CONTROLLED BIOSYNTHESIS OF NEOVIRIDOGRISEINS, NEW HOMOLOGUES OF VIRIDOGRISEIN

II. PRODUCTION, BIOLOGICAL PROPERTIES AND STRUCTURE OF NEOVIRIDOGRISEIN II*

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Our isolate of *Streptomyces griseoviridus*, which produced three minor factors in addition to viridogrisein and griseoviridin, was more sensitive to the addition of L-proline than the type culture of *Streptomyces griseoviridus* (NRRL2427). Yield improvement of these minor factors was attempted by the so-called controlled biosynthesis with L-proline. Addition of L-proline increased the production of neoviridogrisein II in which *allo*-hydroxy-D-proline in viridogrisein was replaced by D-proline.

Neoviridogriseins, the first biochemical homologues of viridogrisein[1], which are composed of eight constituent moieties, are produced by *Streptomyces* sp. P8648[2]. In a previous paper[2], we reported that *Streptomyces* sp. P8648 was identical with *Streptomyces griseoviridus* (NRRL 2427)[3], though some minor physiological differences were observed between the type culture and our isolate. *Streptomyces* sp. P8648 produces five antibiotics under normal fermentation conditions. Among them two factors, viridogrisein[4] and griseoviridin[4], are produced in large quantities, whereas the other three factors are only minor compounds. The three minor factors are named neoviridogriseins I, II and III. They differ from viridogrisein in the *allo*-hydroxy-D-proline and/or L-alanine moiety(ies).

As the new factors were minor products under normal fermentation conditions, the yield improvement of them was tried by the so-called controlled biosynthesis with amino acids. As it is described in a subsequent paper, several amino acids have been found to exert some effects on the composition of neoviridogriseins. Among them, proline and α-amino-*n*-butyric acid seemed to be directly incorporated into neoviridogriseins. This paper deals with the production, structure and some antimicrobial activities of neoviridogrisein II in which *allo*-hydroxy-D-proline of viridogrisein is replaced by D-proline.

Materials and Methods

1. Materials

(1) Antibiotics and reagents

Viridogrisein and griseoviridin were kindly supplied by Parke, Davis & Co., Detroit 32, Mich., U.S.A. and etamycin by Bristol Laboratories Inc., Syracuse, New York, U.S.A. Pre-coated silica gel plates (Pre-coated Silica Gel TLC Plates Silica Gel 60 F-254) and silica gel for column chromatography (Kieselgel 60 Art. 7734) were obtained from E. Merck, Darmstadt, Germany; pre-coated cellulose sheets (Eastman Chromagram Sheet No. 6065) from Eastman Kodak Co., Rochester, New York,

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U.S.A.; and Sephadex LH-20 from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Authentic amino acids were purchased from Sigma Chemicals, St. Louis, Mo., U.S.A.; L-amino acid oxidase (Snake venom, Cat. No. 109792), D-amino acid oxidase (Hog kidney, Cat. No. 102776), catalase (Beef liver, Cat. No. 106810), peroxidase (Horse-radish, Cat. No. 108081) and FAD (Cat. No. 104728) were from Boehringer Mannheim Japan Co., Tokyo, Japan, and brain heart infusion broth from Difco Laboratories, Detroit, Mich., U.S.A. Millipore filter (Cat. No. GSWP 01300) was purchased from Nihon Millipore Ltd., Tokyo, Japan. Other materials were obtained from commercial sources.

(2) Microorganisms

Staphylococcus aureus FDA 209P, *S. aureus* Smith, *Sarcina lutea*, *Bacillus subtilis* (ATCC6633), *Escherichia coli* K-12, *Pseudomonas aeruginosa* P-1, *Salmonella gallinarum* (ATCC9184) and *Candida albicans* were from our collection of cultures. *Diplococcus pneumoniae* Type I and *Streptococcus pyogenes* NY-5 were kindly supplied by The Institute of Medical Science, University of Tokyo, Tokyo, Japan; *Proteus vulgaris* GN76 was obtained from Prof. S. YAMAGISHI, Chiba University, Chiba, Japan; and various strains of *S. aureus* from Dr. H. KAWAGUCHI, Bristol-Banyu Co., Tokyo, Japan.

