BIOSYNTHESIS OF VALANIMYCIN

MASAYUKI YAMATO*†, TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

NOBUO SAKATA, HIDEMI HAYASHI and MAKOTO HORI

Showa College of Pharmaceutical Sciences,
5-1-8 Tsurumaki, Setagaya-ku, Tokyo 154, Japan

(Received for publication March 11, 1986)

In the biosynthesis of valanimycin, valine is mostly incorporated into the isobutyl moiety of the antibiotic. The α-substituted acrylic moiety of valanimycin is derived from alanine. However, a part of alanine is metabolized into valine, thus incorporated also into the isobutyl moiety. Anaerobic conditions and some reducing agents strongly inhibit the biosynthesis.

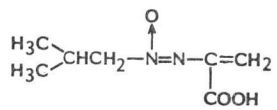
Valanimycin is a new azoxy antibiotic with antibacterial and antitumor activity[1]. Its structure (Fig. 1) suggested that valine and alanine are the precursors. The name valanimycin is derived from this assumption, which was tested in the present study. Attempts were also made to characterize the biosynthetic pathway of valanimycin.

Materials and Methods

Culture Conditions

The culture medium consisted of maltose 2%, peptone 0.05%, meat extract 0.05%, yeast extract 0.03%, NaCl 0.3% and $MgSO_4 \cdot 7H_2O$ 0.1%, the pH being adjusted to 7.2 before sterilization. This medium is referred to as the nitrogen restricted medium (NR medium). A piece of mycelial mat from a slant culture of the producing strain *Streptomyces viridifaciens* MG456-hF10 was inoculated into 100 ml of NR medium in each 500-ml shaker flask. The flasks were incubated at 30°C with shaking at 180 rpm for 24 hours and, after adding 100 mg each of the amino acids or other additives as indicated, incubated again for another 20 hours. The antibiotic titer was determined as reported[1].

Fig. 1. The structure of valanimycin.



Identification of Valanimycin in the Culture Broth

To a 1.0 ml sample of the culture broth, 0.1 ml of 2 N HCl and 0.5 ml of ethyl acetate were added, vortexed for 30 seconds and centrifuged at 2,500 rpm for 5 minutes. From the upper layer, 50 μl was taken, spotted on a plate of Kieselgel $60F_{254}$ and developed with $CHCl_3$ - MeOH - AcOH (200 : 40 : 1). Valanimycin was detected on the plate by UV light and bioautography. An authentic preparation of valanimycin was chromatographed simultaneously for comparison.

Incorporation of Radioactive Precursors into Valanimycin

Cultivation of the valanimycin producer was initiated as described above. At 24 hours of cultivation, the flasks were divided into groups of each 2 flasks. Each group received a mixture of L-[3,4(n)-

† Present address: Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machida-shi, Tokyo 194, Japan.

^{3}H]valine (62.5 μCi), L-[*U*-^{14}C]alanine (5 μCi), and a test additive which was specific to each group. Incubation continued for an additional 20 hours. The culture filtrates of two flasks of each group were combined and valanimycin was extracted with 100 ml of EtOAc at pH 3.0. The upper layer was taken, EtOAc was evaporated *in vacuo* and the residue was dissolved in 12 ml of 50% MeOH in 10 mM phosphate buffer, pH 7.0. The solution was referred to as "labeled valanimycin fraction" and stored at −20°C until analysis. To determine the incorporation ratios of radioactive precursors, a 25 μl sample of the labeled valanimycin fraction was spotted on a plate of Kieselgel 60F_{254} and developed with $CHCl_3$ - MeOH - AcOH (200: 40: 1). Valanimycin was localized on the chromatogram under UV light and silica gels of the corresponding area were scrapped off, suspended in 5.0 ml of a scintillation solution (Atomlight, New England Nuclear) and submitted to radioactivity measurement in a Beckman liquid scintillation counter (Model LS9800). The distributions of ^{3}H and ^{14}C in structural moieties of valanimycin were analyzed as follows. A 250 μl sample of the labeled valanimycin fraction containing 200 μg of valanimycin carrying 0.17 μCi of ^{3}H and 8 nCi of ^{14}C was mixed with 10 μl of 2 N HCl and 2 mg of PtO_2 and submitted to PtO_2-catalyzed reduction at 25°C for 3 hours under 2.8 kg/cm^2 of H_2 gas. A 25 μl sample of the solution was spotted on a thin-layer plate of Kieselgel 60F_{254} and developed with BuOH - AcOH - H_2O (4 : 1 : 1). Isobutylamine and alanine were located on the chromatogram by the ninhydrin reaction. From the corresponding areas, the gels were scrapped off and their radioactivities were determined as described above.

