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Studies on Peptide Antibiotic "Gratisin"

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According to a primary structure proposed for an antibiotic peptide, gratisin, four peptides containing respectively partial sequences, L-Phe-L-Pro-L-Tyr, D-Phe-L-Pro-L-Tyr, L-Phe-L-Pro-D-Tyr, and D-Phe-L-Pro-D-Tyr, were synthesized by a liquid phase method. Among them, *cyclo*(-L-Val-L-Orn-L-Leu-D-Phe-L-Pro-D-Tyr-)$_2$ showed the strongest activity against the Gram-positive microorganisms tested. The activity toward *Bacillus subtilis* was similar to that of gramicidin S. Therefore, it is quite possible that natural gratisin is identical to this peptide. To facilitate a further investigation of the structure-activity relationship, four additional analogs were prepared. The results of their antibiotic activities showed that an interchange of D-Phe residues with D-Tyr residues caused the decrease of activity, and that the hydroxyl group of D-Tyr residues had no important role in this activity. The peptides possessing partial sequences, D-Phe-D-Tyr-L-Pro and L-Pro-D-Phe-D-Phe, exhibited strong activity. These facts show that the positions of Pro residues are not restricted to 5 and 5′ for the exhibition of the activity. The CD spectra of these synthetic peptides in an aqueous solution reflected the partial sequences around Pro residues, which affect largely the conformation of these synthetic antibiotics. The temperature dependence of these CD spectra indicated that the conformation of biologically active peptides is more stable than those of less active peptides.

An antibiotic peptide, gratisin, showing activity toward *Bacillus subtilis* 720 was isolated from *Bacillus brevis* Y-33 by Silaev *et al.*[1] It is a cyclododecapeptide composed of two each of Val, Orn, Leu, Phe, Pro, and Tyr residues. From an analogy of the primary structure of gramicidin S (GS) produced by *Bacillus brevis* ATCC 9999, the primary structure of gratisin was proposed (as shown in Fig. 1).[2,3] However, the configuration of each amino acid residue has not yet been established.

In this paper, the synthesis of several gratisin peptides shown in Fig. 2[4] and the relationship between structure and antibiotic activity will be described.

In the first of a series of studies on gratisin, gratisin peptide **I** consisted of only L-amino acid residues and gratisin peptide **II**, which has a structure analogous to GS containing D-Phe residues at positions 4 and 4′, were synthesized and the antibiotic activities of these peptides were examined.[5,6] Peptide **II** showed an activity against the Gram-positive microorganisms tested, but was less potent than GS. On the other hand, peptide **I** showed little activity. To study configurations of amino acid residues following Pro residues, peptide **III** having a Phe-Pro-D-Tyr partial sequence and peptide **IV** having a D-Phe-Pro-D-Tyr partial sequence were synthesized.[7,8] Peptide **III** was almost inactive. On the other hand, peptide **IV** showed a marked activity against the Gram-positive microorganisms tested. Its activity against *Bacillus subtilis* was similar to that of GS and stronger than that of peptide **II**. Peptide **V** having a D-Tyr-Pro-D-Phe partial sequence and peptide **VI** having a D-Phe-Pro-D-Phe partial sequence were synthesized in order to investigate the role of the side chain of D-Phe residues at 4 and 4′, and D-Tyr residues at 6 and 6′. A Pro residue is frequently found in β-turns in naturally occuring proteins[9] and peptides[10–12], and is known to play an important role in stabilizing their conformations. To investigate the roles of Pro residues in gratisin, peptide **VII** having Pro residues at positions 6 and 6′, and peptide **VIII** having Pro residues at 4 and 4′ were synthesized.

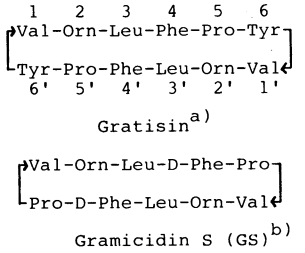


Fig. 1. Proposed structure of gratisin and structure of gramicidin S.
a) The configuration of each amino acid residue has not been established yet. b) Abbreviations with no prefix show L-amino acid residue.