2. Methods

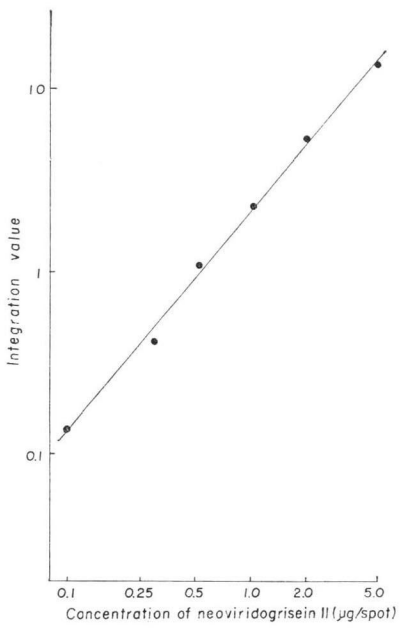
(1) Fermentation

One loopful of *Streptomyces* sp. P8648 maintained on a glucose-yeast extract-malt extract agar slant was inoculated in a 250-ml Erlenmeyer flask containing 50 ml of the medium described in a previous paper[2]. The flask was incubated at 28°C for 72 hours on a rotary shaker. Two milliliters of the seed culture were transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium GYM: 0.4% glucose, 0.4% yeast extract, 1.0% malt extract and the indicated concentration of L-proline. The flask was incubated at 28°C for 144 hours on a rotary shaker at 220 rpm.

(2) Quantitative component analysis

Extraction and thin-layer chromatography of neoviridogriseins were described in a previous paper[2]. Neoviridogriseins were separated on a silica-gel thin-layer plate and then quantitatively determined by fluorometry. Under UV-light (365 nm), neoviridogriseins emitted brilliant fluorescence (400 nm). Intensity of the fluorescence, which was proportional to the amount of neoviridogriseins, was recorded with a Shimadzu CS-900 Chromato-Scanner. Analytical conditions were as follows: Apparatus: Dual-wavelength TLC scanner, Shimadzu Co., Type CS-900. Slit: 10 mm × 0.8 mm. Excitation: 365 nm. Interference filter: 400 nm. Scanning speed: 8 cm/min. The standard curve of neoviridogrisein II is shown in Fig. 1.

Fig. 1. Standard curve of neoviridogrisein II by fluorometry.



(3) Physico-chemical analysis

Melting points, IR spectra, UV spectra, mass spectra and optical rotations were measured with Yazawa's micro-hot stage, Hitachi Infrared Spectrophotometer, model 260-30, Hitachi Spectrophotometer, model 220-20, Hitachi RMU-7L mass spectrometer, and JASCO DIP-180 polarimeter, respectively.

(4) Amino acid analysis

Neoviridogrisein II was hydrolyzed in 6 N HCl at 120°C for 16 hours in a sealed tube. The hydrolysate was evaporated to dryness and subjected to two-dimensional thin-layer chromatography on a cellulose sheet. The first dimension was developed by electrophoresis at 50 V/cm for 60 min in formate-acetate buffer (HCOOH - CH_3COOH - H_2O = 25 : 75 : 900, pH 1.8) and the second dimension by chromatography in *n*-butanol - CH_3COOH - H_2O (4 : 1 : 1). The amino acids were made visible with ninhydrin or isatin reagent spray and iden-

tified by comparison with an authentic viridogrisein hydrolysate and authentic amino acids.

(5) Configuration of proline

The configuration of proline in neoviridogrisein II was determined by the enzymatic procedure.

Total acid hydrolysate of neoviridogrisein II was oxidized by L-amino acid oxidase or D-amino acid oxidase according to the procedure of WELLNER and LICHTENBERG[5]. Before and after oxidation, the content of proline was determined by the method of TROLL and LINDSLEY[6].

Two milligrams of neoviridogrisein II were hydrolyzed in 6 N HCl at 120°C for 16 hours in a sealed tube. The hydrolysate was evaporated to dryness. Half the amount of the hydrolysate was dissolved in 1 ml of Tris-HCl buffer (pH 7.5 at 37°C, 0.4 M) for L-amino acid oxidase reaction and the rest in 1 ml of sodium pyrophosphate buffer (pH 8.3, 0.1 M) for D-amino acid oxidase reaction.

