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Synthesis and Properties of Gramicidin S Analogs Containing D-Phe-L-Pro-D-Val or L-Phe-L-Pro-D-Val Sequences in Place of D-Phe-L-Pro-L-Val Sequence in the β-Turn Part of the Antibiotic

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In order to investigate the contribution of configurations of amino acid residues around Pro residues in gramicidin S to its activity and conformation, [D-Val$^{1,1'}$]- and [D-Val$^{1,1'}$ L-Phe$^{4,4'}$]-gramicidin S were synthesized by a liquid-phase method. The CD spectra of these analogs and gramicidin S in aqueous solutions differ from each other, indicating that these peptides have different conformations. These analogs have practically no activity against the Gram-positive microorganisms tested, indicating the importance of the presence of the D-Phe-Pro-Val sequence in gramicidin S regarding activity.

Gramicidin S (GS)[1] is an antibiotic cyclodecapeptide isolated from *Bacillus brevis*. Its secondary structure has been established as an antiparallel β-sheet conformation (Fig. 1).[2] This conformation is characteristically featured (with the orientation of side chains) in such a way that the charged Orn side chains are situated on one side of the molecule and the hydrophobic Val and Leu side chains are situated on the other side. The side-chain arrangement is apparently held together by a rigid conformation containing two D-Phe-Pro type II′ β-turns.[2,3] In studies regarding the structure-activity relationship of GS, it has been proposed that this specific conformation is necessary in order to exhibit an antibiotic activity.[4]

Recently, we reported in studies of an antibiotic cyclododecapeptide, gratisin, that the configurations of amino acid residues around Pro residues in synthetic isomers greatly affect their conformation, and that the activity of *cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)$_2$ was similar to that of GS against *Bacillus subtilis* and stronger than that of *cyclo*(-Val-Orn-Leu-D-Phe-Pro-Tyr-)$_2$.[5] In connection with these results, it is of interest to study the relationship between configurations of amino acid residues around Pro residues in GS and its secondary structure or antibiotic activity.

Kawai *et al.*[6] synthesized [L-Ala$^{4,4'}$]-GS, in which D-Phe-Pro-Val sequences in GS were replaced with Ala-Pro-Val sequences, and elucidated the structure-activity relationship.

In the present paper, we wish to describe the synthesis and antibiotic activity of [D-Val$^{1,1'}$]- and [D-Val$^{1,1'}$, L-Phe$^{4,4'}$]-GS (Fig. 2), in which the D-Phe-Pro-Val sequence in GS is replaced, respectively, with D-Phe-Pro-D-Val and Phe-Pro-D-Val. We also wish to discuss the relationship between partial sequences around Pro residues and CD patterns in GS and the analogs.

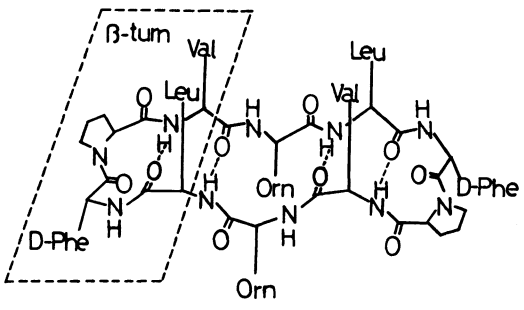


Fig. 1. β-Sheet conformation of gramicidin S.

The synthetic routes of [D-Val$^{1,1'}$]-GS (**7**) and [D-Val$^{1,1'}$, L-Phe$^{4,4'}$]-GS (**13**) are shown in Schemes 1 and 2. In the synthesis of protected [D-Val$^{1,1'}$]-GS (**6**), the Boc-dipeptide ester (**1**) was synthesized with DCC and HOBt, and then converted into the hydrazide (**2**). The Z-tripeptide benzyl ester (**3**) was obtained with DCC and HOBt by stepwise elongation as an oil. The hydrogenolysis of **3** gave the tripeptide (**3′**). The coupling of **2** and **3′** was carried out using an azide method and afforded the Boc-pentapeptide (**4**), which in part was converted into the Boc-pentapeptide succinimido ester (**4′**) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSCD) and HOSu. The coupling of **4′** and the pentapeptide derived from **4** afforded the Boc-decapeptide (**5**) which was converted into the decapeptide succinimido ester (**5′**). The cyclodecapeptide (**6**) was derived by the cyclization of **5′** in pyridine at 60°C for 2 h in a 25% yield. In the synthesis of