I. cyclo(-Val-Orn-Leu-Phe-Pro-Tyr-)$_2$
II. cyclo(-Val-Orn-Leu-D-Phe-Pro-Tyr-)$_2$
III. cyclo(-Val-Orn-Leu-Phe-Pro-D-Tyr-)$_2$
IV. cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)$_2$
V. cyclo(-Val-Orn-Leu-D-Tyr-Pro-D-Phe-)$_2$
VI. cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Phe-)$_2$
VII. cyclo(-Val-Orn-Leu-D-Phe-D-Tyr-Pro-)$_2$
VIII. cyclo(-Val-Orn-Leu-Pro-D-Phe-D-Phe-)$_2$

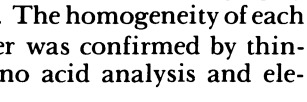
Fig. 2. Structure of synthesized peptides **I**—**VIII**.
Abbreviations with no prefix show L-amino acid residue.

Results and Discussion

Synthesis of Gratisin Peptides I—VIII. Each cyclododecapeptide **I**—**VIII** consists of two identical hexapeptide sequences (Fig. 2). Therefore, these peptides were synthesized according to the following three steps: 1) synthesis of hexapeptides by stepwise elongation; 2) synthesis of dodecapeptides by condensation between two of the fragment hexapeptides; 3) cyclization of the dodecapeptides by an azide or active-ester method. In the synthesis of these peptides, Pro residue was chosen as the C-terminal amino acid in order to avoid racemization in the fragment

condensation and the cyclization step. The benzyl (Bzl) group was used as the protector of the carboxyl groups. α-amino groups were protected by a *t*-butoxycarbonyl (Boc) group. The δ-amino group of Orn residue was protected by a benzyloxycarbonyl (Z), and the hydroxyl group of Tyr residue by a benzyl (Bzl) group or a 2,6-dichlorobenzyl ($BzlCl_2$) group. In the synthesis of gratisin peptides **I—VIII**, each Boc-hexapeptide benzyl ester was synthesized by stepwise elongation using dicyclohexylcarbodiimide (DCC) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSCD) and 1-hydroxybenzotriazole (HOBt) (Scheme 1). Throughout the synthesis of each Boc-hexapeptide benzyl ester, intermediates were purified by only washing with 5% citric acid and 5% Na_2CO_3. The purities of the intermediates were confirmed by thin-layer chromatography with several solvent systems. Finally, each Boc-hexapeptide benzyl ester was purified by silica-gel column chromatography followed by recrystallization. The homogeneity of each Boc-hexapeptide benzyl ester was confirmed by thin-layer chromatography, amino acid analysis and elemental analysis.

i) Synthesis of Gratisin Peptide I. Three different procedures were examined for the synthesis of gratisin peptide **I**. In the first trial, Pro residue was chosen as the C-terminal amino acid of the dodecapeptide, and cyclization was carried out by an azide method. However, the cyclization of H-[Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-]$_2$-N_3, did not proceed under the usual conditions. In the pathway of the biosynthesis of GS and tyrocidine in *Bacillus brevis*, they are produced by cyclization of the linear decapeptide with a Leu residue at the C-terminal.[13,14] In the imitation of the biosynthesis, a Leu residue was chosen as the C-terminal residue and the cyclization was also tried using an azide method *via* H-[Phe-Pro-Tyr(Bzl)-Val-Orn(Z)-Leu-]$_2$-N_3. Though this trial was also unsuccessful, it afforded a ninhydrin-negative compound. In an amino acid analysis of the acid hydrolysate of this product, each value of Val, Orn, Phe, Pro, and Tyr was in good agreement with the value expected for the gratisin peptide, but the value obtained by Leu was one-half of the value. The results of amino acid and elemental analyses of this product can be explained by giving it the following tentative structure; *cyclo*[-Phe-Pro-Tyr-(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-Tyr(Bzl)-Val-Orn(Z)-



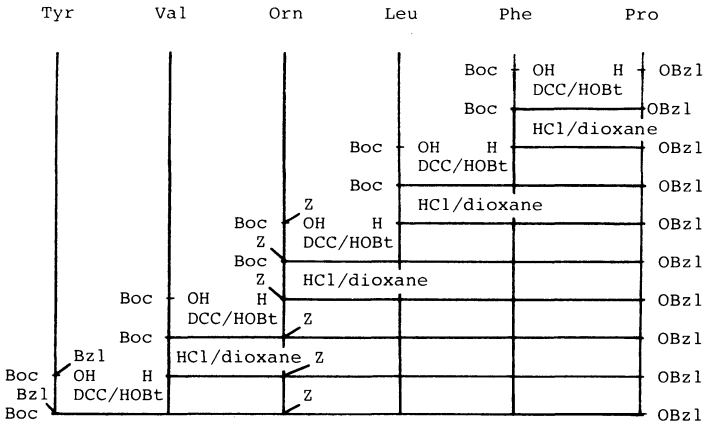
NH-CH-NH-CO-].

