CONTROLLED BIOSYNTHESIS OF NEOVIRIDOGRISEINS, NEW HOMOLOGUES OF VIRIDOGRISEIN

III. PRODUCTION, STRUCTURES AND BIOLOGICAL PROPERTIES OF NEOVIRIDOGRISEINS I AND III

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The high response of our isolate of *Streptomyces griseoviridus* was exploited to provoke the synthesis of new viridogrisein homologues by adding various amino acids to the culture medium in an attempt to replace the alanine, sarcosine, leucine and phenylsarcosine moieties of viridogrisein. Among the amino acids added, L- and DL-α-amino-*n*-butyric acid and L-methionine gave new TLC spots which we named neoviridogriseins I and III and neoviridogrisein VII, respectively. The structures of neoviridogriseins I and III were elucidated: In both compounds, the alanine moiety of viridogrisein is replaced by a L-α-amino-*n*-butyric acid residue; furthermore, in neoviridogrisein I, the *allo*-hydroxy-D-proline is replaced by D-proline.

In a previous paper[1], we reported the proline-controlled biosynthesis of neoviridogrisein II in which the *allo*-hydroxy-D-proline moiety of viridogrisein[2] was replaced by D-proline. In this connection, it can be anticipated that the alanine, sarcosine, leucine and/or phenylsarcosine moieties of viridogrisein could be replaced by related amino acids; therefore the effects of exogenous amino acids on *Streptomyces* sp. P8648[3] were studied by TLC analysis for possible biosynthesis of new viridogrisein homologues.

Among many amino acids tested, DL-α-amino-*n*-butyric acid was found specific to give new homologues named neoviridogriseins I and III. In this paper, the α-amino-*n*-butyric acid-controlled biosynthesis, structures and some antimicrobial activities of neoviridogriseins I and III are described.

Materials and Methods

Reagents

All amino acids were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Other chemicals were obtained from commercial sources.

Microorganisms

All strains of bacteria used in this paper were same as described previously[1].

Effects of exogenous amino acids on the production of viridogrisein and neoviridogriseins

One loopful of *Streptomyces* sp. P8648 maintained on a glucose-yeast extract-malt extract agar slant was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of LL-3 medium (0.5% glucose, 0.5% soy bean meal, 0.5% soluble starch, 0.5% cotton seed meal and 0.5% dry yeast, pH 6.5 before sterilization) and 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 GYM medium (0.4% glucose, 0.4% yeast extract and 1% malt extract and various amino acids at indicated concentrations,

pH 6.5 before sterilization). After incubation under shaking at 28°C for 6 days, the broth filtrate was extracted with ethyl acetate and the extract was subjected to product analysis by TLC.

Physico-chemical analysis

The methods for TLC analysis of neoviridogrisein components, determination of physico-chemical properties and amino acid analysis were detailed in a previous paper[1,3].

Configurations of proline and α-amino-*n*-butyric acid

Configurations of proline and α-amino-*n*-butyric acid in neoviridogrisein I and of α-amino-*n*-butyric acid in neoviridogrisein III were determined by the enzymatic procedures described in a previous paper[1]. Three milligrams of neoviridogrisein I were hydrolyzed in a sealed tube with 6 N hydrochloric acid at 110°C for 16 hours. The hydrolysate was evaporated to dryness under reduced pressure: One half 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 and the other half in 1 ml of sodium pyrophosphate buffer (pH 8.3, 0.1 M) for D-amino acid oxidase. Before and after enzymatic oxidation, the contents of proline and α-amino-*n*-butyric acid were measured by the methods of TROLL and LINDSLEY[4], and of NAFTALIN[5], respectively. Six milligrams of neoviridogrisein III were hydrolyzed in a similar manner: One half of the hydrolysate was dissolved in 2 ml of Tris-HCl buffer (pH 7.5 at 37°C, 0.4 M) for L-amino acid oxidase and the other half in 2 ml of sodium pyrophosphate buffer (pH 8.3, 0.1 M) for D-amino acid oxidase.