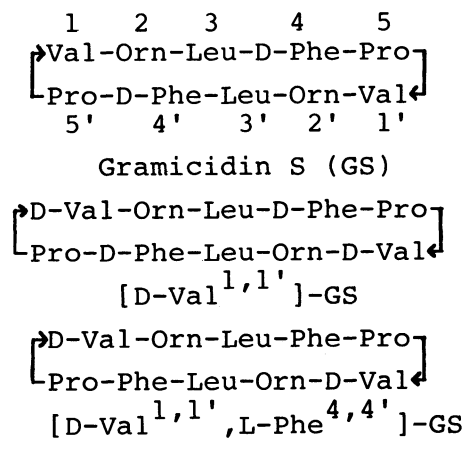
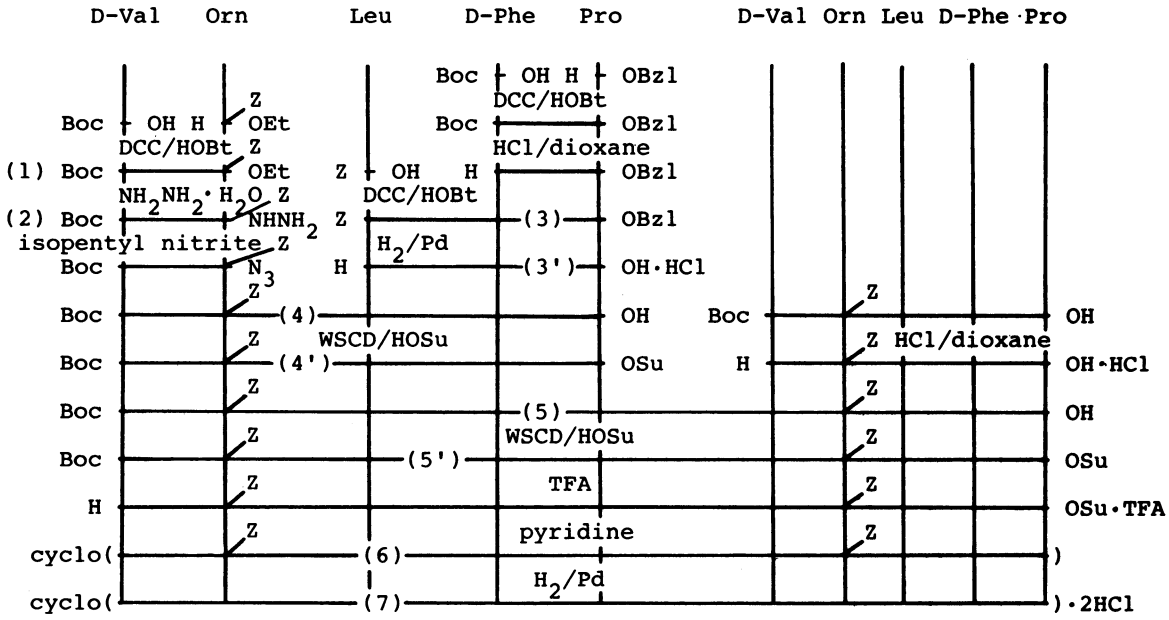