The cyclization to give peptide **I** was finally achieved using an active-ester method, according to Scheme 2. The active ester (**Id'**) was prepared by the following procedure. The coupling of Boc-hexapeptide hydrazide (**Ib**) with H-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-OH derived from **Ic** was carried out by an azide method. The product was purified by Sephadex LH-20 gel filtration, which was very effective for the removal of the contaminating amine and acid components. The Boc-dodecapeptide (**Id**) was converted into the corresponding active ester by WSCD and *N*-hydroxysuccinimide (HOSu). The active ester (**Id'**) was deprotected with trifluoroacetic acid (TFA) in the presence of anisole and then subjected to cyclization at 60 °C for 3 h under high dilution in pyridine ($<3\times10^{-3}$ M 1 M=1 mol dm^{-3}) to minimize intermolecular reactions. The product was purified by chromatography on a silica-gel column followed by recrystallization. The yield of the cyclization was 29%. The removal of the protecting groups of cyclododecapeptide (**Ie**) by hydrogenolysis in methanol containing hydrogen chloride afforded peptide **I**.

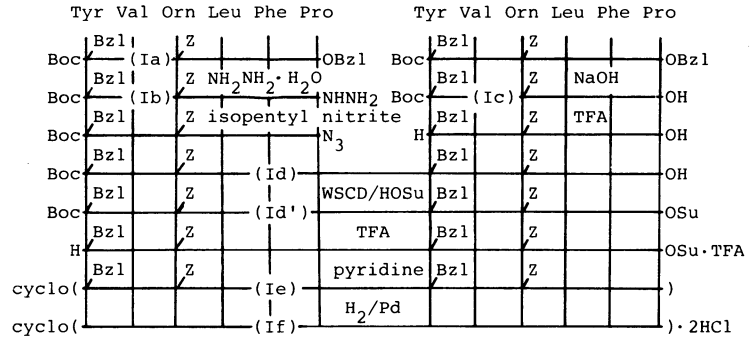
In the synthesis of gratisin peptide **III**, cyclization *via* the azide, H-[D-Tyr($BzlCl_2$)-Val-Orn(*Z*)-Leu-Phe-Pro-]$_2$-N_3, did not proceed. Hence, cyclization was carried out using an active-ester method similar to that described in the synthesis of gratisin peptide **I**. The desired peptide was obtained at a yield of 60%.

Peptides **VII** and **VIII** were also synthesized in a similar manner. The yields of cyclization by an active-ester method were 62% (peptide **VII**) and 57% (**VIII**).

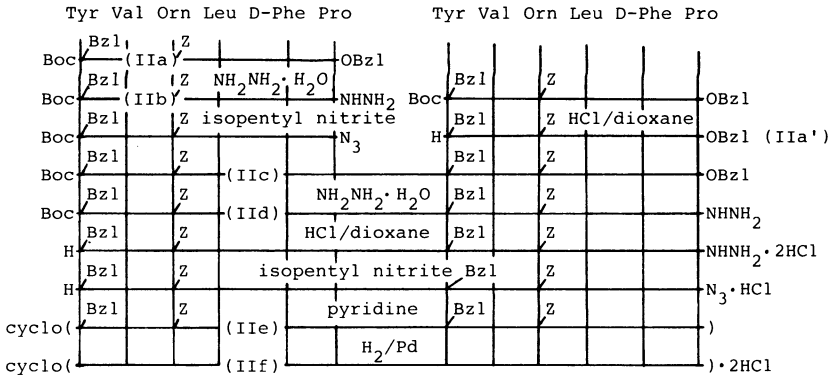
ii) Synthesis of Gratisin Peptide II. Peptide **II** was prepared according to Scheme 3. Boc-dodecapeptide benzyl ester (**IIc**) was obtained by the coupling of two hexapeptides (**IIa'** and **IIb**). The product was



Scheme 1. Synthesis of Boc-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-OBzl (**Ia**).
* Each hexapeptide (**IIa—VIIIa**) was synthesized by a similar method.
WSCD was used the coupling reagent in synthesis of **IVa—VIIIa** instead of DCC.



