: C 54.32, H 5.05, N 5.88.

N-p-Nitrophenyl[3-^{2}H]-3-*epi*-valienamine tetraacetate: mp 64~66°C; $[\alpha]_D^{25}$ +66.8° (*c* 1.0, $CHCl_3$); MS *m/z* 465 (M^+). *Anal* Calcd for $C_{21}H_{23}N_2DO_{10}$: C 54.19, H 4.98, N 6.01. Found: C 54.11, H 4.94, N 5.94.

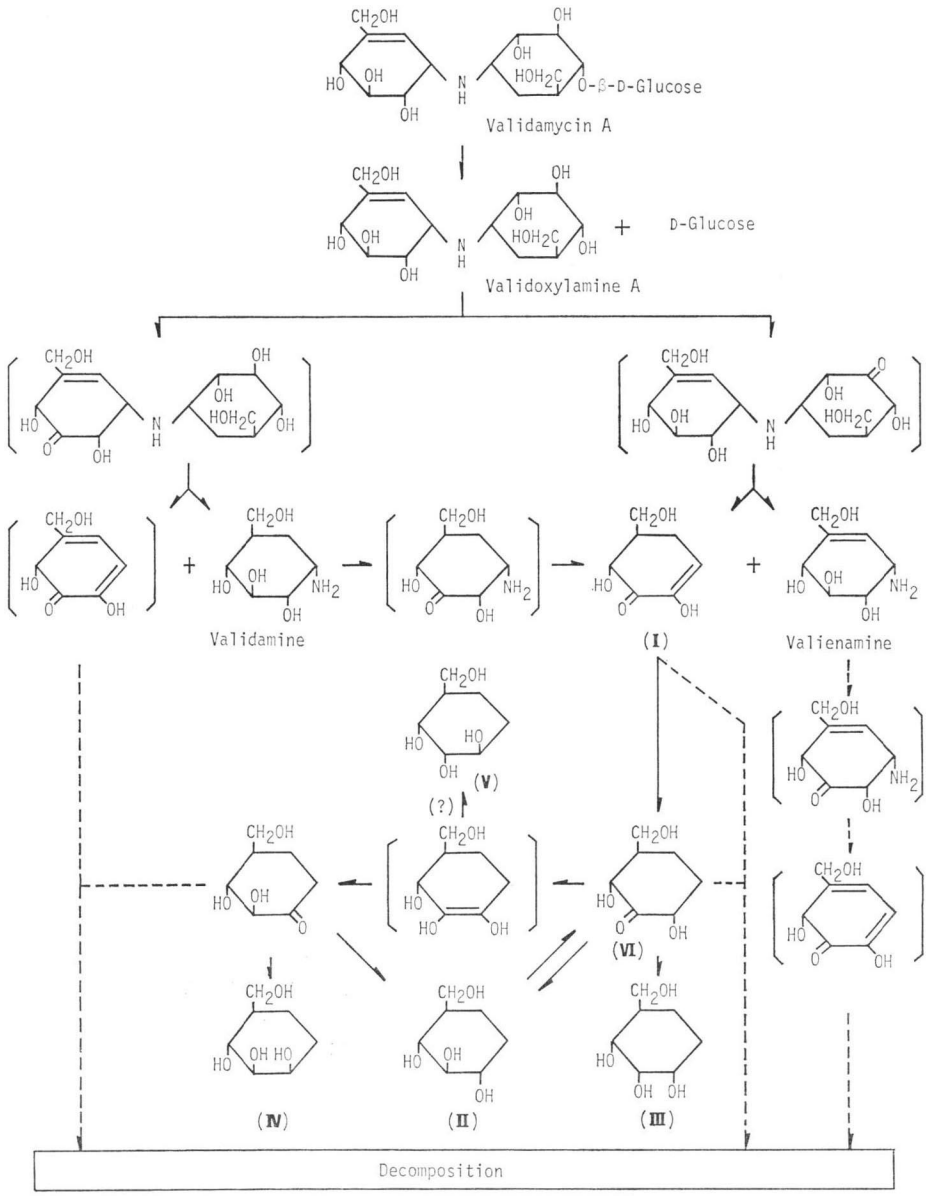
The ^{1}H NMR spectra of *N-p*-nitrophenylvalienamine and ^{2}H-derivative tetraacetates are shown in Fig. 7 and Table 3. The signals for H-3 where the deuterium substitution has taken place disappeared. The neighboring H-2 and H-4 signals were a doublet with $J_{1,2}$=4.5 and a singlet, respectively. These data established the structure of intermediate B as *N-p*-nitrophenyl-3-ketovalienamine (Fig. 6).

The existence of an enzyme which converts *N-p*-nitrophenylvalidamine to intermediate A and *N-p*-nitrophenylvalienamine to intermediate B was assured and the enzyme, identified as a dehydrogenase, facilitate the next cleavage of C–N linkage.