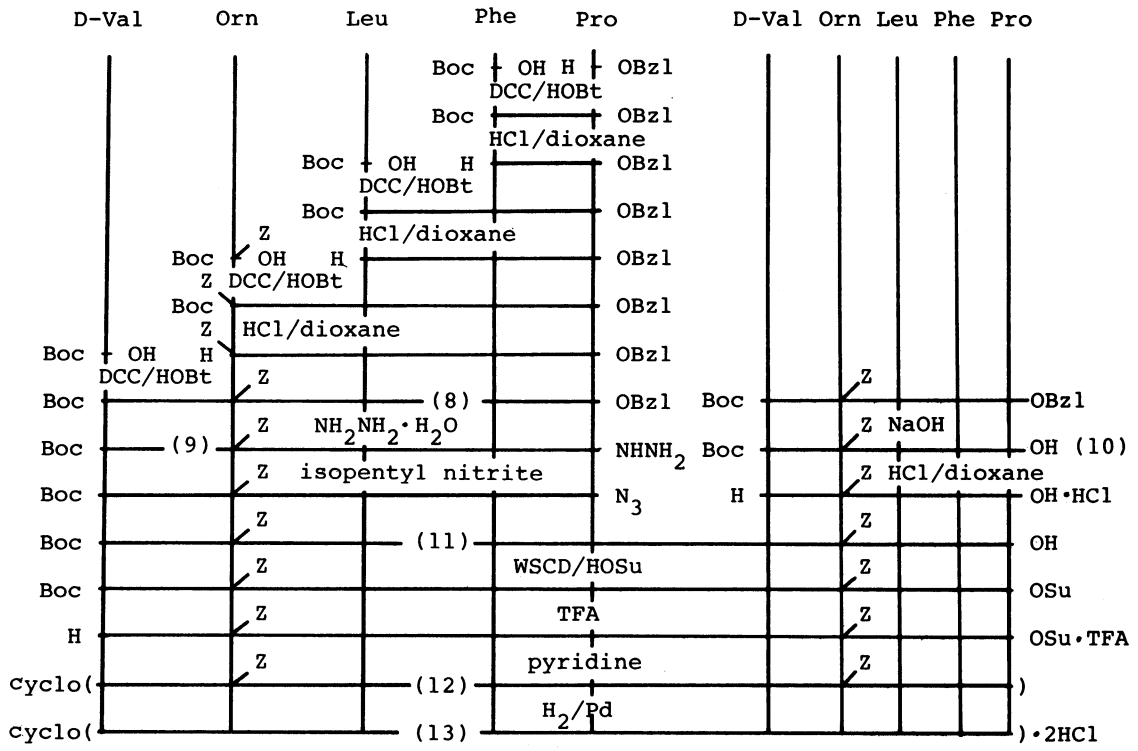