Preparation of Washed Cells

The producing strain was cultured as described previously[1] until the packed cell volume in the culture broth reached 10% (about 20 hours). The cells were harvested by centrifugation at 4°C for 15 minutes at 5,000 rpm. The supernatant was removed, the pellet was suspended in ten volumes of 0.85% NaCl solution and centrifuged as above (washing). After two more washings, the cells were suspended in two volumes of 0.85% NaCl solution and tested for biosynthetic activity of valanimycin.

Biosynthesis of Valanimycin with Washed Cells

The reaction mixture consisted of valine 0.1%, alanine 0.1%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ (unless otherwise indicated) 0.1% and indicated test components in 50 mM sodium phosphate (pH 7.0). In experiments testing effects of metal ions, 50 mM sodium phosphate was replaced by 50 mM Tris-HCl (pH 7.0) to avoid formation of insoluble complexes. To a 50-ml aliquot of the reaction mixture in a 300-ml flask, 5 ml of the cell suspension (equiv to 90 mg dry weight of cells) was added and incubation was conducted at 30°C for 24 hours at 220 rpm. Valanimycin was assayed as described previously[1].

Chemicals

L-[*U*-^{14}C]Alanine (>150 mCi/mmol), L-[3,4(n)-^{3}H]valine (25~50 mCi/mmol), L-[*U*-^{14}C]valine (290 mCi/mmol) and L-[1-^{14}C]valine (55 mCi/mmol) were purchased from Amersham International. All unlabeled amino acids were of L-form.

Results and Discussion

Production of Valanimycin in NR Medium Supplemented with either Valine or Alanine, or with their Combination

Addition of valine at 0.1% to NR medium was markedly effective in increasing the production of valanimycin; the accumulation of 330 μg/ml was 20 times that produced in the unsupplemented NR medium (Table 1). This productivity was even 2 to 3 times that obtained in the enriched production medium as reported previously[1]. Interestingly, the packed cell volume in the valine-supplemented NR medium was only 1/4 that in the enriched production medium, implying efficient conversion of valine into valanimycin rather than into the cell mass. Alanine was also effective for valanimycin production, but to a lesser extent than valine (12-fold for alanine instead of 20-fold for valine, Table 1). However, the effects of the two amino acids were not additive; the combination of valine with alanine showed only

the same effect as that of valine alone, suggesting that the production of valanimycin was depending mostly on the supply of valine and that a rate limiting step for valanimycin biosynthesis should be located before valine. The possibility was confirmed by another experiment where the effects of increasing concentration of alanine with a fixed concentration of valine on the production of valanimycin (Fig. 2A) and the effects of the reverse combination (Fig. 2B) were investigated. The results clearly demonstrated that the supply of valine was important for the valanimycin production. For a better understanding of the biosynthetic pathway of valanimycin, studies were extended to determine the effects of various amino acids, either alone or in combination with valine or alanine, on valanimycin production in NR medium. As shown in Table 1 (column a), threonine and some other amino acids, if added alone, were stimulatory to valanimycin production, but to lesser extents than valine or alanine. It should be noticed that no valanimycin analogs were found in which valine was replaced by isoleucine or threonine (see below). Pyruvate, an intermediate of valine biosynthesis, was not stimulatory due possibly to a difficulty for the

Table 1. Effect of amino acids and related compounds on the production of valanimycin.