(a) Oxidation by L-amino acid oxidase: To 0.5 ml of a solution containing Tris-HCl buffer (40 μmoles, pH 7.5), peroxidase (30 units) and L-amino acid oxidase (1 unit), 10 μl of the hydrolysate solution was added and incubated at 37°C for 30 minutes. Before and after oxidation, the content of proline was measured by the method of TROLL and LINDSLEY[6].

(b) Oxidation by D-amino acid oxidase: A reaction mixture contained, in a total volume of 0.5 ml, sodium pyrophosphate buffer (10 μmoles, pH 8.3), catalase (30 units), FAD (5 μg), D-amino acid oxidase (0.5 unit) and 10 μl of the hydrolysate solution. The reaction was carried out at 37°C for 30 minutes. The concentration of proline was determined before and after reaction by the method of TROLL and LINDSLEY[6].

(6) Minimum inhibitory concentration

Test antibiotics were dissolved in a small amount of methanol and suitably diluted in distilled water. Minimum inhibitory concentrations were determined by the broth dilution technique: 0.5 ml of brain heart infusion broth was distributed into small test tubes and autoclaved at 120°C for 15 minutes. Test antibiotic solutions were sterilized by ultra-filtration using Millipore filter (Cat. No. GSWP 01300) and added to the test tubes at the indicated concentrations. The test microorganisms were inoculated at approximately 10^6 viable cells/ml into the test tubes and incubated at 37°C for 24 hours. The minimum inhibitory concentration was the concentration of an antibiotic at which no growth could be detected. When the minimum inhibitory concentrations against *Streptococcus pyogenes* NY-5 and *Diplococcus pneumoniae* Type I were determined, brain heart infusion broth was supplemented with 10% of defibrinated horse blood. Malt extract-yeast extract medium was used for the measurement of the minimum inhibitory concentration against *Candida albicans*.

Results

1. Production of Neoviridogrisein II

The effect of L-proline on production of neoviridogrisein II was examined. Generally, the synthesis of neoviridogriseins begins *ca.* 48 hours after inoculation and continues for 6 days. The neoviridogriseins mixture was subjected to component analysis by thin-layer chromatography followed by fluorometry.

As it is apparent from Table 1, the production of neoviridogrisein II increased in parallel with the concentration of L-proline added in the medium. The clear effect of L-proline on production of neoviridogrisein II was observed at concentrations above 0.2%, but not below 0.1%. But, for neoviridogrisein II to be produced in a substantial amount compared with viridogrisein, there seemed to be a minimum concentration of L-proline under specified fermentation conditions.

Time course of fermentation of *Streptomyces* sp. P8648 in the medium containing 0.4% L-proline is shown in Fig. 2. Neoviridogriseins I and III were hardly detected in this fermentation.

Neoviridogrisein II was recovered from the broth in the manner described in a previous paper[2].

Table 1. Effect of L-proline on production of neoviridogriseins.

Concentration of L-proline (%)	5 days			6 days		
	pH	NVG II* (μg/ml)	VG** (μg/ml)	pH	NVG II* (μg/ml)	VG** (μg/ml)
0	6.80	trace	57.0	7.20	trace	64.5
0.05	6.80	1.70	68.5	7.25	1.95	63.5
0.10	6.75	3.36	64.4	7.30	3.92	60.0
0.20	6.60	24.8	62.0	7.30	28.4	66.0
0.40	6.85	38.0	63.0	7.33	45.0	78.0

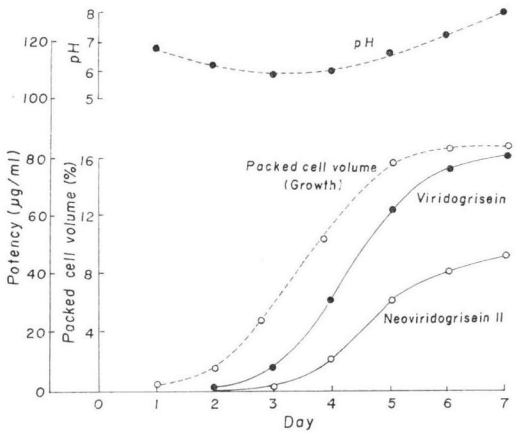
Basal medium (GYM): glucose 0.4%, yeast extract 0.4% and malt extract 1.0% (pH 6.5)
*NVG II: neoviridogrisein II
**VG: viridogrisein (=neoviridogrisein IV)

2. Physico-chemical Properties of Neoviridogrisein II

Physico-chemical properties of neoviridogrisein II are summarized in Table 2. IR and mass spectra of neoviridogrisein II are shown in Figs. 3 and 4, respectively.