Minimum inhibitory concentration

Minimum inhibitory concentrations were determined by the broth dilution method described before[1].

Results

1. Effect of exogenous amino acids

The following amino acids were added at concentrations of 0.1% and 0.4%: L-, D- and DL-alanine, L-, D- and DL-α-amino-*n*-butyric acid, L-cysteine, L-glutamic acid, glycine, L- and DL-homoserine, L- and D(*allo*)-hydroxylysine, L-, D- and DL-isoleucine, L-, D- and DL-leucine, L- and DL-methionine, DL-norleucine, DL-norvaline, L-, D- and DL-phenylalanine, DL-phenylglycine, L-, D- and DL-threonine, L-, D- and DL-tryptophan, L-, D- and DL-tyrosine, L-, D- and DL-valine and sarcosine.

Among these amino acids, L- and DL-α-amino-*n*-butyric acid and L-methionine were found to yield new antimicrobial components in addition to viridogrisein and neoviridogrisein II, as evidenced in the thin-layer chromatogram of Fig. 1.

The two new TLC spots produced in the presence of L- and DL-α-amino-*n*-butyric acid were easily identified as homologues of viridogrisein (not of griseoviridin) on account of their characteristic spectroscopic properties and were

Fig. 1. New viridogrisein homologues produced by the exogenous addition of α-amino-*n*-butyric acid and methionine.

Plate: Pre-coated Silica gel 60 F-254 (E. Merck)
Solvent system: $CHCl_3$ - MeOH (30: 1)
Detection: Irradiation by UV-light (365 nm)
Sample: 1. viridogrisein
2. neoviridogrisein II
3. ethyl acetate extract from broth filtrate of GYM medium containing 0.4% L-α-amino-*n*-butyric acid
4. ethyl acetate extract from broth filtrate of GYM medium containing 0.4% L-methionine

VG: viridogrisein, II: neoviridogrisein II, I: neoviridogrisein I, III: neoviridogrisein III, VII: neoviridogrisein VII

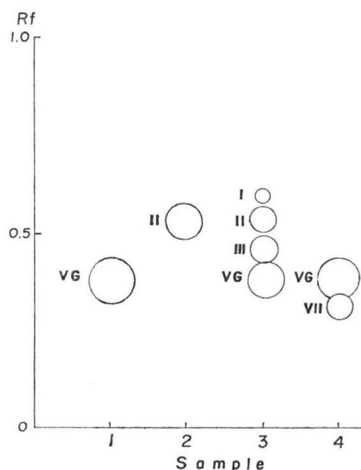


Table 1. Effect of L- and D-α-amino-*n*-butyric acid on the production of neoviridogriseins and viridogrisein.

α-Amino-*n*-butyric acid (%)		pH	PCV (%)†	Potency (μg/ml)			
				NVG I*	NVG II**	NVG III***	VG****
L-Form	0.05	7.50	15.0	0	1.27	1.47	48.39
	0.10	7.70	15.0	1.10	3.55	10.81	40.00
	0.20	7.90	15.0	2.94	9.27	18.24	35.92
	0.40	7.80	15.5	3.09	7.41	15.75	28.85
D-Form	0.05	6.80	12.0	0	1.61	0	61.85
	0.10	6.80	11.0	0	2.05	0	40.60
	0.20	6.90	12.0	0	2.47	0	38.15
	0.40	6.00	8.0	0	2.12	0	20.27
None		6.60	16.0	0	0	0	63.12

Basal medium (GYM): glucose 0.4%, yeast extract 0.4% and malt extract 1.0% (pH 6.5); incubation: 120 hours. NVG I*: neoviridogrisein I, NVG II**: neoviridogrisein II, NVG III***: neoviridogrisein III, VG****: viridogrisein. PCV†: packed cell volume.

named neoviridogriseins I and III according to the decreasing order of their Rf values.

The addition of L-methionine gave a new viridogrisein homologue which could hardly be differentiated from viridogrisein by TLC: it was named neoviridogrisein VII.

Generally, the addition of the D-form of an amino acid, except alanine, inhibited not only cell growth but also antibiotic production.