Additive	Alone (a)	In combination with	
		Valine (b)	Alanine (c)
None	1.0	—	—
Valine	20	—	20
Alanine	12	20	—
Serine	4	3.6	—
Threonine	10	—	13.4
Glycine	5.6	—	—
Cysteine	0	7	0
Glutamic acid	3.4	10	—
Aspartic acid	2.6	7.6	—
Lysine	2	7	—
Arginine	4	13.6	—
Histidine	5	10	—
Tyrosine	2.4	10	—
Proline	4	7	—
Leucine	1	7	1
Isoleucine	0.6	20	9
Norvaline	1.6	20	14
β-Alanine	4	—	—
Homocysteine	0	—	0
α-Aminobutyric acid	0	—	0
α,γ-Diaminobutyric acid	6.6	—	6.6
Isobutylamine	5	—	—
Sodium pyruvate	1.6	—	—

Productivities of valanimycin with various additives are shown in relative values based on the productivity of the "none" shaker (15 μg/ml) being expressed as 1.0.

Fig. 2. Effect of valine and alanine on the production of valanimycin.
(A) Valine was added at 0.05% in combination with varing concentrations of alanine.
(B) In the reverse combination.

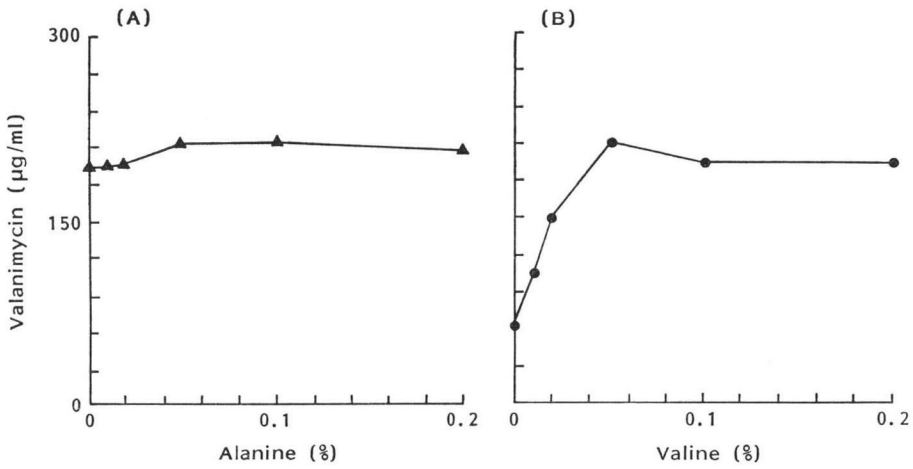
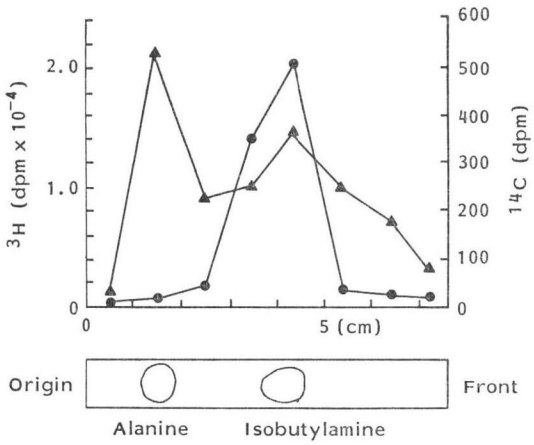


Fig. 3. Distribution of the radioactivity derived from [^{3}H]valine and [^{14}C]alanine in the moieties of valanimycin.

Valanimycin doubly labeled with [^{3}H]valine and [^{14}C]alanine was submitted to PtO_2-catalyzed reduction and decomposed into isobutylamine and alanine. The reaction product (mixture) was spotted onto a plate of Kieselgel $60F_{254}$ and developed with BuOH - AcOH - H_2O (4 : 1 : 1). Isobutylamine and alanine were detected by the ninhydrin reaction. Silica gels in the corresponding areas on the chromatogram were scrapped off and their radioactivities were determined.

●; ^{3}H, ▲; ^{14}C.



exogenous pyruvate in entering into the metabolic pool. Although valine was found to be the precursor of the isobutylamine moiety of valanimycin (see below), isobutylamine did not replace valine for valanimycin production. This observation was in contrast to the case of elaiomycin[2], another azoxy antibiotic, where the corresponding amine (*n*-octylamine) was efficiently incorporated into the antibiotic[3]. An unexpected finding was that some amino acids were rather inhibitory to valanimycin production. The inhibition could be due to interference with the biosynthetic pathway of valanimycin and/or to blocking the entry of valine or alanine into the pathway. In this respect, the effect of leucine is worth noticing; leucine was strongly inhibitory only in the combination experiments (b and c), as opposed to no significant effect when added alone (a). To explain this, a simple interpretation would be that leucine competes with valine for a common membrane transport system while it inhibits metabolic conversion of alanine to valine as an end-product of the common pathway for branched-chain amino acids.