UV spectrum of neoviridogrisein II was almost identical with that of viridogrisein. That is, λ_{max} 305 nm (in MeOH, $E^{1\%}_{1cm}$ 90) and λ_{max} 340 nm (in MeOH - 0.1 N NaOH, $E^{1\%}_{1cm}$ 84) were shown. IR spectrometry also indicated its similarity to viridogrisein, except that the intensity of absorption derived from the hydroxyl groups was less in the former. At this point, the smaller number of hydroxyl groups in neoviridogrisein II was suggested. Molecular weight of neoviridogrisein II was determined to be 862 by mass spectrometry. The difference of molecular weight(16) also supported the above assumption.

Fig. 2. Time course of fermentation of *Streptomyces* sp. P8648 in medium GYM containing 0.4% L-proline.



Neoviridogrisein II was compared with viridogrisein in the total acid-hydrolysate by two-dimensional thin-layer chromatography (Fig. 5).

Table 2. Physico-chemical properties of neoviridogrisein II and viridogrisein.

	Neoviridogrisein II	Viridogrisein
Melting point	145°C	160°C
Optical rotation	$[\alpha]_D^{23}$ −39.3° (*c* 1, MeOH)	$[\alpha]_D^{25}$ +50° (*c* 5, $CHCl_3$)
Molecular weight (MS*)	862	878
UV Absorption	λ_{max} 305 nm (in MeOH)	λ_{max} 304 nm (in EtOH)
($E^{1\%}_{1cm}$)	(90)	(92)
	λ_{max} 340 nm (in MeOH - 0.1 N NaOH)	λ_{max} 335 nm (in EtOH - 0.1 N NaOH)
($E^{1\%}_{1cm}$)	(84)	(82)

MS*: mass spectrometry

Fig. 3. IR spectrum of neoviridogrisein II (KBr).

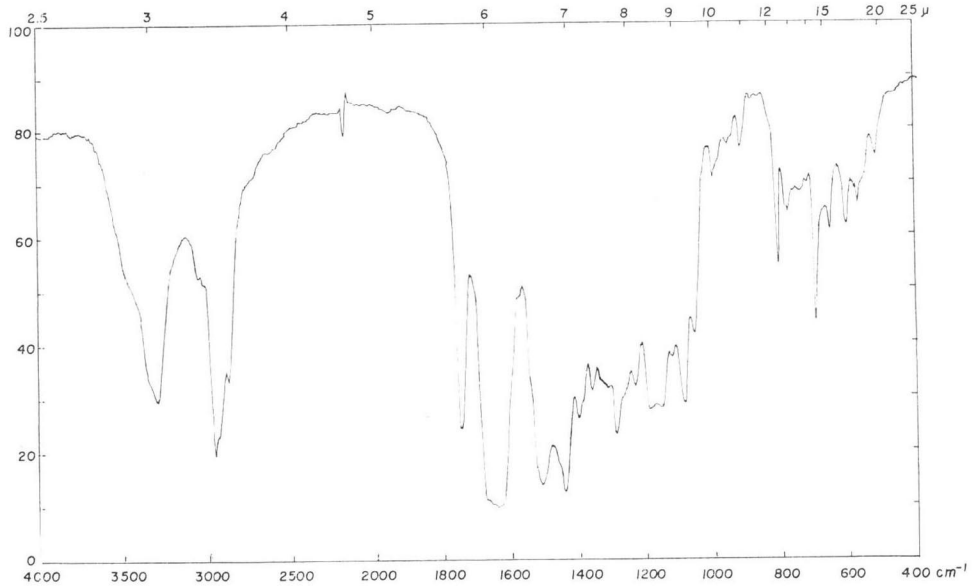
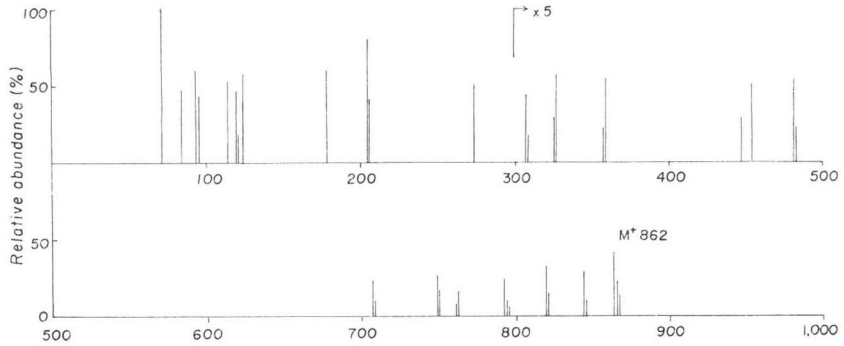


