Studies of a Peptide Antibiotic "Gratisin". II

Makoto TAMAKI,* Michiaki TAKIMOTO, and Ichiro MURAMATSU†
Department of Chemistry, Faculty of Science, Toho University, Miyama, Funabashi-shi, Chiba 274
†Department of Chemistry, College of Science, Rikkyo University, Nishi-ikebukuro, Tokyo 171
(Received November 29, 1986)

In order to investigate the structure-activity relationship of the "gratisin"-related peptide, *cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)$_2$ (GR-I), its two analogs, *cyclo*(-Val-Orn-Leu-D-Ala-Pro-D-Tyr-)$_2$ (**6a**) and *cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Ala-)$_2$ (**6b**), were synthesized. The CD spectra of these synthetic peptides in an aqueous solution were similar to that of GR-I, indicating that these peptides have similar conformations in an aqueous solution. The activity of **6b** was half that of GR-I, whereas **6a** did not show any antibiotic activity. These results indicated that the phenyl group of the D-Phe residue preceding the Pro residue is essential for exhibiting the activity, while the *p*-hydroxyphenyl group of the D-Tyr residue following the Pro residue is not essential.

A peptide antibiotic, gratisin, which shows activity against *Bacillus subtilis* 720, was isolated from *Bacillus brevis* Y-33 by Silaev et al.[1,2] In order to determine the primary structure of natural gratisin, we have ourselves synthesized several peptides on the basis of the primary structure proposed by Silaev et al. In these synthetic studies,[3] we found that *cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)$_2$ (GR-I)[4] possesses the strongest activity against the gram-positive microorganisms tested and that it shows the same activity as that of gramicidin S-1 (GS)[5] toward *Bacillus subtilis;* we proposed that the amino-acid sequence of GR-I is most probable as that of natural peptide. We also reported that *cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Phe-)$_2$, in which D-Tyr residues at positions 6 and 6′ of GR-I are replaced with D-Phe residues, shows almost the same activity as does GR-I, indicating that the hydroxyl groups of the D-Tyr residues at positions 6 and 6′ are not necessary for exhibiting antibiotic activity.[3]

In order to investigate further the structure-activity relationship of GR-I, we synthesized two analogs, *cyclo*(-Val-Orn-Leu-D-Ala-Pro-D-Tyr-)$_2$ (**6a**) and *cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Ala-)$_2$ (**6b**) (Fig. 1), in which D-Phe-Pro-D-Tyr sequences in GR-I are replaced with D-Ala-Pro-D-Tyr and D-Phe-Pro-D-Ala sequences respectively. The roles of the side chains of the D-Phe residues at positions 4 and 4′ and of the D-Tyr residues at positions 6 and 6′ in GR-I for exhibiting the antibiotic activity were elucidated.

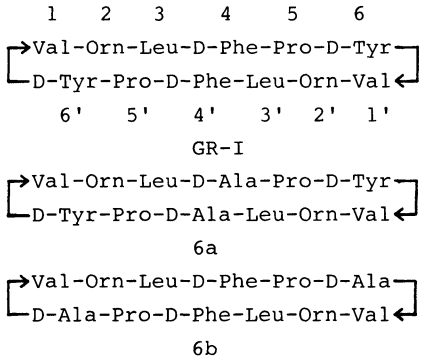

Fig. 1. Primary structures of GR-I and its analogs (**6a** and **6b**).

The routes of the syntheses of these peptides are shown in Fig. 2. In the synthesis of peptide **6a**, the Boc-hexapeptide benzyl ester (**1a**) was synthesized from the Pro benzyl ester by step-by-step elongation using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt). Then, a part of the **1a** was converted into the corresponding hydrazide (**2a**). The coupling of **2a** with the hexapeptide benzyl ester derived from **1a** was carried out using the azide method; this afforded the Boc-dodecapeptide benzyl ester (**3a**). This ester **3a** was treated with hydrazine hydrate to give the corresponding hydrazide (**4a**), and then the Boc-group of **4a** was removed by the action of trifluoroacetic acid. The dodecapeptide hydrazide trifluoroacetate (**4a′**) thus obtained was changed to the azide by using isopentyl nitrite at −20 °C for 15 min. The azide could be cyclized under a high dilution in pyridine at 0 °C for 3 d. The resulting product was purified by means of silica-gel column chromatography, followed by recrystallization. The cyclododecapeptide (**5a**) was obtained in a 53% yield. The removal of all the masking group of **5a** by hydrogenolysis yielded **6a**. Peptide **6b** was synthesized in a similar manner. In the synthesis of **1b**, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCD·HCl) was used as the coupling reagent instead of DCC. The yield of cyclization by the azide method was 67%. The homogeneities of the products were confirmed by means of thin-layer chromatography (TLC), amino-acid analysis, high-performance liquid chromatography (HPLC), and fast-atom-bombardment (FAB) mass spectrum.