Fig. 2. Primary structures of gramicidin S and its analogs.



Scheme 1. Synthesis of [D-Val$^{1,1'}$]-GS.



Scheme 2. Synthesis of [D-Val$^{1,1'}$, L-Phe$^{4,4'}$]-GS.

protected [D-Val$^{1,1'}$, L-Phe$^{4,4'}$]-GS (**12**), the Boc-pentapeptide benzyl ester (**8**) was synthesized by stepwise elongation using DCC and HOBt from the Pro benzyl ester. A part of **8** was converted into the hydrazide (**9**), and another part was saponified to obtain the Boc-pentapeptide (**10**). The coupling of **9** and the pentapeptide derived from **10** was carried out using an azide method and afforded the Boc-decapeptide (**11**). The cyclization of **11** was performed using an active ester method in a similar manner as described regarding the synthesis of [D-Val$^{1,1'}$]-GS. The desired peptide was obtained in a 47% yield. The masking groups of **6** and **12** were removed by hydrogenolysis. Their homogeneity was confirmed by thin-layer chromatography, cellulose plate electrophoresis, elemental analysis, amino acid analysis, high-performance liquid chromatography (HPLC) and secondary ion mass spectrometry (SIMS).

The CD spectra of [D-Val$^{1,1'}$]-GS, [D-Val$^{1,1'}$, L-Phe$^{4,4'}$]-GS, and GS in aqueous solutions are shown in Fig. 3. The spectral shapes of these peptides differ from each other, indicating that they have different conforma-

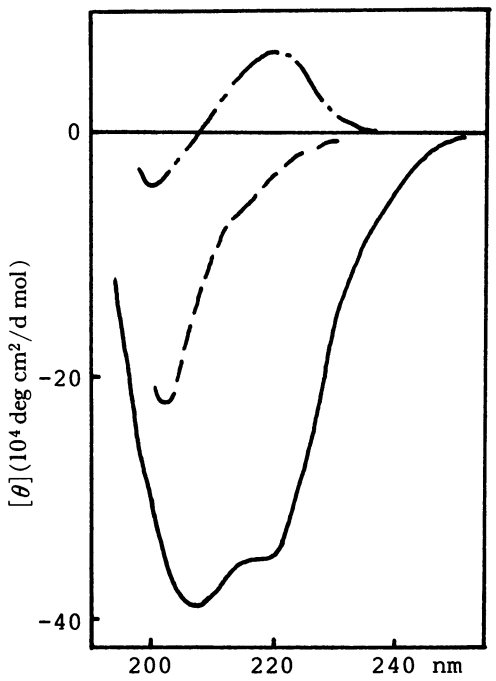


Fig. 3. CD spectra of GS and its analogs in aqueous solution.
——, GS: ---, [D-Val$^{1,1'}$]-GS: --, [D-Val$^{1,1'}$, L-Phe$^{4,4'}$]-GS.

tions in aqueous solutions. Recently, in CD spectroscopic studies of cyclododecapeptide gratisin, we reported that the CD spectra of synthetic isomers could be classified into four groups, and that the isomers belonging to each group possess analogous partial sequences around the Pro residues. That is, L-X-Pro-L-Y, D-X-Pro-L-Y, L-X-Pro-D-Y, and D-X-Pro-D-Y.[5] Each CD spectrum of [D-Val$^{1,1'}$]-GS, [D-Val$^{1,1'}$, L-Phe$^{4,4'}$]-GS and GS showed a similar pattern to that of the gratisin isomer having the corresponding partial sequences around the Pro residues (D-X-Pro-D-Y, L-X-Pro-D-Y and D-X-Pro-L-Y, respectively). The CD pattern of [L-Ala$^{4,4'}$]-GS[6] also resembles that of the gratisin isomer having a L-X-Pro-L-Y partial sequence. It is interesting that the CD spectra of these peptides related with both GS and gratisin are dependent on the particular sequence around the Pro residue, although these peptides have a different number of ring members. The CD spectra of [Pro$^{4,4'}$, Asn$^{5,5'}$]-GS and [Pro$^{4,4'}$, D-Ala$^{5,5'}$]-GS reported by Sato *et al.*[7] also showed a similar sequence dependence, although the Pro residues occupy positons different from 5 and 5′ in GS. Moreover, the CD spectrum of *cyclo*(-Leu-Orn-Leu-Orn- Leu-D-Phe-Pro-)$_2$ synthesized by Ando *et al.*[8] was also similar to that of GS. This may result from the presence of a D-X-Pro-L-Y partial sequence in the peptide. These results indicate that the CD spectra of these peptides in aqueous solutions reflect the partial sequences around the Pro residues, which affect (largely) the conformation of these peptides.

[D-Val$^{1,1'}$]-GS showed antibiotic activity only against *Corynebacterium diphtheriae* at a concentration of 25 μg/ml, and no activity toward the other Gram-positive microorganisms tested. [D-Val$^{1,1'}$, L-Phe$^{4,4'}$]-GS showed no antibiotic activity against all the Gram-positve microorganisms tested. That is, the D-Phe-Pro-Val sequences in GS can not be substituted by Phe-Pro-D-Val or D-Phe-Pro-D-Val sequence without affecting the activity. In gratisin analogs, several isomers containing different sequences in the corresponding part were more or less active.[5] The significant difference between GS and gratisin in this regard may result from the distinction of their rings, in other words, the flexibility of the β-turn parts.