Fig. 4. Mass spectrum of neoviridogrisein II.



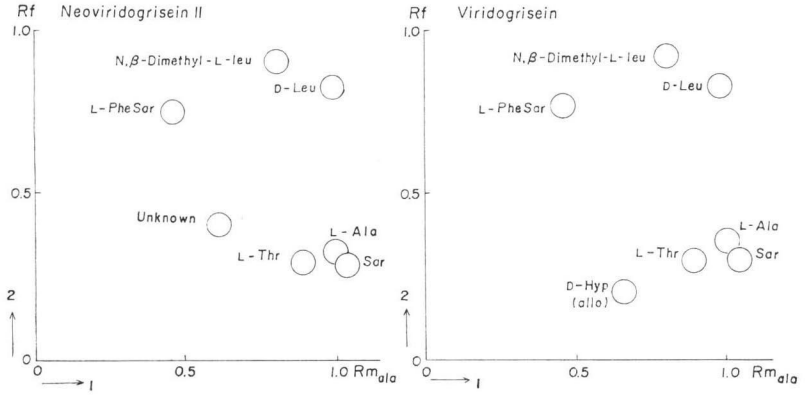
The following constituent amino acids of neoviridogrisein II were identical with those of viridogrisein: 3-Hydroxypicolinic acid, L-threonine, D-leucine, sarcosine, N,β-dimethyl-L-leucine, L-alanine and L-phenylsarcosine. In neoviridogrisein II, *allo*-hydroxy-D-proline was absent, and instead there was one unknown isatin-positive amino acid. It was compared with authentic amino acids, and particularly with proline, by the chromatographic procedure and finally identified as proline.

3. Configuration of Proline

The proline moiety of neoviridogrisein II which replaced *allo*-hydroxy-D-proline in viridogrisein was subjected to the determination of configuration by the enzymatic procedure described in Materials and Methods. Based on the results that proline in neoviridogrisein II was oxidized by D-amino acid oxidase but not L-amino acid oxidase, we concluded that the configuration of proline was the D-form. From all the physico-chemical (and biological) data described above, the structure of neoviridogrisein II was determined as shown in Fig. 6.

Fig. 5. Two-dimensional thin-layer chromatograms of the acid-hydrolysates of neoviridogrisein II and viridogrisein.

Plate: cellulose sheet (Eastman Chromagram Sheet No. 6065)
First dimension: electrophoresis at 50 V/cm for 60 minutes in formate-acetate buffer ($HCOOH - CH_3COOH - H_2O = 25 : 75 : 900$, pH 1.8)
Second dimension: chromatography (*n*-butanol - CH_3COOH - $H_2O = 4 : 1 : 1$)
Coloration: ninhydrin or isatin reagent spray



4. Antimicrobial Properties

The antimicrobial activity of neoviridogrisein II was compared with that of viridogrisein using the broth dilution technique. The results are shown in Table 3.

Like viridogrisein, in general, neoviridogrisein II was active against Gram-positive bacteria only. It drew our attention that neoviridogrisein II looked more active than viridogrisein on

Fig. 6. Structure of neoviridogrisein II.