Possible Biosynthesis of Valanimycin Analogs

As shown in Table 1 (column c), the combination of norvaline and alanine for instance showed an antibiotic titer comparable to that of the combination of valine and alanine. A question arose as to whether the former combination yielded an analog which could be named "norvalanimycin". However, the antibiotic produced was solely valanimycin which was confirmed by the ^{1}H NMR spectrum of its ammonia adduct. The same was true with other combinations, being confirmed by TLC of each product. Generally, enzymes involved in the biosynthesis of secondary metabolites have broad specificity for substrates, as exemplified by the enzymes synthesizing tyrocidins[4], tetracyclines[5] and some aminoglycosides[6~10]. In this respect, the strict substrate-specificity of the enzyme(s) responsible for valanimycin biosynthesis appears to be unique.

Distribution of the Radioactivities Derived from Labeled Valine and Alanine

Valanimycin doubly labeled with L-[3,4(n)-^{3}H]valine and L-[*U*-^{14}C]alanine was prepared as described in Materials and Methods, decomposed to isobutylamine and alanine by catalytic reduction, and examined for the distribution of radioactivities between the two moieties. As shown in Fig. 3, ^{3}H was detected only in the isobutylamine fraction while ^{14}C was both in the isobutylamine and alanine fractions. Incorporation rates for [^{3}H]valine and [^{14}C]alanine were 6.4% and 4.5%, respectively. In order to trace

Fig. 4. Distribution of radioactivities derived from L-[*U*-^{14}C] and L-[1-^{14}C]valine in valanimycin.

The valanimycin producing strain was cultured in 10 ml aliquots of NR medium in 50 ml test tubes under shaking at 30°C. At 24 hours of cultivation, 10 μCi of L-[*U*-^{14}C] or L-[1-^{14}C]valine was added to a tube and incubation was resumed for additional 20 hours. Labeled valanimycin was isolated and treated as described in Materials and Methods.

(A) The EtOAc extract of the labeled culture filtrate was concd, spotted onto a plate of Kieselgel $60F_{254}$ and developed with $CHCl_3$ - MeOH - AcOH (200 : 40 : 1). Valanimycin was detected by UV absorption.

○ L-[*U*-^{14}C]Valine, ● L-[1-^{14}C]valine.

(B) ^{14}C-Labeled valanimycin was treated as described in the legend to Fig. 3. Silica gels of the corresponding areas were scrapped off and radioactivities were determined.

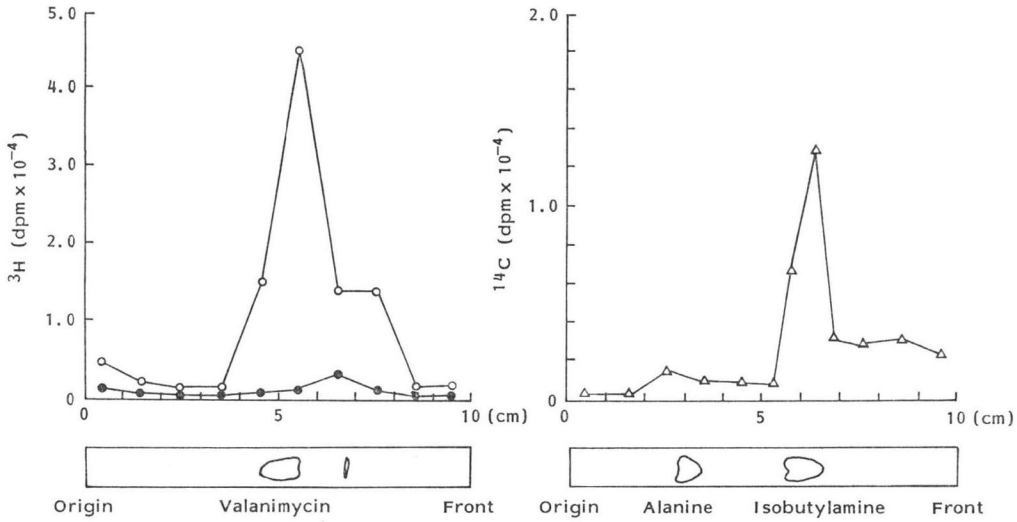
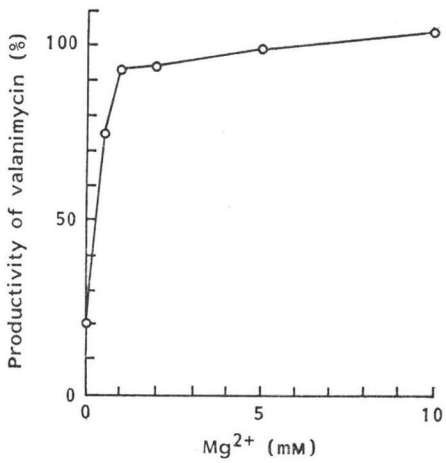