2. Effect of L- and D-α-amino-*n*-butyric acid on the production of neoviridogriseins

The effect of L- and D-α-amino-*n*-butyric acid (0.05~0.4%) on the composition of neoviridogriseins and viridogrisein is reported in Table 1.

It is evident from Table 1 that the percent production of neoviridogriseins I and III rose, while that of viridogrisein decreased with increasing concentrations of L-α-amino-*n*-butyric acid. It will be shown below that the alanine moiety of viridogrisein is replaced by L-α-amino-*n*-butyric acid in neoviridogriseins I and III. It is interesting to observe that the production of neoviridogrisein II, which contains no L-α-amino-*n*-butyric acid, also increases upon addition of L-α-amino-*n*-butyric acid.

In contrast to L-α-amino-*n*-butyric acid, the D-isomer inhibited both cell growth and production of neoviridogrisein II and viridogrisein, and completely abolished the synthesis of neoviridogriseins I and III (Table 1). The inhibitory effect of D-α-amino-*n*-butyric acid becomes clearer as its concentration is higher. The nonspecific induction of the production of neoviridogrisein II is again observed.

3. Time course of production of neoviridogriseins I and III in the presence of L-α-amino-*n*-butyric acid

The time course of fermentation in the presence of 0.4% L-α-amino-*n*-butyric acid is shown in Fig. 2. Viridogrisein and neoviridogriseins I, II and III are biosynthesized in a similar manner throughout fermentation and reach their maxima at 5 days of incubation.

4. Physico-chemical properties of neoviridogriseins I and III

From 10 liters of fermentation broth, 10.7 mg of neoviridogrisein I and 74.0 mg of neoviridogrisein III were obtained.

Physico-chemical data of neoviridogriseins I and III are summarized in Table 2. For comparison,

Table 2. Physico-chemical properties of neoviridogriseins I, II and III and viridogrisein.

	NVG I*	NVG II**	NVG III***	VG****
Melting point	143°C	145°C	152°C	160°C
Optical rotation	$[\alpha]_D^{23}$ +13° (*c* 1 in MeOH)	$[\alpha]_D^{23}$ −39.3° (*c* 1 in MeOH)	$[\alpha]_D^{25}$ +73.7° (*c* 1 in MeOH)	$[\alpha]_D^{25}$ +59° (*c* 5 in $CHCl_3$)
Molecular weight (MS†)	876	862	892	878
UV Absorption	λ_{max} 305 nm (in MeOH)	λ_{max} 305 nm (in MeOH)	λ_{max} 305 nm (in MeOH)	λ_{max} 304 nm (in EtOH)
($E_{1cm}^{1\%}$)	(84)	(90)	(90)	(92)
	λ_{max} 340 nm (in MeOH/ 0.1 N NaOH)	λ_{max} 340 nm (in MeOH/ 0.1 N NaOH)	λ_{max} 340 nm (in MeOH/ 0.1 N NaOH)	λ_{max} 335 nm (in EtOH/ 0.1 N NaOH)
($E_{1cm}^{1\%}$)	(70)	(84)	(84)	(82)

NVG I*: neoviridogrisein I, NVG II**: neoviridogrisein II, NVG III***: neoviridogrisein III
VG****: viridogrisein (cited from reference 2)
MS†: mass spectrometry

neoviridogrisein II and viridogrisein are also included. IR and mass spectra of neoviridogriseins I and III are shown in Figs. 3, 4 and 5 respectively.

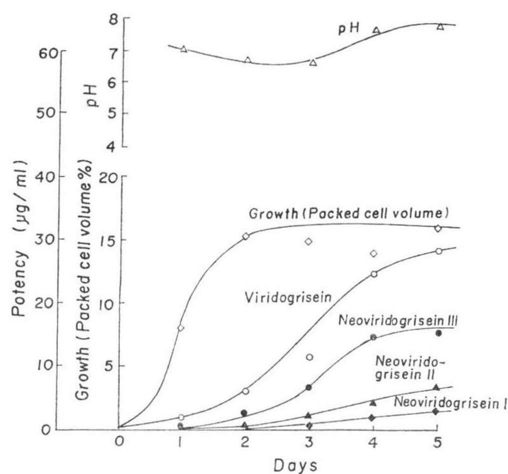


Fig. 2. Time course of fermentation of *Streptomyces* sp. P8648 in medium GYM containing 0.4% L-α-amino-*n*-butyric acid.