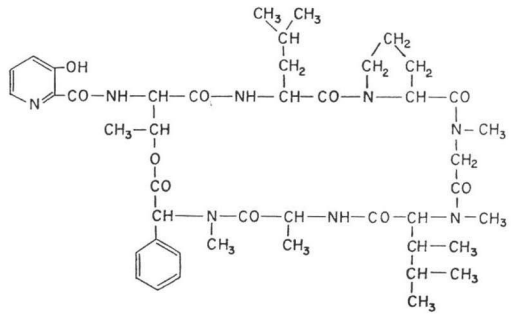


Table 3. Minimum inhibitory concentrations of neoviridogrisein II and viridogrisein.

Microorganism	Medium	Neoviridogrisein II (μg/ml)	Viridogrisein (μg/ml)
Staphylococcus aureus FDA 209P	(1)	0.1	0.2
Staphylococcus aureus Smith	(1)	0.2	0.4
Diplococcus pneumoniae Type I	(2)	0.4	0.4
Streptococcus pyogenes NY-5	(2)	0.2	0.4
Sarcina lutea	(1)	0.4	0.4
Bacillus subtilis ATCC6633	(1)	0.4	0.8
Salmonella gallinarum	(1)	>25	>25
Escherichia coli K-12	(1)	>25	>25
Pseudomonas aeruginosa P-1	(1)	>25	>25
Proteus vulgaris GN 76	(1)	>25	>25
Candida albicans	(3)	>25	>25

Medium (1): Brain heart infusion broth (pH 7.0)
(2): Brain heart infusion broth containing 10% horse blood (pH 7.0)
(3): Malt extract-yeast extract medium
Inoculum size: 10^6 cells/ml

Table 4. Comparison of neoviridogrisein II with viridogrisein in the antimicrobial activity against resistant strains of *Staphylococcus aureus*.

Microorganism	Minimum inhibitory concentration (μg/ml)	
	Neoviridogrisein II	Viridogrisein
Staph. aureus FDA209P	0.078	0.133
(EM, CP, SM, PC, TC)^r	0.125	0.334
(TC, CP, PC)^r	0.094	0.334
BX-1633 (PC)^r	0.125	0.267
Russell (PC)^r	0.125	0.267
Smith	0.125	0.267

Medium: brain heart infusion broth (pH 7.0)
Inoculum size: 10^6 cells/ml
Abbreviation: ( )^r: resistant, EM: erythromycin, CP: chloramphenicol, SM: streptomycin, PC: penicillin G, TC: tetracycline.

Staphylococcus aureus FDA 209P, *Streptococcus pyogenes* NY-5 and *Bacillus subtilis* (ATCC 6633). For further confirmation, we compared in detail the antimicrobial activities of neoviridogrisein II and viridogrisein on several strains of *Staphylococcus aureus* which were resistant to various antibiotics (Table 4). Table 4 clearly shows that neoviridogrisein II possessed 1.7 to 3.5 times higher activity than viridogrisein on the resistant strains of *Staphylococcus aureus*. Moreover, though data are not shown, the synergistic effect of neoviridogrisein II and mikamycin A group antibiotics was proved to be superior to that of viridogrisein on the strains listed in Table 3.

Discussion

There have been many analogues reported in the mikamycin B group of antibiotics[7] which consist of seven moieties, that is, 3-hydroxypicolinic acid, L-threonine, L-proline, L-phenylglycine and three amino acids. But no homologues of viridogrisein[1] (=etamycin[8]) which consists of eight moieties have been reported.

The controlled biosynthesis of actinomycin has extensively been studied for the purpose of obtaining more useful and less toxic analogues. As far as the proline moieties in actinomycin are concerned, azetidine-2-carboxylic acid[9], hydroxyproline[10], methylproline[11], pipecolic acid[12] and sarcosine[13] are known to replace proline. The mechanism of biosynthesis of hydroxyproline by *Streptomyces griseoviridus* (strain 04955)[14,15] seems different from that in animals[16]. In animal collagen, the ribosome-dependent synthesis of proline-rich polypeptide precursors precedes the hydroxylation reaction. By contrast, the synthesis of viridogrisein is considered to be independent of the ribosome system, because chloramphenicol did not inhibit the biosynthesis[15]. Furthermore, free hydroxyproline can not be a precursor of hydroxyproline moieties in collagen, whereas, in the microbial system, L-hydroxyproline-^{3}H-(G) was reported to be readily and specifically incorporated into viridogrisein[14]. HOOK and VINING[14] observed that labeled 4-hydroxy-L-proline was incorporated more specifically and with less dilution than labeled L-proline into the *allo*-hydroxy-D-proline moiety of viridogrisein and concluded that the initial step of hydroxyproline synthesis was probably a hydroxylation reaction followed by inversion of configuration. But, an alternative explanation for their results would be that L-proline was far more easily utilized for protein synthesis or metabolized by the organism than L-hydroxyproline. As their incorporation experiments were not carried out under the conditions where the protein synthesis in the organism was stopped by an inhibitor of protein synthesis such as chloramphenicol, their conclusion must be appreciated with some limitation.