Experimental

All the melting points are uncorrected. The CD spectra were obtained using a JASCO spectropolarimeter (model J-500). The CD spectroscopy of GS and its analogs was carried out with an aqueous solution of their dihydrochlorides. The molecular weights of GS analogs and several intermediary products were determined by secondary ion mass spectrometry (SIMS) using a Hitachi M-80B mass spectrometer and fast atom bomberdment (FAB) mass spectrometry using a JMS D-300 mass spectrometer. Amino acid analyses were carried out using a Hitachi 835 amino acid analyzer, after hydrolysis of the peptides in 6 M HCl (1 M=1 mol dm^{-3}) at 110 °C for 24 h. HPLC was performed by an ODS column (φ 4.6×250 mm) using MeOH-5% $NaClO_4$ aq (5:1)[1] as eluting solvent. Thin-layer chromatography was performed on Merck silica-gel F_{254} plates with the following solvent systems (v/v): R_f^1, $CHCl_3$-MeOH (9:1); R_f^2, $CHCl_3$-MeOH-AcOH (95:5:3); R_f^3, *n*-BuOH-AcOH-H_2O (4:1:1); R_f^4, *n*-BuOH-pyridine-AcOH-H_2O (4:1:1:2).

Boc-D-Val-Orn(Z)-OEt (1). DCC (2.9 g, 14 mmol) was added to a solution of Boc-D-Val (3.0 g, 14 mmol), HOBt (2.1 g, 15.5 mmol), Orn(Z)-OEt·TsOH (6.5 g, 14 mmol) and TEA (2 ml, 14 mmol) in $CHCl_3$ (70 ml) at 0 °C. This solution was stirred for 3 h at 0 °C and overnight at room temperature. After the reaction mixture was concentrated *in vacuo*, AcOEt (20 ml) was added to the residue, and insoluble substance was removed by filtration. The filtrate was then diluted with AcOEt (150 ml). The solution was washed (successively) with 5% citric acid, water, 5% Na_2CO_3 and water, and then dried over sodium sulfate. After removal of the drying agent, the solvent was evaporated *in vacuo*. The product was recrystallized from hot AcOEt; yield, 4.56 g (65%); mp 130—132 °C; $[\alpha]_D^{29}$ −4.0° (*c* 1, DMF); R_f^1 0.88, R_f^2 0.68. Found: C, 61.25; H, 7.98; N, 8.53%. Calcd for $C_{25}H_{39}O_7N_3$: C, 60.83; H, 7.96; N, 8.51%.

Boc-D-Val-Orn(Z)-NHNH$_2$ (2). A solution of **1** (4.0 g, 8.1 mmol) and hydrazine hydrate (5 ml) in DMF (15 ml) was allowed to stand for 24 h at room temperature. The solution was concentrated, and then water was added to the residue. The resulting solid was collected by filtration; yield, 3.5 g (90%); mp 179—180 °C; $[\alpha]_D^{29}$ −9.7° (*c* 1, DMF); R_f^1 0.41; R_f^2 0.44. MS (FAB), *m/z* 480 (MH^+). Found: C, 57.65; H, 7.82; N, 14.32%. Calcd for $C_{23}H_{37}O_6N_5$: C, 57.60; H, 7.78; N, 14.60%.

Z-Leu-D-Phe-Pro-OBzl (3). The coupling of Boc-D-Phe (5.4 g, 20 mmol) and Pro-OBzl·HCl (5.15 g, 20 mmol) were performed by the method described in the preparation of **1**. The product was dissolved in 4 M HCl/dioxane

(30 ml) containing anisole (0.5 ml) at 0 °C. After stirring for 30 min at room temperature, the solution was concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (30 ml). The solution was washed by 5% Na_2CO_3 and water under cooling with an ice bath. To this solution were added Z-Leu (5.3 g, 20 mmol), HOBt (2.96 g, 22 mmol) and DCC (5.15 g, 20 mmol). The mixture was treated in a similar way to that described in the preparation of **1**. The crude tripeptide was purified by chromatography on a silica-gel column (2×40 cm) using a solvent system of $CHCl_3$–MeOH (50:1). The fractions containing the desired product were combined and concentrated. The product was obtained as an oil in a yield of 3.18 g (27% calculated from Pro-OBzl as the starting material). R_f^1 0.80, R_f^2 0.65.