Fig. 5. Mg^{2+}-Dependency of valanimycin production with washed cells.

Productivity of valanimycin is expressed in relative values where the amount produced at 5 mM $MgSO_4$ was 100%.



the fate of the carboxyl moiety of valine, L-[*U*-^{14}C] or L-[1-^{14}C]valine was added to the culture and the ^{14}C-labeled valanimycin was isolated and treated likewise for the determination of radioactivity distribution. The [*U*-^{14}C]precursor was incorporated into the isobutyl moiety of valanimycin at an incorporation rate of 7.5% whereas the [1-^{14}C] precursor was incorporated only slightly. These results indicated that valine was incorporated efficiently into the isobutyl moiety of valanimycin after decarboxylation at some stage while alanine was incorporated in part into the α-substituted acrylic moiety and in part into the isobutyl moiety, possibly *via* metabolic conversion to valine.

Requirements for Valine and Alanine

To further characterize the valanimycin biosynthesis, some additional experiments were conducted with "washed cells" (see Materials and Methods) of the producing strain. In contrast to the results based on the studies using NR

Table 2. Inhibitory effects of various metal ions.

Metal ions (1 mM)	Relative amount of valanimycin produced (%)
Mg^{2+}	100
Mg^{2+} plus	
Mn^{2+}	0
Zn^{2+}	0
Cu^{2+}	0
Co^{2+}	0
Fe^{2+}	0
Fe^{3+}	80
Ca^{2+}	67

Metal salts used were $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$.

Table 3. Effects of various compound on the production of valanimycin.

Compound added	Concentrations		Valanimycin produced (relative value)
	mM or %		
None			100
NaCN	0.01		10
NaN_3	1		4
Cysteine	1		0
Ascorbic acid	10		0
Hydroquinone	5		182
	10		255
Triton X-100		0.1	0
EtOH		1	20

Table 4. Consumption of valine and alanine in washed cells under conditions where valanimycin biosynthesis was inhibited.

	Before incubation	After incubation with an inhibitor						
		None (control)	Cys	Asc	NaN_3	Mn^{2+}	Cu^{2+}	Co^{2+}
Val	909 μg/ml	347	940	633	878	322	729	910
Ala	909	4	—	30	518	17	—	806

Concentrations of compounds added were as follows:
Cys (cysteine) 5 mM, Asc (ascorbic acid) 10 mM, NaN_3 5 mM, Mn^{2+} 1 mM, Cu^{2+} 1 mM, Co^{2+} 1 mM.
The amino acid analysis was performed with a Jeol JLC-200A amino acid analyzer. Cysteine was converted to cystine after incubation.

medium (Table 1), the requirement for the simultaneous presence of valine and alanine was demonstrated with "washed cells" for the best production of valanimycin; the amounts produced were 80 μg/ml for the combination, 7 μg/ml for valine alone and 4 μg/ml for alanine alone. The obvious requirement for alanine should reflect the depletion of endogenous alanine in washed cells. This experiment again confirmed that valine and alanine are the direct precursors of the antibiotic.