Scheme 2. Synthesis of *cyclo*(–Tyr–Val–Orn–Leu–Phe–Pro–)$_2$·2HCl (**If**).
* Gratisin peptides **III**, **VII**, and **VIII** were synthesized by a similar manner.



Scheme 3. Synthesis of *cyclo*(–Tyr–Val–Orn–Leu–D-Phe–Pro–)$_2$·2HCl (**IIf**).
* Gratisin peptides **IV**—**VI** were synthesized by a similar manner.

purified by Sephadex LH-20 gel filtration and silica-gel column chromatography. **IIc** was converted into hydrazide **IId**. Then, it was treated with 4M HCl/dioxane in the presence of anisole, and converted into an azide with isopentyl nitrite at −20 °C for 15 min. The azide could be cyclized under high dilution in pyridine at 0 °C for 3 d. The resulting product was purified by silica-gel column chromatography following recrystallization. The yield of cyclization was 43%. The removal of all masking groups by hydrogenolysis yielded peptide **II**.

Peptides **IV**—**VI** were synthesized by a method similar to that described for peptide **II**. The yields of cyclization by the azide method were 63 (peptide **IV**), 29 (**V**), and 41% (**VI**).

The homogeneity of peptides **I**—**VIII** was confirmed by means of thin-layer chromatography, electrophoresis, amino acid analysis and elemental analysis (Table 1 and 3). The molecular weight of the fully protected cyclic peptides were measured by a vapor-pressure osmometer and were in good agreement with the respective calculated values.

In the synthesis of peptides **I**—**IV**, cyclization was carried out by an azide method in the first trial. In the case of peptides **II** and **IV**, both having a D-Phe-Pro partial sequence, the cyclization proceeded satisfactorily. On the other hand, the cyclization of peptides **I** and **III**, both having a L-Phe-Pro partial sequence, did not proceed well by this method. The investigation of CPK molecular models of these four sequences elucidated that the β-methylene group

Table 1. Amino acid analysis of gratisin peptides **I**—**VIII**

	Val	Orn	Leu	Phe	Pro	Tyr
I.	1.04	1.04	1.06	1.02	0.94	0.91
II.	1.04	1.03	1.04	1.03	0.96	0.91
III.	1.07	1.03	1.01	0.94	0.94	0.94
IV.	1.03	1.09	1.02	0.92	0.95	0.90
V.	0.95	1.07	1.08	0.93	1.06	0.90
VI.	1.01	0.99	1.04	1.93	1.02	—
VII.	1.00	1.02	1.00	1.00	1.02	0.95
VIII.	1.00	0.99	1.03	1.99	0.99	—

of L-Phe residue preceding Pro residue collides with the δ-methylene group of the Pro residue if the L-Phe-Pro sequence take GS-like β-turn. On the other hand, the D-Phe-Pro sequence seems to favor the formation of β-turn. A similar tendency to β-turn formation was also reported for cyclic hexapeptides containing a D-Phe-Pro sequence by Kopple *et al.*[15], and on GS analogs and linear tetrapeptides with partial sequences related to the β-turn part of GS by Nagai *et al.*[16,17] The feasibility of the cyclization in the preparation of peptides **II** and **IV** by an azide method might result from such a β-turn preference in their primary structure containing a D-Phe-Pro sequence. The synthesis of peptides **I** and **III** was finally accomplished by an active-ester method. Under the conditions of this method, the degree of conformational freedom of the corresponding linear peptides

Table 2. Antibiotic activities of GS and synthetic peptides I—VIII[a]