The CD spectra of GR-I and its two analogs were measured in aqueous solutions (Fig. 3). Peptide **6a** showed almost the same CD curve as GR-I, although this analog does not contain aromatic side chains at positions 4 and 4′. In the CD spectrum of peptide **6b** we observed a curve similar to that of GR-I, but its trough was shallower than the troughs of GR-I. These results indicated that, in aqueous solutions, these synthetic peptides possess a conformation similar to that of GR-I; they also show that the aromatic side chains of the D-Phe residues at positions 4 and 4′ make only a

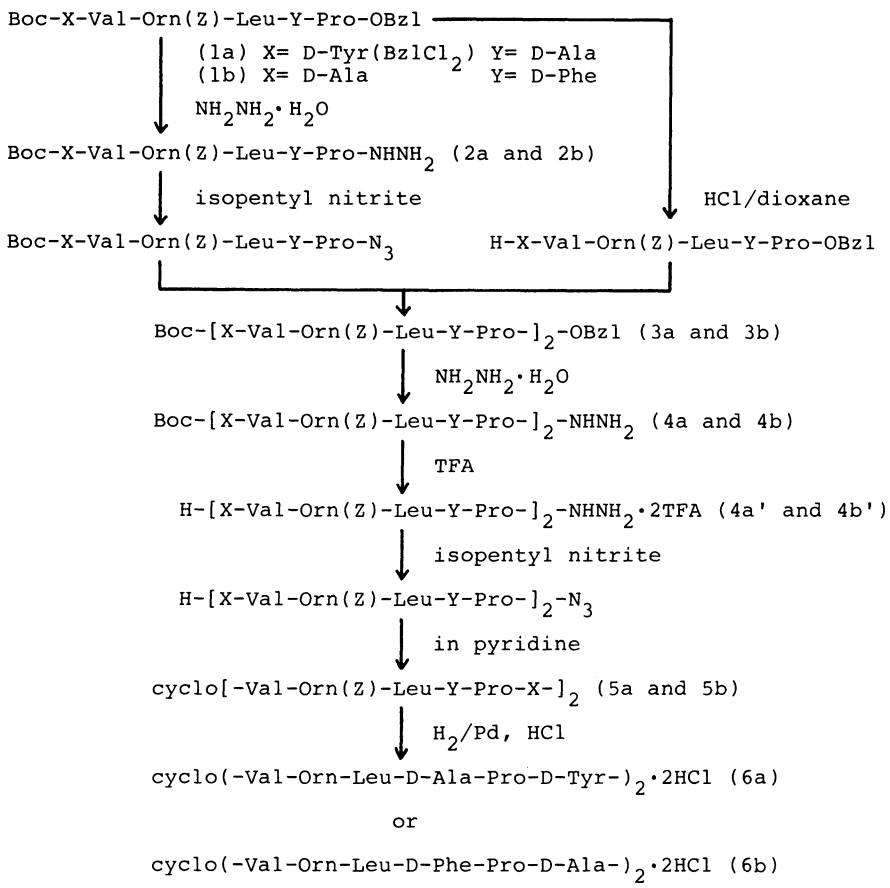

Fig. 2. Synthesis of peptides **6a** and **6b**.

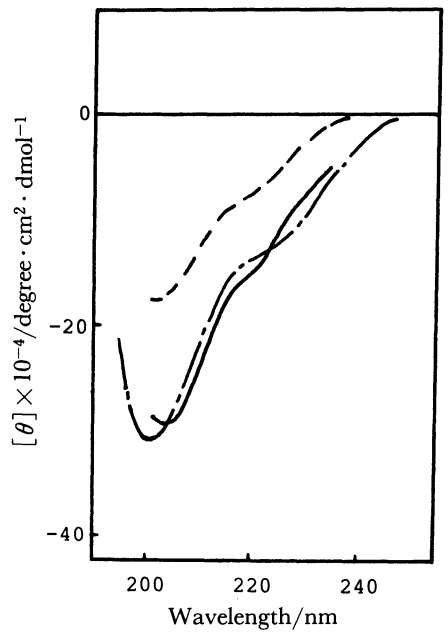

Fig. 3. CD spectra of **6a** ——, **6b**----, GR-I -·-·- in water.

small contribution to the conformation in an aqueous solution.