Boc-D-Val-Orn(Z)-Leu-D-Phe-Pro-OH (4). Compound **3** (2.64 g, 4.4 mmol) was dissolved in 90% MeOH (30 ml), and then 1 M HCl (4.4 ml) was added to the solution. The mixture was hydrogenated in the presence of Pd black for 10 h. After removal of the catalyst, the filtrate was concentrated *in vacuo*. To a solution of **2** (2.1 g, 4.4 mmol) in DMF (20 ml) were added 9 M HCl/dioxane (1.4 ml, 12.6 mmol) and isopentyl nitrite (0.7 ml, 5.0 mmol) at −40 °C. After stirring at −20 °C for 15 min, TEA (1.8 ml, 12.6 mmol) was added at −40 °C. This mixture was combined with a solution of the total amount of the H-Leu-D-Phe-Pro-OH prepared above and TEA (1.25 ml, 8.8 mmol) in DMF (12 ml). The mixture was stirred at 0 °C for 3 d, and concentrated. The residue was dissolved in AcOEt (100 ml). The solution was washed with 5% citric acid, water, 5% Na_2CO_3 and water, and dried. After the removal of the drying agent, the solution was evaporated. The product was recrystallized from MeOH-ether; yield, 3.05 g (84%); mp 191—192 °C; $[\alpha]_D^{29}$ −36.7° (*c* 1, DMF); R_f^1 0.37, R_f^2 0.39. MS (FAB), *m/z* 823 (MH^+). Found: C, 62.57; H, 7.81; N, 10.14%. Calcd for $C_{43}H_{62}O_{10}N_6$: C, 62.76; H, 7.59; N, 10.21%.

Boc-[D-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-OH (5). To a solution of **4** (1.17 g, 1.4 mmol) in DMF (7 ml) was added HOSu (0.35 g, 3 mmol) and WSCD·HCl (0.52 g, 2.7 mmol) at 0 °C, and then the mixture was stirred for 48 h at room temperature. The solution was evaporated, and then water was added to the residue. The resulting solid, Boc-D-Val-Orn(Z)-Leu-D-Phe-Pro-OSu (**4'**), was collected by filtration, and washed with water and dried. Another crop of **4** (1.17 g, 1.4 mmol) was dissolved in 4 M HCl/dioxane (10 ml) containing anisole (0.5 ml) at 0 °C. After stirring at room temperature for 30 min, the solution was concentrated *in vacuo*. To a solution of H-D-Val-Orn(Z)-Leu-D-Phe-Pro-OH prepared above and TEA (0.4 ml, 2.8 mmol) in DMF (8 ml) at 0 °C was added **4'** derived from **4**. The mixture was left to stand for 2 h at 0 °C and overnight at room temperature. The solution was evaporated, and the residue was dissolved into AcOEt (200 ml). The solution was washed with 5% citric acid and water, and was evaporated. The product was recrystallized from MeOH-ether; yield, 1.37 g (64%); mp 146—148 °C; $[\alpha]_D^{29}$ −38.3° (*c* 1, DMF); R_f^1 0.32, R_f^2 0.42. Found: C, 62.91; H, 7.67; N, 10.75%. Calcd for $C_{81}H_{114}O_{17}N_{12}\cdot H_2O$: C, 62.93; H, 7.56; N, 10.87%.