In this paper, the addition of L-proline significantly affected the synthesis of neoviridogrisein II while the production of viridogrisein was not influenced at least under the specified experimental con-

ditions (Table 1). It is interesting to note that D-proline did not affect the synthesis of neoviridogrisein II. This suggests that the enzyme system of neoviridogriseins biosynthesis had strict specificity for the L-configuration. The effect of D-proline and other D-amino acids will be reported in a subsequent paper. If the initial step of synthesis of hydroxyproline was the hydroxylation of free proline, the addition of L-proline would result in increased production of viridogrisein without neoviridogrisein II. Our data seem to indicate the opposite effect. That is, the initial step of synthesis of the hydroxyproline moiety from proline would not be the hydroxylation of proline. Presumably exogenous L-proline would be incorporated into the cells and would compete with endogenous L-proline for production of neoviridogriseins. Intracellular L-proline would first be converted to peptidyl-L-proline, peptidyl-D-proline or neoviridogrisein II before proline is hydroxylated to provide viridogrisein. In other words, though not yet proved, neoviridogrisein II will probably be a precursor of viridogrisein. A plausible explanation for the significant incorporation of exogenous L-hydroxyproline into viridogrisein would be that the enzyme system catalyzing the activation and incorporation of proline into the antibiotic peptide possessed such a low or broad specificity that hydroxyproline would not be distinguished from proline in the neoviridogriseins formation.

Neoviridogrisein II differs from viridogrisein solely in the site of the imino acid. But neoviridogrisein II was proved to be 1.7~3.5 times more active than viridogrisein against some bacteria (Tables 3 and 4). In actinomycins, there occurs a profound reduction in antimicrobial activity when the proline moieties are replaced by proline analogues such as hydroxyproline[10], methylproline[11], pipecolic acid[12] and sarcosine[13], but not azetidine-2-carboxylic acid[9]. For example, actinomycin II (in which two moles of proline are replaced by two moles of sarcosine) and actinomycin III (in which one mole of proline is replaced by one mole of sarcosine) possess 2~4 times less activity of actinomycin IV on *Staphylococcus aureus* and *Bacillus subtilis*[18]. In actinomycins, the imino acid moieties must occupy one of the important positions to express antimicrobial activity. On the other hand, the antimicrobial activity of gramicidin S[17] is not affected by the replacement of the proline moieties. IZUMIYA *et al.*[18] chemically synthesized new gramicidin S homologues in which proline moieties were replaced by sarcosine, leucine or phenylalanine; they were biologically as active as natural gramicidin S and very similar in the ORD curves. In contrast, the site of the imino acid in viridogrisein was proved to play an important role in antimicrobial activity. In viridogrisein, as is shown by the negative optical rotation of neoviridogrisein II ($-39.3°$) compared with the positive optical rotation of viridogrisein ($+59°$), the replacement of *allo*-hydroxy-D-proline by D-proline seems to cause the conformational change which might result in a change in affinity of binding to the ribosomes or a change in permeability for the antibiotic. Anyway, it should be noted that the imino acid in viridogrisein has a critical importance in respect to the interaction of the antibiotic molecule with pathogens.

P. S.

In the April issue of this journal, CHOPRA *et al.* reported production of a new etamycin analogue which was tentatively named etamycin B. Based on the $[\alpha]_D$ data (neoviridogrisein II: $-39.3°$ *versus* etamycin B: $+20°$), neoviridogrisein II was considered to be different from etamycin B.

Acknowledgement

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