Neoviridogriseins I and III show a λ_{max} at 305 nm ($E_{1cm}^{1\%}$ = 84 and 90) in MeOH and a λ_{max} at 340 nm ($E_{1cm}^{1\%}$ = 70 and 84) in MeOH - 0.1 N NaOH. These UV spectra are almost identical with that of viridogrisein, suggesting that neoviridogriseins I and III also have the 3-hydroxypicolinic acid moiety. Furthermore, the IR spectra indicate their similarity to viridogrisein. Neoviridogriseins I and III also have hydroxyl groups, phenyl groups, amide bonds and ester bonds. The weaker absorption intensity of the hydroxyl group in neoviridogrisein I suggests that the number of hydroxyl groups is less in neoviridogrisein I than in neoviridogrisein III.

Fig. 4 shows the mass spectra of neoviridogriseins I and III in which molecular ions are located at *m/e* 876 and 892(M^+), respectively. The fragmentation patterns of neoviridogriseins I and III are very similar to that of viridogrisein in that the azomethine type of fragmentation[6], but not the CO_2 type[7], is confirmed.

5. Amino acid analysis

The total acid hydrolysates of neoviridogriseins I and III were subjected to amino acid analysis by two-dimensional thin-layer chromatography, and compared with that of viridogrisein (Fig. 6).

In neoviridogrisein I the following constituent amino acids are identical with those of viridogrisein: 3-hydroxypicolinic acid, L-threonine, D-leucine, sarcosine, N,β-dimethyl-L-leucine and L-phenylsarcosine. Neoviridogrisein I gave no *allo*-hydroxy-D-proline and no L-alanine, but two un-