Test organisms	GS	I	II	III	IV	V	VI	VII	VIII
Staph. aureus ATCC 6538P	1.6	>50	50	50	6.3	25	6.3	3.1	12.5
Strept. pyogenes N.Y. 5	1.6	>50	12.5	>100	3.1	12.5	6.3	6.3	6.3
Micrococcus flavus ATCC 10240	0.8	25	12.5	12.5	3.1	6.3	1.6	1.6	6.3
Corynebact. diphtheriae P.W. 8	0.8	25	3.1	100	3.1	6.3	1.6	1.6	3.1
Bac. subtilis ATCC 6633	3.1	>50	50	>100	3.1	25	6.3	3.1	3.1
E. coli NIHJ-JC2	>100	>50	>50	>100	>50	>50	>100	>100	>50
Proteus vulgaris OX 19	>100	>50	>50	>100	>50	>50	>100	>100	>50

a) Minimum inhibitory concentration in μg/ml: The minimum amount of the compounds necessary for the complete inhibition of the growth was determined by an agar dilution method with 10^6 organisms per milliter.

was enhanced as compared with that in an azide method, because the reaction was performed at a higher temperature. The increase of a chance collision between N- and C-terminals in the linear peptides might explain why cyclization by an active-ester method proceeded better than with an azide method.

Antibiotic Activity. The antibiotic activities of synthetic peptides **I—VIII** are shown in Table 2. Peptides **I—IV** have the amino acid sequence proposed by Silaev *et al.*, although the configurations of the amino acid residues preceding and following the Pro residue differ from each other. The extent of the activities of these pepides is influenced by the configuration of the amino acid residues. Peptide **I** and **III** having, respectively, L-Phe-Pro-L-Tyr and L-Phe-Pro-D-Tyr partial sequences showed little activity. Peptide II containing a D-Phe-Pro-L-Tyr partial sequence was active toward Gram-positive miroorganisms tested, but its activity against *Bacillus subtilis* was 1/16 of that of GS. On the other hand, peptide **IV** possessing a D-Phe-Pro-D-Tyr partial sequence was most active in peptides **I—IV**, and its activity against *Bacillus subtilis* was similar to that of GS. These results indicated that the presence of D-Phe residues at 4 and 4′ is important for activity, and D-residues at positions 6 and 6′ also contribute to activity. Although we have had no opportunity to directly compare our synthetic peptides with natural gratisin, it is quite possible that synthetic peptide **IV** possessing D-residues in positions 4, 4′, 6, and 6′ is identical with the natural antibiotic.

The activity of peptide **V** was 1/2—1/8 of that of peptide **IV**. This result points out the significance of the D-Phe residues at 4 and 4′ regarding activity. Similar results have been obtained for GS analogs.[18-20] For example, [D-Tyr$^{4,4'}$]-GS containing D-Tyr residues in place of D-Phe residues displayed only about 1/3 of the activity of GS.[18] On the other hand, peptide **VI** showed a similar activity to that of peptide **IV**. The result suggests that the hydroxyl groups of D-Tyr residues in the 6 and 6′ positions are not necessary for activity.

Peptide **VII** showed the highest activity among peptides **I—VIII**, and its activity against *Bacillus subtilis* was the same as that of GS. The activity of peptide **VIII** against *Bacillus subtilis* was also the same as that of GS, but the activity against other microorganisms was a little less compared to peptides **IV** and **VII**. It is an unexpected result that peptides **VII** and **VIII**, in which the Pro residues occupy positions different from 5 and 5′, showed a strong antibiotic activity, considering the restriction of the sequence in GS for its activity.[21] Supposing that the action mode of gratisin is the same as that of GS[22], this result suggests that the presence of additional amino acid residures, D-Tyr or D-Phe, in gratisin peptides make them more likely to adopt a conformation suitable for the antibiotic action regardless of the transfer of Pro residue, in comparison with rigidity of the structure of GS.