The antibiotic activity of these synthetic peptides is summarized in Table 1. Peptide **6a**, which has a D-Ala-Pro-D-Tyr sequence, showed no activity against the microorganisms tested, although this peptide possesses almost the same CD curve as GR-I in an aqueous solution. Lee et al.[6] reported that [D-Ala$^{4,4'}$]GS containing D-Ala residues in place of D-Phe residues preceding Pro residues possesses about 1/8 of the activity of the natural GS against *Bacillus subtillis* and *Staphylococcus aureus*, and that its ORD spectrum is similar to that of GS. The difference in activity between **6a** and [D-Ala$^{4,4'}$]GS may result from the difference in their ring sizes, in other words, the difference in the rigidity of their ring conformations. The activity of **6b**, which has a D-Phe-Pro-D-Ala sequence, was half that of GR-I. These results indicated that, in GR-analog peptides, the phenyl group of the D-Phe residue preceding the Pro residue is essential for the activity, while the *p*-hydroxyphenyl group of the D-Tyr residue following the Pro residue is not essential. Further, no relationship between the CD spectra in aqueous solution and the antibiotic activities could be found.

As for the mode of action of gratisin and GS, it is considered that the most significant role for their antibiotic action is played by each ring conformation induced during the binding of their antibiotics to the bacterial cell.[7-9] In the present studies, it is suggested that peptides **6a** and **6b** in aqueous solutions have conformations similar to that of GR-I, but that, when these peptides interact with the bacterial cell, peptide

Table 1. Antibiotic Activity of GR-I and Its Analogs[a)]

Test organism	GR-I	6a	6b
Staph. aureus ATCC 6538 P	6.3	>50	12.5
Strept. pyogenes N.Y. 5	3.1	>50	6.25
Micrococcus flavus ATCC 10240	3.1	>50	6.25
Corynebact. diphtheriae P.W. 8	3.1	>50	3.13
Bac. subtilis ATCC 6633	3.1	>50	6.25
E. coli NIHJ-JC2	>50	>50	>100
Proteus vulgaris OX 19	>50	>50	>100

a) The minimum inhibitory concentration ($\mu g\,ml^{-1}$) was determined by means of an agar-dilution method with 10^6 organisms per milliliter.

6b can take an active conformation similar to that of GR-I, while peptide **6a** can not take the active conformation. This great contrast between the two peptides is caused by the removal of the aromatic ring of the residues at positions 4 and 4'.

Experimental[4)]

All the melting points are uncorrected. The CD spectra were obtained by use of a JASCO spectropolarimeter, model J-500, using a 1-mm quartz cell at room temperature. The CD spectroscopy of GR-I and its two analogs was carried out with an aqueous solution of their dihydrochlorides at the concentration of $1.1—1.5\times10^{-4}$ M (1 M=1 mol dm^{-3}). The molecular weights of these synthetic peptides were determined by FAB mass spectrometry using a JEOL D-300 mass spectrometer. Amino-acid analyses were carried out by means of a Hitachi 835 amino-acid analyzer after the hydrolysis of the peptides in 6 M HCl at 110 °C for 24 h. HPLC was performed by means of an ODS column (ϕ 4.6×250 mm), using MeOH-5% $NaClO_4$ aq (5:1) as the elution solvent. TLC 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-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Ala-Pro-OBzl (1a). DCC (2.06 g, 10 mmol) was added to a solution of Boc-D-Ala (1.90 g, 10 mmol), HOBt (1.49 g, 11 mmol), Pro-OBzl·HCl (2.42 g, 10 mmol), and NMM (1.1 ml, 10 mmol) in $CHCl_3$ (50 ml) at 0 °C. This solution was stirred for 2 h at 0 °C and then overnight at room temperature. After the reaction mixture has then been concentrated in vacuo, AcOEt (20 ml) was added to the residue and insoluble substances were removed by filtration. The filtrate was then diluted with AcOEt (200 ml). The solution was washed successively with 5% citric acid, water, 5% Na_2CO_3 and water, and dried over sodium sulfate. After the removal of the drying reagent, the solvent was evaporated in vacuo. The residue was dissolved in 4 M HCl/dioxane (20 ml) containing anisole (0.2 ml) at 0 °C. After stirring for 30 min at room temperature, the solution was concentrated in vacuo. The residue was dissolved in $CHCl_3$ (50 ml). The solution was washed with 10% Na_2CO_3 and water under cooling with an ice bath. To this solution we then added Boc-Leu (2.40 g, 10 mmol), HOBt (1.49 g, 11 mmol), and DCC (2.06 g, 10 mmol) at 0 °C. The same procedure as has been described above was repeated for this reaction mixture. Further, Boc-Orn(Z), Boc-Val, and Boc-D-Tyr(BzlCl$_2$) were successively coupled by the same method. All the reactions were followed by TLC on a silica-gel plate. The crude, protected hexapeptide obtained from the final reaction mixture was purified by chromatography on a silica-gel column (ϕ 2×50 cm), using a solvent system of $CHCl_3$-MeOH (50:1). The fractions containing the desired product were combined and concentrated. The product was reprecipitated from AcOEt-ether; overall yield, 3.28 g (28% from Pro-OBzl); mp 161—163 °C; $[\alpha]_D^{29}$ −13.2° (*c* 1, DMF); R_f^1 0.63, R_f^2 0.55.