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

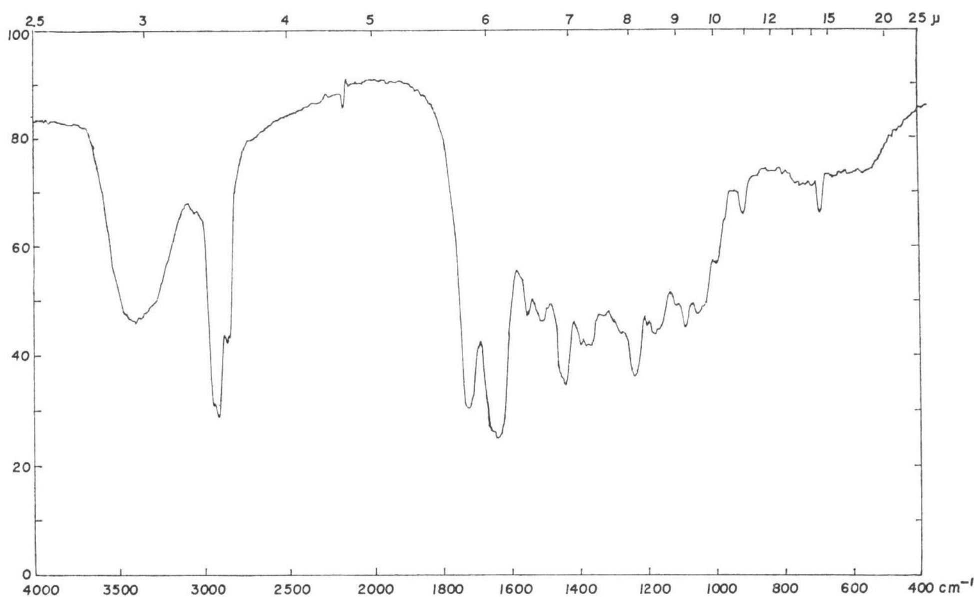
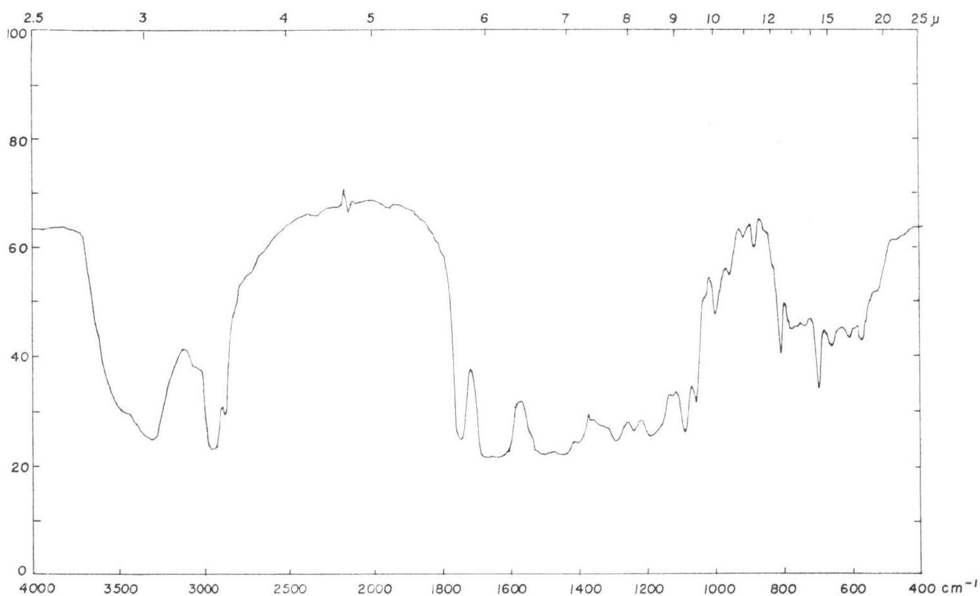


Fig. 4. IR spectrum of neoviridogrisein III (KBr).



known amino acids. After comparison with authentic amino acids by high-voltage paper electrophoresis and thin-layer chromatography followed by the ninhydrin or isatin reagent spray, the unknown spots were identified as proline and α-amino-*n*-butyric acid, respectively.

Neoviridogrisein III yielded the following amino acids: 3-hydroxypicolinic acid, L-threonine, D-leucine, *allo*-hydroxy-D-proline, sarcosine, N,β-dimethyl-L-leucine, L-phenylsarcosine and an un-

Fig. 5. Mass spectra of neoviridogriseins I and III.