Secondary Structure-Activity Relationship. CD spectra of GS and synthetic peptides **I—VIII** (dihydrochloride) in aqueous solution are shown in Figs. 3 and 4. These spectra are classified into four groups according to their configuration around the Pro residue (Fig. 3). The spectrum of gratisin peptide **I** was characterized by a small negative band near 211 nm and a shoulder near 230 nm (Fig. 3-a), and resembles that of [L-Ala$^{4,4'}$]-GS.[16] The spectrum of peptide **II** showed double-minima in a similar region to that of GS, although its ellipticity was less (Fig. 3-b). Since peptide **II** has a D-Phe-Pro partial sequence similar to GS, these two peptides seem to adopt an analogous conformation to give similar CD spectra. A similar result was obtained by Takiguchi *et al.*[23] in a study on an analog of GS with a larger ring size. Their analog (*iso*-GS) involves two amide bonds between the carboxyl groups of Val residues and the δ-amino groups of Orn residues and, hence, the number of ring members of this analog is equal to that of peptide **II**. This analog showed an ORD spectrum similar to that of GS, but its trough was shallower than that of GS. The CD pattern of peptide **VII** is also similar to that of GS. (Fig. 3-b) and might result from the presence of a D-X-Pro-L-Y partial sequence in the peptide. The spectrum of peptide **III** showed negative bands near 199 nm and 230 nm, and a shoulder near 215 nm (Fig. 3-c). The CD spectrum of peptide **VIII** was characterized by a positive band at 217 nm and a shoulder near 205 nm, but was similar in shape to that of peptide **III**. The spectra of peptides **IV—VI** resembled each other and were characterized by negative bands near 200 nm and shoulders near 220 nm (Fig. 3-d). Some differences were found in ellipticities near 200 nm. However, no relationship between the depth of the trough in these CD spectra and antibiotic activity could be found.

Table 3. Yields, physical properties and analytical data of gratisin peptides **I**—**VIII**

	Yield %	Mp θ_m/°C	$[\alpha]_D^{25}$ (c 1, DMF)		Elemental analysis/%				R_f^1	R_f^2	R_f^3	R_f^4
						C	H	N				
Gratisin Peptide **I**												
a) Boc-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-OBzl	47	196—199	−27.7	$C_{66}H_{83}N_7O_{12}$	C:	67.96	7.17	8.41	0.94	0.67		
					F:	67.66	7.26	8.39				
b) Boc-Try(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-$NHNH_2$	71	174—178	−27.4	$C_{59}H_{79}N_9O_{11}$	C:	64.99	7.30	11.56	0.69	0.62		
					F:	64.89	7.56	11.46				