Enzymatic Cleavage of *N-p*-Nitrophenyl-3-ketovalidamine

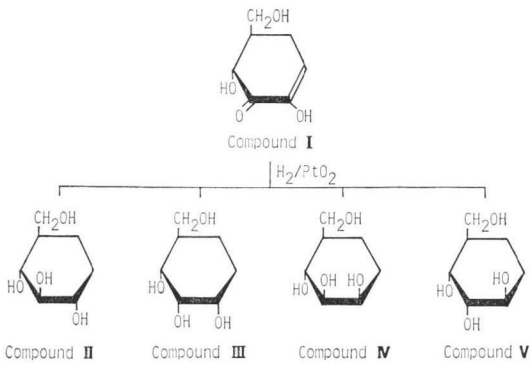
In order to isolate a product containing a 3-ketovalidamine fragment from the cleavage of the C–N linkage, the following experiment was carried out. A mixture of *N-p*-nitrophenyl-3-ketovalidamine (0.6 g) and 0.05 M phosphate buffer (pH 6.5) was incubated with the membrane fraction (from 100 g of wet cells) at 37°C for 24 hours. The incubation mixture was dialyzed against water at 4°C. The outer aqueous solution was applied to a column of Amberlite XAD-4 (50 ml) and washed with water. The

Fig. 8. Proposed degradation pathway of validamycin A by *Flavobacterium saccharophilum*.



effluent was then applied to a column of activated carbon (50 ml) and eluted with 10% EtOH. Concentration of the EtOH eluate afforded a carbonyl compound as compound **I** (210 mg). Since the compound was too unstable for identification, it was subjected rapidly to catalytic hydrogenation with Pt/H_2. When a part of the product was trimethylsilylated and subjected to the GLC analysis, four peaks appeared. The hydrogenation products were applied to Dowex 50W X8 (Ca^{2+} form, 50 ml) and Dowex 1 X2 (OH^- form, 50 ml) column chromatography with water as an eluent, to give four substances, compound **II**, **III**, **IV** and **V** (Scheme 1). These compounds were identified respectively with authentic samples, epimers of validatol which were previously reported. These findings indicate that compound

Scheme 1. Hydrogenation of compound I.



I is 5D-(5/6)-5-*C*-(hydroxymethyl)-2,6-dihydroxy-2-cyclohexen-1-one. That is, the enzyme which cleaved the C–N linkage is *p*-nitrophenyl-3-keto-validamine *p*-nitroaniline lyase.

The same experiment was carried out with *N*-*p*-nitrophenyl-3-ketovalienamine. In this case, the product could not be isolated because of its instability. *p*-Nitroaniline was recovered.

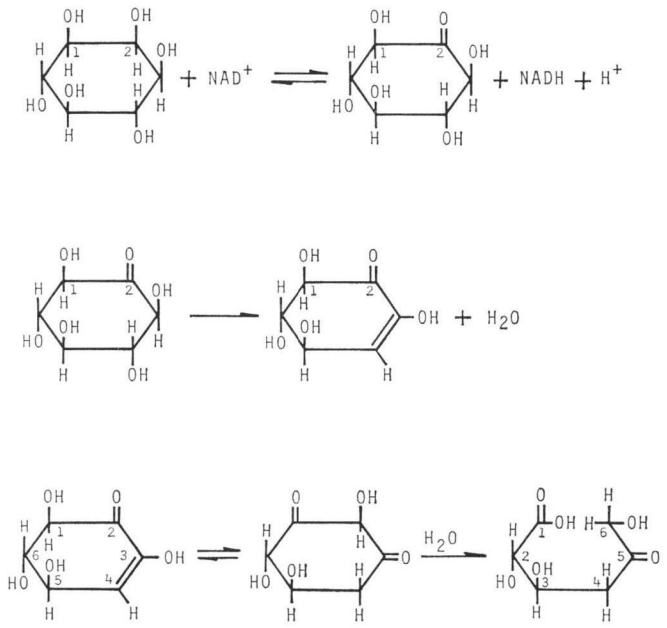
Discussion

In the beginning of this study, we presumed the participation of an unknown, rare, enzyme responsible for the C–N cleavage. However, the experimental results established that the cleavage reaction consists of two steps catalyzed by a dehydrogenase and a lyase, and moreover, the end products of the steps are the keto-enol compounds shown in Fig. 6.

The above results, together with those of the previous study, allow us to propose a degradation pathway of validamycin A by *Flavobacterium saccharophilum*, which is illustrated in Fig. 8. Validamycin A is first hydrolyzed to D-glucose and validoxylamine A. Validoxylamine A undergoes oxidation at the C-3 position of the validamine or valienamine moiety by a 3-dehydrogenase to form two 3-keto compounds. Further, elimination in each ketocyclitol moiety occurs to form two keto-enol compounds, in addition to validamine and valienamine. Validamine and valienamine are also deaminated in the similar way as mentioned above, to form the keto-enol compounds. The resulting keto-enol compounds, as BERMAN and MAGASANIK have reported on *myo*-inositol degradation in *Aerobacter aerogenes* (Scheme 2)[8,9], could be degradated by the some hydrolase into open chain compounds and further into low molecular weight compounds.

In addition, one of the keto-enol compounds, 5D-(5/6)-5-*C*-(hydroxymethyl)-2,6-dihydroxy-2-

Scheme 2. *myo*-Inositol degradation in *Aerobacter aerogenes*.[8,9]



cyclohexen-1-one (**I**), is partially reduced by some dehydrogenase to 1-*epi*-3-ketovalidatol (**VI**) and further to four epimers of validatol (**II**, **III**, **IV** and **V**).

To confirm the above hypothesis, further experiments are underway to isolate the enzymes catalyzing the individual steps of the degradation.

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