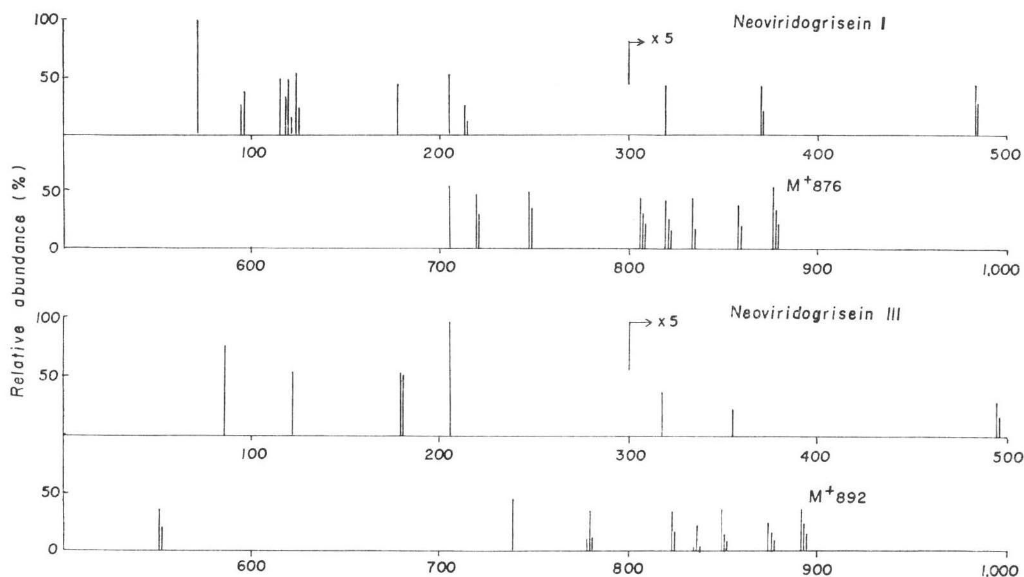
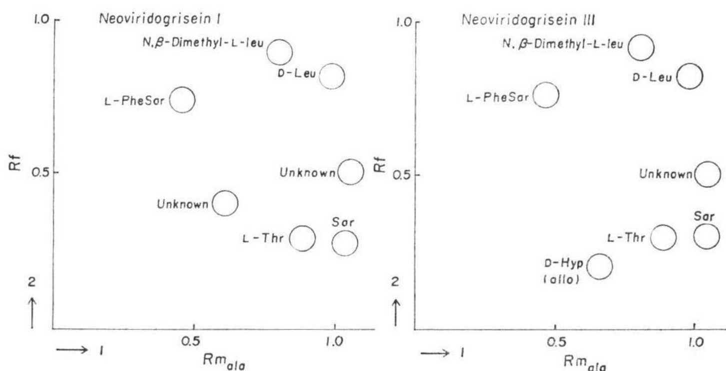


Fig. 6. Two-dimensional thin-layer chromatograms of the acid-hydrolysates of neoviridogriseins I and III.
Plate: cellulose sheet (Eastman Chromagram Sheet No. 6065)
First dimension: electrophoresis at 50 V/cm for 60 minutes in a formate-acetate buffer ($HCOOH - CH_3COOH - H_2O=25 : 75 : 900$, pH 1.8)
Second dimension: *n*-butanol - $CH_3COOH - H_2O=4 : 1 : 1$
Coloration: ninhydrin or isatin reagent spray



known amino acid. In neoviridogrisein III, L-alanine was absent; the unknown amino acid was later identified as α-amino-*n*-butyric acid.

The Rf and Rm_{ala} values of the constituent amino acids of neoviridogriseins I and III are given in Table 3.

6. Configuration of α-amino-*n*-butyric acid and proline in neoviridogriseins I and III

The α-amino-*n*-butyric acid and proline moieties of neoviridogrisein I were subjected to the enzymatic assay. Proline in neoviridogrisein I could be oxidized by D-amino acid oxidase but not by L-amino acid oxidase, and α-amino-*n*-butyric acid by L-amino acid oxidase but not by D-amino acid

oxidase. Therefore, we concluded that the configurations of proline and α-amino-*n*-butyric acid in neoviridogrisein I were the D-form and the L-form, respectively. The configuration of α-amino-*n*-butyric acid in neoviridogrisein III was similarly established to be the L-form.

From all the physico-chemical and enzymological data described above, the structures in Fig. 7 are proposed for neoviridogriseins I and III.

7. Antimicrobial properties

The antimicrobial activities of neoviridogriseins I and III were measured and compared with those of neoviridogrisein II and viridogrisein using the broth dilution technique. The results are presented in Table 4.

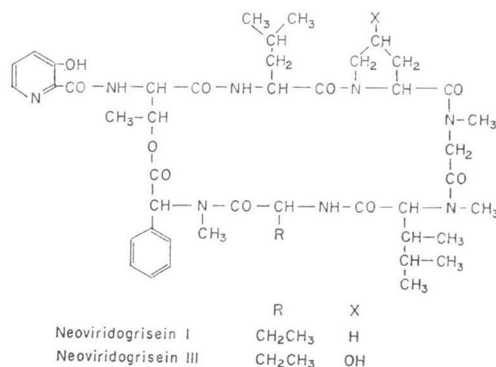
In general, neoviridogriseins I and III like viridogrisein are active against Gram-positive bacteria such as *Staphylococcus aureus* FDA 209P, *S. aureus* Smith, *Streptococcus pneumoniae* Type I, *Streptococcus pyogenes* NY-5, *Sarcina lutea* and *Bacillus subtilis* ATCC 6633. Neoviridogrisein I looks more active than neoviridogrisein III against *S. aureus* Smith, *Streptococcus pyogenes* NY-5 and *Bacillus subtilis* ATCC 6633. The structure-antimicrobial activity relationship between neoviridogriseins I and III is the same as that between neoviridogrisein II and viridogrisein, that is, the increased antimicrobial activity of neoviridogrisein I is due to the replacement of hydroxyproline by proline.