Found: C, 62.20; H, 6.81; N, 8.82%. Calcd for $C_{60}H_{77}O_{12}N_7Cl_2$: C, 62.17; H, 6.69; N, 8.46%.

Boc-D-Ala-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl (1b). This compound was prepared from Pro-OBzl·HCl (1.45 g, 6 mmol) in a manner similar to that described for the preparation of **1a**. In the synthesis of this compound, WSCD·HCl was used instead of DCC as the coupling reagent, and the reaction mixture was washed successively in a $CHCl_3$ solution with 5% citric acid and 5% Na_2CO_3; yield, 3.84 g (64%); mp 188—193 °C; $[\alpha]_D^{29}$ −33.5° (*c* 1, DMF); R_f^1 0.76, R_f^2 0.53.

Found: C, 64.54; H, 7.47; N, 10.05%. Calcd for $C_{53}H_{73}O_{11}N_7$: C, 64.68; H, 7.48; N, 9.96%.

Boc-D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Ala-Pro-NHNH$_2$ (2a). A solution of **1a** (1.17 g, 1 mmol) and hydrazine hydrate (1.5 ml) in DMF (10 ml) was allowed to stand for 2 d at room temperature. The solution was concentrated in vacuo, and then water was added to the residue. The resulting solid product was collected by filtration; yield, 0.96 g (88 %); mp 167—170 °C; $[\alpha]_D^{29}$ −17.6° (*c* 1, DMF); R_f^1 0.49, R_f^2 0.33.

Found: C, 58.44; H, 6.92; N, 11.69%. Calcd for $C_{53}H_{73}O_{11}N_9Cl_2$: C, 58.77; H, 6.79; N, 11.64%.

Boc-D-Ala-Val-Orn(Z)-Leu-D-Phe-Pro-NHNH$_2$ (2b). Compound **1b** (1.81 g, 1.84 mmol) was treated with hydrazine hydrate in the manner described for the preparation of **2a**; yield, 1.20 g (72%); mp 171—173 °C; $[\alpha]_D^{29}$ −34.2° (*c* 1, DMF); R_f^1 0.54, R_f^2 0.49.

Found: C, 60.64; H, 7.74; N, 13.56 %. Calcd for $C_{46}H_{69}O_{10}N_9$: C, 60.84; H, 7.66; N, 13.88%.