cyclo[-D-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$ (6). Boc-[D-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-OH (0.94 g, 0.6 mmol) was converted into the succinimido ester (**5'**) by the method described for **5**. It was dissolved in TFA (10 ml) containing a few drops of anisole at 0 °C. The mixture were stirred for 30 min at room temperature and then concentrated. The residue, the trifluoroacetate of the decapeptide active ester, was triturated with ether, collected by filtration and dissolved in DMF (8 ml). The solution was added dropwise into pyridine (400 ml) at 60 °C. After stirring for 3 h at 60 °C, the solvent was evaporated, and then water was added to the residue. The resulting solid was collected by filtration, washed with water and dried. The product was purified by reprecipitaion from MeOH-ether; yield, 0.21 g (25%); mp 146—148 °C; $[\alpha]_D^{29}$ −58.0° (*c* 0.5, DMF); R_f^1 0.73, R_f^2 0.70. Found: C, 63.89; H, 7.56; N, 11.81%. Calcd for $C_{76}H_{104}O_{14}N_{12}\cdot H_2O$: C, 63.93; H, 7.48; N, 11.77%.

cyclo(-D-Val-Orn-Leu-D-Phe-Pro-)$_2$·2HCl (7). **6** (100 mg, 0.07 mmol) was hydrogenated in 90% MeOH (35 ml) containing 1 M HCl (0.14 ml) in the presence of Pd black for 8 h. After removal of the catalyst, the filtrate was concentrated *in vacuo*. The product was purified by gel filtration on a Sephadex LH-20 column (1×150 cm) using MeOH as solvent, and by reprecipitation from MeOH-ether; yield, 40 mg (47%); mp 241—243 °C (dec.); $[\alpha]_D^{29}$ −55.1° (*c* 0.2, EtOH); R_f^3 0.55, R_f^4 0.71. Amino acid ratios: Val, 0.93; Orn, 0.90; Leu, 1.05; Phe, 1.07; Pro, 1.07. MS (SIMS), *m/z* 1141 ($C_{60}H_{93}O_{10}N_{12}$; MH^+) Found: C, 55.93; H, 7.68; N, 12.83%. Calcd for $C_{60}H_{92}O_{10}N_{12}\cdot 2HCl\cdot 4H_2O$; C, 56.01; H, 7.99; N, 13.06%.

Boc-D-Val-Orn(Z)-Leu-Phe-Pro-OBzl (8). This product was synthesized with DCC and HOBt by stepwise elongation from Pro-OBzl·HCl (2.57 g, 10 mmol) as described for the preparation of **3**. The product was purified by a silica-gel column (2.2×40 cm) using $CHCl_3$–MeOH (50:1) as a solvent, and the fractions containing the desired product were combined and concentrated. The product was recrystallized from MeOH-ether; yield, 4.6 g (50%); mp 92—96 °C; $[\alpha]_D^{29}$ −37.9° (*c* 1, DMF); R_f^1 0.80, R_f^2 0.59. Found: C, 66.08; H, 7.55; N, 9.09%. Calcd for $C_{50}H_{68}O_{10}N_6$: C, 65.77: H, 7.51, N, 9.20%.

Boc-D-Val-Orn(Z)-Leu-Phe-Pro-NHNH$_2$ (9). Compound **8** (1.49 g, 1.63 mmol) was treated with hydrazine hydrate (1.5 ml) as described for the preparation of **2**; yield, 0.95 g (70%); mp 132—135 °C; $[\alpha]_D^{29}$ −32.0° (*c* 0.5, DMF); R_f^1 0.55, R_f^2 0.44. Found: C, 60.54; H, 7.75; N, 13.03%. Calcd for $C_{43}H_{64}O_9N_8\cdot H_2O$: C, 60.40; H, 7.77; N, 13.10%.

Boc-D-Val-Orn(Z)-Leu-Phe-Pro-OH (10). In a solution of **8** (1.01 g, 1.1 mmol) in MeOH (24 ml) and dioxane (12 ml), 1 M NaOH (2.3 ml) was added. The solution was stirred for 5 h at room temperature. After the addition of water (10 ml), the solution was evaporated, and then 10% citric acid was added to the residue. The resulting solid was collected by filtration, washed with water and dried. The product recrystallized from MeOH-ether; yield, 0.68 g (75%); mp 134—136 °C; $[\alpha]_D^{29}$ −28.6° (*c* 0.5, DMF); R_f^1 0.50, R_f^2 0.44. Found: C, 62.42; H, 7.71: N, 10.48%. Calcd for $C_{43}H_{62}O_{10}N_6$: C, 62.76; H, 7.59; N, 10.21%.