Table 3. Rf and Rm values of the constituent amino acids of neoviridogriseins I and III.

		Rf†	Rm_{ala}††
NVG I*	3-Hydroxypicolinic acid	0.62	—
	L-Threonine	0.30	0.85
	D-Leucine	0.85	0.95
	Sarcosine	0.29	1.03
	N,β-Dimethyl-L-leucine	0.90	0.81
	L-Phenylsarcosine	0.82	0.47
	Unknown 1	0.48	1.05
	Unknown 2	0.35	0.62
NVG III**	3-Hydroxypicolinic acid	0.62	—
	L-Threonine	0.30	0.86
	D-Leucine	0.84	0.95
	allo-Hydroxy-D-proline	0.22	0.65
	Sarcosine	0.29	1.03
	N,β-Dimethyl-L-leucine	0.91	0.80
	L-Phenylsarcosine	0.82	0.47
	Unknown 1′	0.48	1.05

NVG I*: neoviridogrisein I
NVG III**: neoviridogrisein III
Rf†: Rf values were determined in the following conditions;
Plate: cellulose sheet (Eastman Chromagram Sheet No. 6065)
Solvent: *n*-butanol - acetic acid - H_2O (4: 1: 1)
Detection: irradiation by UV-light (365 nm), ninhydrin or isatin reagent spray
Rm_{ala}††: Rm_{ala} values were measured in the following conditions;
Plate: cellulose sheet (Eastman Chromagram Sheet No. 6065)
Electrophoresis: 50 V/cm for 60 minutes in a formate-acetate buffers (pH 1.8)
Detection: same as for the Rf determination

Fig. 7. Structures of neoviridogriseins I and III



Discussion

Neoviridogriseins I and III were obtained in substantial amounts in the presence of exogenous

Table 4. Minimum inhibitory concentrations of neoviridogriseins and viridogrisein.

Microorganism	Medium	Minimum inhibitory concentration (μg/ml)			
		NVG I*	NVG II**	NVG III†	VG††
Staphylococcus aureus FDA 209P	(1)	0.2	0.1	0.2	0.2
Staphylococcus aureus Smith	(1)	0.2	0.2	0.4	0.4
Streptococcus pneumoniae Type I	(2)	0.4	0.4	0.4	0.4
Streptococcus pyogenes NY-5	(2)	0.2	0.2	0.4	0.4
Sarcina lutea	(1)	0.4	0.4	0.4	0.4
Bacillus subtilis ATCC 6633	(1)	0.4	0.4	0.8	0.8
Salmonella gallinarum	(1)	>25	>25	>25	>25
Escherichia coli K-12	(1)	>25	>25	>25	>25
Pseudomonas aeruginosa P-1	(1)	>25	>25	>25	>25
Proteus vulgaris GN 76	(1)	>25	>25	>25	>25
Candida albicans	(3)	>25	>25	>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.
NVG I*: neoviridogrisein I, NVG II**: neoviridogrisein II, NVG III†: neoviridogrisein III, VG††: viridogrisein.
Inoculum size: 10^6 cells/ml.