Boc-[D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Ala-Pro-]$_2$-OBzl (3a). Compound **1a** (0.95 g, 0.82 mmol) was dissolved in 4 M HCl/dioxane (15 ml) containing anisole (0.5 ml) at 0 °C. After stirring at room temperature for 30 min, the solution was concentrated in vacuo. Ether was added to the residue, and the resulting solid, H-D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Ala-Pro-OBzl·HCl, was collected by filtration. To a solution of **2a** (0.86 g, 0.8 mmol) in DMF (20 ml) we added 4 M HCl/dioxane (0.6 ml, 2.4 mmol) and isopentyl nitrite (0.12 ml, 0.9 mmol) at −40 °C. After stirring at −20 °C for 15 min, the solution was cooled again to −40 °C and neutralized with TEA (0.34 ml, 2.4 mmol). This solution was combined with the chilled solution of H-D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Ala-Pro-OBzl·HCl mentioned above and TEA (0.12 ml, 0.82 mmol) in DMF (12 ml). The mixture was stirred at 0 °C

for 3 d and then concentrated. The residue was dissolved in AcOEt (200 ml), and the solution was washed with 5% citric acid, water, 5% Na_2CO_3, and water, and dried over sodium sulfate. After the solution has been concentrated, the residue was purified by chromatography on a silica-gel column (ϕ 1.8×30 cm), using a solvent system of $CHCl_3$-MeOH (20 : 1), and the fractions containing the desired product were combined and concentrated. Further purification was performed by reprecipitation from $CHCl_3$-ether; yield, 0.90 g (53%); mp 202—205 °C; $[\alpha]_D^{29}$ −9.36° (*c* 1, DMF); R_f^1 0.55, R_f^2 0.77.

Found: C, 61.19; H, 6.81; N, 9.43%. Calcd for $C_{108}H_{138}O_{21}N_{14}Cl_4$: C, 61.47; H, 6.59; N, 9.29%.

Boc-[D-Ala-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-OBzl (3b). This compound was prepared from **1b** (1.08 g, 1.1 mmol) and **2b** (1.00 g, 1.1 mmol) in a manner similar to that described for the preparation of **3a**. Purification was performed by silica-gel column chromatography, followed by recrystallization from AcOEt-ether; yield, 1.16 g (60%); mp 213—216 °C; $[\alpha]_D^{29}$ −35.8° (*c* 1, DMF); R_f^1 0.53, R_f^2 0.68.

Found: C, 64.02; H, 7.67; N, 11.13%. Calcd for $C_{94}H_{130}O_{19}N_{14}$: C, 64.14; H, 7.44; N, 11.14%.

Boc-[D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Ala-Pro-]$_2$-NHNH$_2$ (4a). Compound **3a** (0.76 g, 0.36 mmol) was treated with hydrazine hydrate as has been described for the preparation of **2a**; yield, 0.61 g (83%); mp 206—210 °C; $[\alpha]_D^{29}$ −15.8° (*c* 1, DMF); R_f^1 0.51, R_f^2 0.26.

Found: C, 59.34; H, 6.83; N, 10.98%. Calcd for $C_{101}H_{134}O_{20}N_{16}Cl_4$: C, 59.64; H, 6.64; N, 11.02%.

Boc-[D-Ala-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-NHNH$_2$ (4b). Compound **3b** (1.00 g, 0.57 mmol) was treated with hydrazine hydrate as has been described for the preparation of **2a**; yield, 0.90 g, (92%); mp 214—218 °C; $[\alpha]_D^{29}$ −36.0° (*c* 1, DMF); R_f^1 0.59, R_f^2 0.49.

Found: C, 61.43; H, 7.61; N, 13.13%. Calcd for $C_{87}H_{126}O_{18}N_{16}\cdot H_2O$: C, 61.39; H, 7.58; N, 13.16%.

***Cyclo*[-Val-Orn(Z)-Leu-D-Ala-Pro-D-Tyr(BzlCl$_2$)-]$_2$ (5a).** Compound **4a** (0.55 g, 0.27 mmol) was dissolved in TFA (10 ml) containing anisole (0.2 ml). The mixture was stirred for 40 min at room temperature and then concentrated in vacuo. The residue was triturated with ether and collected by filtration. To a solution of the trifluoroacetate of the dodecapeptide hydrazide in DMF (5 ml), we then added 4 M HCl/dioxane (0.2 ml) and isopentyl nitrite (0.041 ml, 0.30 mmol) at −40 °C. After stirring at −20 °C for 15 min, the mixture was poured, drop by drop, into pyridine (300 ml) at 0 °C. After stirring for 3 d at 0 °C, the solution was concentrated. The addition of water to the residue afforded a precipitate, which was then filtered and washed with water. Purification was performed by recrystallization from MeOH-ether; yield, 272 mg (53%); mp 270—275 °C (decomp); $[\alpha]_D^{29}$ −9.1° (*c* 0.8, DMF); R_f^1 0.72, R_f^2 0.55.