Boc-[D-Val-Orn(Z)-Leu-Phe-Pro-]$_2$-OH (11). This compound was prepared from **9** (0.62 g, 0.73 mmol) and **10** (0.6 g, 0.73 mmol) as described for the preparation of **4**. The product was purified by gel filtration on a Sephadex LH-20 column (2.5×120 cm) using MeOH as solvent, and recrystallization from MeOH-ether; yield, 0.74 g (66%); mp 150—151 °C; $[\alpha]_D^{29}$ −37.1° (*c* 0.5, DMF); R_f^1 0.40, R_f^2 0.40. Found: C, 62.86; H, 7.35; N, 10.84%. Calcd for $C_{81}H_{114}O_{17}N_{12}\cdot H_2O$: C, 62.93; H, 7.56; N, 10.87%.

cyclo[-D-Val-Orn(Z)-Leu-Phe-Pro-]$_2$ (12). This compound was prepared from **11** (0.41 g, 0.27 mmol) as described for the preparation of **6**. The product was purified

by silica-gel column chromatography, and recrystallization from MeOH–ether; yield, 179 mg (47%); mp 218—219 °C; $[\alpha]_D^{29}$ −22.0° (*c* 0.5, DMF); R_f^1 0.66, R_f^2 0.41. Found: C, 64.31; H, 7.59; N, 12.23%. Calcd for $C_{76}H_{104}O_{14}N_{12}$: C, 64.75; H, 7.43; N, 11.92%.

cyclo(-D-*Val-Orn-Leu-Phe-Pro-*)$_2$·*2HCl* (*13*). This compound was prepared from **12** (105 mg, 0.074 mmol) as described for the preparation of **7**; yield, 66 mg (74%); mp 239—241 °C; $[\alpha]_D^{29}$ −31.8° (*c* 0.2, EtOH); R_f^3 0.68, R_f^4 0.65. Amino acid ratios: Val, 0.95; Orn, 1.03; Leu, 1.03; Phe, 1.00; Pro, 1.00. MS(SIMS), *m/z* 1141 ($C_{60}H_{93}O_{10}N_{12}$, MH^+). Found: C, 56.03; H, 7.78; N, 13.08%. Calcd for $C_{60}H_{92}O_{10}N_{12}$·2HCl·4H_2O: C, 56.01; H, 7.99; N, 13.06%.

Cellulose Plate Electrophoresis. It was carried out with cellulose (Avicel) plate and with a solvent system of HCOOH–AcOH–MeOH–H_2O (1:3:6:10 v/v, PH 1.4) for 2 h at 500 V/20 cm. Each of analogs revealed a single spot, the mobility being the same as that of GS.

Microbiological Assays. The microorganisms employed were *Stapylococcus aureus* ATCC 6538, *Streptomyces pyogenes* N.Y.5, *Corynebacterium diphtheriae* P.W.8, *Micrococcus pyogenes* ATCC 10240, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NIHJ-JC2 and *Proteus vulgaris* OX 19. The minimum concentration of the compounds necessary for the complete inhibition of growth of these microorganisms was determined by an agar dilution method with 10^6 organisms per milliter.

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References

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3) Amino acid residues with no plefix are of L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of IUPAC-IBU Commission of Biological Nomencalture. Abbreviations used are as follows: Boc, *t*-butoxycarbonyl; Z, benzyloxycarbonyl; OBzl, benzyloxy; OEt, ethoxy; DCC, dicyclohexylcarbodiimide; WSCD, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; TFA, trifluoroacetic acid; TEA, triethylamine; NMM, *N*-methylmorpholine; DMF, *N,N*-dimethylformamide; AcOEt, ethyl acetate.

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