c) Boc-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-OH	88	163—166	−23.2	$C_{59}H_{77}N_7O_{12}$ $\cdot 1/2H_2O$	C:	65.29	7.24	9.04	0.60	0.62		
					F:	65.24	7.38	9.22				
d) Boc-[Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-]$_2$-OH	67	216—219 (dec.)	−32.0	$C_{113}H_{144}N_{14}O_{21}$ $\cdot 1/2H_2O$	C:	66.42	7.15	9.60	0.52	0.65		
					F:	66.42	7.39	10.04				
e) *cyclo*[-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-]$_2$	29	268—269 (dec.)	−47.5	$C_{108}H_{134}N_{14}O_{18}$ $\cdot H_2O$	C:	67.06	7.09	10.14	0.65	0.50		
					F:	66.77	7.02	10.14				
f) *cyclo*(-Tyr-Val-Orn-Leu-Phe-Pro-)$_2$·2HCl	94	236—241 (dec.)	−78.0 (EtOH c 0.15)	$C_{78}H_{110}N_{14}O_{14}$ $\cdot 2HCl \cdot 7H_2O$	C:	56.20	7.62	11.77			0.78	0.81
					F:	56.00	7.47	12.24				
Gratisin Peptide **II**												
a) Boc-Tyr(Bzl)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl	64	205—207	−27.7	$C_{66}H_{83}N_7O_{12}$	C:	67.96	7.17	8.14	0.94	0.64		
					F:	67.68	7.36	7.93				
b) Boc-Tyr(Bzl)-Val-Orn(Z)-Leu-D-Phe-Pro-$NHNH_2$	100	180—182	−30.4	$C_{59}H_{79}N_9O_{11}$	C:	64.99	7.30	11.56	0.65	0.54		
					F:	64.57	7.58	11.68				
c) Boc-[Tyr(Bzl)-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-OBzl	60	180—183	−42.8	$C_{120}H_{150}N_{14}O_{21}$	C:	67.84	7.12	9.23	0.93	0.64		
					F:	67.51	7.13	9.33				
d) Boc-[Tyr(Bzl)-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-$NHNH_2$	94	153—157	−42.0	$C_{113}H_{146}N_{16}O_{20}$ $\cdot H_2O$	C:	65.68	7.22	10.84	0.77	0.60		
					F:	65.28	7.21	10.88				
e) *cyclo*[-Tyr(Bzl)-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$	43	169—173	−77.5 (c 0.2)	$C_{108}H_{134}N_{14}O_{18}$ $\cdot H_2O$	C:	67.06	7.09	10.14	0.72	0.60		
					F:	67.04	7.21	10.34				
f) *cyclo*(-Tyr-Val-Orn-Leu-D-Phe-Pro-)$_2$·2HCl	90	267—269 (dec.)	−130.0 (EtOH c 0.15)	$C_{78}H_{110}N_{14}O_{14}$ $\cdot 2HCl \cdot 7H_2O$	C:	56.20	7.62	11.77			0.69	0.79
					F:	56.51	7.14	11.42				
Gratisin peptide **III**												
a) Boc-D-Tyr($BzlCl_2$)-Val-Orn(Z)-Leu-Phe-Pro-OBzl	63	170—173	−17.9	$C_{66}H_{81}N_7O_{12}Cl_2$ $\cdot H_2O$	C:	63.25	6.67	7.82	0.73	0.64		
					F:	63.31	6.58	7.81				
b) Boc-D-Tyr($BzlCl_2$)-Val-Orn(Z)-Leu-Phe-Pro-$NHNH_2$	97	138—142	−12.3	$C_{59}H_{77}N_9O_{11}Cl_2$ $\cdot 1/2H_2O$	C:	60.65	6.73	10.79	0.48	0.41		
					F:	60.89	6.41	10.67				
c) Boc-D-Tyr($BzlCl_2$)-Val-Orn(Z)-Leu-Phe-Pro-OH	99	146—150	−13.3	$C_{59}H_{75}N_7O_{12}Cl_2$ $\cdot H_2O$	C:	60.92	6.67	8.43	0.30	0.45		
					F:	61.34	6.58	7.82				
d) Boc-[D-Tyr($BzlCl_2$)-Val-Orn(Z)-Leu-Phe-Pro-]$_2$-OH	43	175—179	−12.9	$C_{113}H_{140}N_{14}O_{21}Cl_4$ $\cdot 2H_2O$	C:	61.46	6.57	8.88	0.28	0.40		
					F:	61.85	6.75	8.34				