Mg^{2+}-Dependency

As shown in Fig. 5, the antibiotic production was dependent on Mg^{2+}. EDTA at 5 mM completely abolished the effect of 5 mM Mg^{2+} (data not shown). Other metal ions were rather inhibitory if present with Mg^{2+} (Table 2). Fe^{2+} was more strongly inhibitory than Fe^{3+}. A possible explanation for the difference would be that Fe^{2+} acts as an electron donor, or a reducing agent, interfering with the oxidative process involved in valanimycin biosynthesis (see below).

Other Characteristics

Valanimycin was not produced at all under anaerobic conditions; the air in an incubation flask was replaced by N_2 and the flask was immediately sealed with a rubber stopper, otherwise the conditions were the same as those for the optimum production (data not shown). Cyanide and azide were inhibitory, as were cysteine and ascorbic acid (Table 3). An oxygenase is likely to be involved in the formation of the *N*-oxide structure and the inhibitory effects of Fe^{2+}, cysteine and ascorbic acid may be due to competition with the hypothetical substrate as an electron donor. Specific inhibition by

cysteine of valanimycin synthesis was demonstrated by the results shown in Table 4. In the presence of cysteine, the metabolic consumption of only valine but not alanine was totally inhibited. During the incubation period, cysteine itself was converted to cystine. Cysteine seems to inhibit the first step of the metabolic conversion of valine, that is oxidation (probably coupled with decarboxylation). The effects of ascorbic acid and Cu^{2+} were similar to that of cysteine (Table 4). Hydroquinone differed from the other reducing agents in its stimulatory effect on valanimycin biosynthesis (Table 3). The cycle between hydroquinone and quinone may facilitate the electron transfer between the substrate and oxygen as an artificial coenzyme. The inhibitory effects of ethanol and Triton X-100 (Table 3) suggest a role of some membraneous structure in the biosynthesis.

References

1) YAMATO, M.; H. IINUMA, H. NAGANAWA, Y. YAMAGISHI, M. HAMADA, T. MASUDA, H. UMEZAWA, Y. ABE & M. HORI: Isolation and properties of valanimycin, a new azoxy antibiotic. J. Antibiotics 39: 184~191, 1986
2) STEVENS, C. L.; B. T. GILLIS, J. C. FRENCH & T. H. HASKELL: Elaiomycin. An aliphatic α,β-unsaturated azoxy compound. J. Am. Chem. Soc. 80: 6088~6092, 1958
3) PARRY, R. J.; H. S. P. RAO & J. MUELLER: Biosynthesis of elaiomycin. 1. Incorporation of labeled forms of *n*-octylamine. J. Am. Chem. Soc. 104: 339~340, 1982
4) ROSKOSKI, R., Jr.; H. KLEINKAUF, W. GEVERS & F. LIPMANN: Isolation of enzyme-bound peptide intermediates in tyrocidin biosynthesis. Biochemistry 9: 4846~4851, 1970
5) NEIDLEMAN, S. L.; E. ALBU & E. BIENSTOCK: Biosynthesis of 7-chloro-6-demethyltetracycline in the presence of certain homocysteine derivatives and methoxinine. Biotechnol. Bioeng. 5: 87~89, 1963
6) TESTA, R. T.; G. H. WAGMAN, P. J. L. DANIELS & M. J. WEINSTEIN: Mutamicins; biosynthetically created new sisomicin analogues. J. Antibiotics 27: 917~921, 1974
7) SHIER, W. T.; K. L. RINEHART, Jr. & D. GOTTLIEB: Preparation of four new antibiotics from a mutant of *Streptomyces fradiae*. Natl. Acad. Sci. 63: 198~204, 1969
8) KOJIMA, M. & A. SATOH: Microbial semi-synthesis of aminoglycosidic antibiotics by mutants of *S. ribosidificus* and *S. kanamyceticus*. J. Antibiotics 26: 784~786, 1973
9) CLARIDGE, C. A.; J. A. BUSH, M. D. DEFURIA & K. E. PRICE: Fermentation and mutation studies with a butirosin-producing strain of *Bacillus circulans*. Dev. Ind. Microbiol. 15: 101~113, 1974
10) NAGAOKA, K. & A. L. DEMAIN: A mutant of *Streptomyces griseus* dependent upon exogenous streptidine for streptomycin production. Bacteriol. Proc. p. 19, 1974