L-α-amino-*n*-butyric acid (Table 1). In these two new homologues, the L-alanine moiety is replaced by L-α-amino-*n*-butyric acid (Fig. 7). This type of replacement seems common in the antibiotics of sub-group B I. For example, under normal fermentation conditions and in the absence of exogenous α-amino-*n*-butyric acid, virginiamycins S, S_2 and S_3, which contain a D-α-amino-*n*-butyric acid moiety, are abundantly produced together with virginiamycin S_4 which contains a D-alanine instead of a D-α-amino-*n*-butyric acid moiety[8]. It is true that the configuration and the intramolecular position of the alanine moiety are different in sub-groups B I and B II. However, it seems probable that sub-group B I producers, such as *Streptomyces virginiae*[8] and *S. ostreogriseus*[9], have a fairly large intracellular pool of α-amino-*n*-butyric acid, because addition of α-amino-*n*-butyric acid is not necessary for the accumulation of virginiamycins S, S_2 and S_3. Another example is the production of vernamycins by *S. loidensis*[10]. The normal composition of vernamycin B complex is far more favorable for vernamycins B_α and B_β, which contain D-α-amino-*n*-butyric acid, than for vernamycin B_γ and B_δ, which contain D-alanine (vernamycins $B_\alpha : B_\beta : B_\gamma : B_\delta = 53 : 24 : 7 : 4$).

On the other hand, *Streptomyces* sp. P8648 produces undetectable amounts of neoviridogriseins I and III under normal fermentation conditions, which might be ascribed to a very small intracellular pool of α-amino-*n*-butyric acid. Another plausible reason for the negligible production of neoviridogriseins I and III under normal fermentation conditions may stem from the higher affinity of the responsible enzyme system for alanine than for α-amino-*n*-butyric acid. In this connection, it should be recalled that the effect of exogenous L-α-amino-*n*-butyric acid on the production of neoviridogriseins I and III was insignificant at 0.05%, but became evident above 0.1% (Table 1). Hence, exogenous α-amino-*n*-butyric acid obviously competes with endogenous alanine for the same substrate site of the viridogrisein-synthesizing enzyme system. Thus, 0.1% of α-amino-*n*-butyric acid is the minimum concentration necessary to suppress the effect of endogenous alanine and favor the formation of neoviridogriseins I and III.

Contrary to our expectation, the addition of L-α-amino-*n*-butyric acid (and other amino acids) promoted the production of neoviridogrisein II (Table 2). As there is no probable biosynthetic relationship between L-α-amino-*n*-butyric acid and proline, this effect of L-α-amino-*n*-butyric acid can presumably be ascribed to the nonspecific sensitivity of *Streptomyces* sp. P8648 to environmental factors.

D-α-Amino-*n*-butyric acid inhibited cell growth and consequently production of neoviridogriseins.

This indicates that *Streptomyces* sp. P8648 has no racemase for α-amino-*n*-butyric acid and can utilize only the L-form of α-amino-*n*-butyric acid.

The addition of L-methionine yielded a new homologue of viridogrisein named neoviridogrisein VII, which was very difficult to isolate from viridogrisein by routine thin-layer chromatography. A small amount of neoviridogrisein VII, which was obtained in low yield by multiple development (×2) of TLC plates in a solvent system consisting of $CHCl_3$ - MeOH (40: 1), seemed slightly less active than viridogrisein against *S. aureus* FDA 209P. Preliminary amino acid analysis did not reveal a difference between neoviridogrisein VII and viridogrisein, but their configurations have not yet been examined. The structural study of neoviridogrisein VII is in progress.

As reported in a previous paper[1], neoviridogrisein II, which contains D-proline instead of *allo*-hydroxy-D-proline, was 2 to 4 times more active against *S. aureus* than viridogrisein. The same relationship exists between neoviridogriseins I and III. Thus, the replacement of L-alanine by L-α-amino-*n*-butyric acid might not affect the antimicrobial activity of viridogrisein. Although a certain conformational difference is suggested by $[\alpha]_D$ between neoviridogriseins I and II (neoviridogrisein I: $+13°$ *versus* neoviridogrisein II: $-39.3°$), and between neoviridogrisein III and viridogrisein (neoviridogrisein III: $+73.7°$ *versus* viridogrisein: $+59°$) respectively, the alanine site of the viridogrisein molecule does not seem to play an important role in the antimicrobial activity.

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