Found: C, 59.91; H, 6.75; N, 9.59 %. Calcd for $C_{96}H_{122}O_{18}N_{14}Cl_4\cdot 2H_2O$: C, 59.50; H, 6.55; N, 10.12%.

***Cyclo*[-Val-Orn(Z)-Leu-D-Phe-Pro-D-Ala-]$_2$ (5b).** This compound was prepared from **4b** (0.70 g, 0.42 mmol) as has been described for the preparation of **5a**. The purification of this compound was performed by chromatography on a silica-gel column (ϕ 1.8×20 cm), using a solvent system of $CHCl_3$-MeOH (50 : 1), followed by recrystallization from MeOH-ether; yield, 438 mg (67%); mp 271—276 °C (decomp); $[\alpha]_D^{29}$ −86.0° (*c* 0.8, DMF); R_f^1 0.72, R_f^2 0.53.

Found: C, 62.87; H, 7.49; N, 12.01%. Calcd for $C_{82}H_{114}O_{16}N_{14}\cdot 1.5\ H_2O$: C 62.37; H, 7.46; N, 12.41 %.

***Cyclo*(-Val-Orn-Leu-D-Ala-Pro-D-Tyr-)$_2\cdot$2HCl (6a).** Compound **5a** (100 mg, 0.052 mmol) was dissolved in 90% MeOH (20 ml) and dioxane (10 ml), and then 1 M HCl (0.11 ml) was added to the solution. The mixture was hydrogenated in the presence of palladium black for 15 h. After the removal of the catalyst, the filtrate was concentrated in vacuo. The product was purified by gel filtration on a Sephadex LH-20 column (ϕ 1.3×120 cm), using MeOH as the solvent, and by reprecipitation from MeOH-ether; yield, 40 mg (55 %); mp 256—259 °C (decomp); $[\alpha]_D^{29}$ −56.0° (*c* 0.2, EtOH); R_f^3 0.76, R_f^4 0.44. Amino acid ratios: Val, 0.98; Orn, 0.97; Leu, 1.00; Ala, 1.11; Pro, 1.01; Tyr, 0.94. MS (FAB), *m/z* 1316 ($C_{66}H_{102}O_{14}N_{14}$, MH^+).

Found: C, 53.75; H, 7.60; N, 13.27%. Calcd for $C_{66}H_{102}O_{14}N_{14}\cdot 2\ HCl\cdot 5\ H_2O$: C, 53.61; H, 7.77; N, 13.26%.

***Cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Ala-)$_2\cdot$2HCl (6b).** This compound was prepared from **5b** (200 mg, 0.13 mmol) as has been described for the preparation of **6a**; yield, 164 mg (93%); mp 276—279 °C (decomp) $[\alpha]_D^{29}$ −134.1° (*c* 0.4, EtOH); R_f^3 0.62, R_f^4 0.65. Amino acid ratios: Val, 1.00; Orn, 0.98; Leu, 1.00; Phe, 1.02; Pro, 1.03; Ala, 1.07. MS(FAB), *m/z* 1283 ($C_{66}H_{102}O_{12}N_{14}$, MH^+).

Found: C, 55.12; H, 7.42; N, 13.23%. Calcd for $C_{66}H_{102}O_{12}N_{14}\cdot 2\ HCl\cdot 4.5\ H_2O$: C, 55.14; H, 7.92; N, 13.64%.

Cellulose-Plate Electrophoresis. The electrophoresis of the products was carried out with a cellulose (Avicel) plate and a solvent system of HCOOH-AcOH-MeOH-H_2O (1 : 3 : 6 : 10 v/v, pH 2.2) for 2 h at 500 V /20 cm. Each of the peptides revealed a single spot, the mobility being the same as that of GS.

We are grateful to the staff of the Research Laboratories of the Toyo Jozo Co. for their elemental analyses, microbiological assays, and the measurement of the FAB Mass.

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4) The amino acid residues with no prefix have the L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission of Biological Nomencalture. The abbreviations used are as follows: Boc, *t*-butoxycarbonyl; Z, benzyloxycarbonyl; OBzl, benzyloxy; $BzlCl_2$ 2,6-dichlorobenzyl; TFA, trifluoroacetic acid; TEA, triethylamine; NMM, *N*-methylmorpholine; DMF, *N,N*-dimethylformamide; AcOEt, ethyl acetate.

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