TABLE 3. Continued.

		Yield %	Mp θ_m/°C	$[\alpha]_D^{25}$ (c 1, DMF)		Elemental analysis/%			R_f^1	R_f^2	R_f^3	R_f^4
							C	H	N				
e)	*cyclo*[-D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-Phe-Pro-]$_2$	60	228—229	+0.9	$C_{108}H_{130}N_{14}O_{18}Cl_4$	C:	63.15	6.38	9.55	0.70	0.60		
						F:	62.79	6.22	9.55				
f)	*cyclo*(-D-Tyr-Val-Orn-Leu-Phe-Pro-)$_2$·2HCl	55	283—287 (dec.)	−44.5 (DMF c 0.25)	$C_{78}H_{110}N_{14}O_{14}$ ·2HCl·6H$_2$O	C:	56.81	7.58	11.89			0.69	0.79
						F:	57.00	7.39	11.64				
	Gratisin peptide **IV**												
a)	Boc-D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl	41	172—176	−15.6	$C_{66}H_{81}N_7O_{12}Cl_2$	C:	64.17	6.67	7.94	0.66	0.59		
						F:	63.85	6.89	7.66				
b)	Boc-D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Phe-Pro-NHNH$_2$	95	144—148	−19.5	$C_{59}H_{77}N_9O_{11}Cl_2$ ·H$_2$O	C:	60.20	6.60	10.70	0.49	0.45		
						F:	60.20	6.82	10.09				
c)	Boc-[D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-OBzl	64	199—201	−21.7	$C_{120}H_{146}N_{14}O_{21}Cl_4$	C:	63.70	6.50	8.68	0.70	0.60		
						F:	63.62	6.25	8.67				
d)	Boc-[D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-NHNH$_2$	94	205—206	−27.1	$C_{113}H_{142}N_{16}O_{20}Cl_4$ ·2H$_2$O	C:	61.07	6.62	10.08	0.46	0.43		
						F:	61.13	6.72	9.58				
e)	*cyclo*[-D-Tyr(BzlCl$_2$)-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$	63	273—276	−24.0 (c 0.2)	$C_{108}H_{130}N_{14}O_{18}Cl_4$	C:	63.15	6.38	9.55	0.65	0.59		
						F:	63.26	6.25	9.51				
f)	*cyclo*(-D-Tyr-Val-Orn-Leu-D-Phe-Pro-)$_2$·2HCl	90	246—249 (dec.)	−72.3 (EtOH c 0.2)	$C_{78}H_{110}N_{14}O_{14}$ ·2HCl·7H$_2$O	C:	56.20	7.62	11.77			0.66	0.75
						F:	56.43	7.64	11.18				
	Gratisin peptide **V**												
a)	Boc-D-Phe-Val-Orn(Z)-Leu-D-Tyr(BzlCl$_2$)-Pro-OBzl	63	152—155	−20.7	$C_{66}H_{81}N_7O_{12}Cl_2$	C:	64.17	6.67	7.94	0.72	0.59		
						F:	63.86	6.75	7.77				
b)	Boc-D-Phe-Val-Orn(Z)-Leu-D-Tyr(BzlCl$_2$)-Pro-NHNH$_2$	90	132—135	−28.0	$C_{59}H_{77}N_9O_{11}Cl_2$	C:	61.13	6.70	10.87	0.59	0.46		
						F:	60.79	6.68	10.58				
c)	Boc-[D-Phe-Val-Orn(Z)-Leu-D-Tyr(BzlCl$_2$)-Pro-]$_2$-OBzl	56	195—196	−22.3	$C_{120}H_{146}N_{14}O_{21}Cl_4$	C:	63.70	6.50	8.68	0.83	0.59		
						F:	63.56	6.74	8.41				
d)	Boc-[D-Phe-Val-Orn(Z)-Leu-D-Tyr(BzlCl$_2$)-Pro-]$_2$-NHNH$_2$	85	188—190	−27.6	$C_{113}H_{142}N_{16}O_{20}Cl_4$	C:	62.08	6.55	10.25	0.64	0.50		
						F:	62.05	6.79	9.96				
e)	*cyclo*[-D-Phe-Val-Orn(Z)-Leu-D-Tyr(BzlCl$_2$)-Pro-]$_2$	29	256—257	−32.0	$C_{108}H_{130}N_{14}O_{18}Cl_4$	C:	63.15	6.38	9.55	0.80	0.59		
						F:	62.90	6.37	9.15				
f)	*cyclo*(-D-Phe-Val-Orn-Leu-D-Tyr-Pro-)$_2$·2HCl	95	234—237 (dec.)	−107.0 (EtOH c 0.2)	$C_{78}H_{110}N_{14}O_{14}$ ·2HCl·7H$_2$O	C:	56.20	7.62	11.77			0.73	0.72
						F:	56.29	7.44	11.12				
	Gratisin peptide **VI**												
a)	Boc-D-Phe-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl	70	181—183	−20.3	$C_{59}H_{77}N_7O_{11}$	C:	66.83	7.32	9.25	0.70	0.58		
						F:	66.35	7.34	8.62				
b)	Boc-D-Phe-Val-Orn(Z)-Leu-D-Phe-Pro-NHNH$_2$	95	174—176	−26.7	$C_{52}H_{73}N_9O_{10}$	C:	63.46	7.48	12.81	0.49	0.40		
						F:	63.04	7.84	12.37				

Table 3. Continued.

		Yield %	Mp θ_m/°C	$[\alpha]_D^{25}$ (c 1, DMF)		Elemental analysis/%			R_f^1	R_f^2	R_f^3	R_f^4
							C	H	N				
c)	Boc-[D-Phe-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-OBzl	51	216—219	−26.3	$C_{106}H_{138}N_{14}O_{19}$	C:	66.58	7.27	10.25	0.85	0.56		
						F:	66.36	7.44	10.06				
d)	Boc-[D-Phe-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$-NHNH$_2$	93	212—216	−24.6	$C_{99}H_{134}N_{16}O_{18}$ ·H_2O	C:	64.13	7.44	12.09	0.63	0.42		
						F:	64.12	7.41	11.98				
e)	*cyclo*[-D-Phe-Val-Orn(Z)-Leu-D-Phe-Pro-]$_2$	41	288—289 (dec.)	−37.3	$C_{94}H_{122}N_{14}O_{16}$	C:	66.25	7.22	11.51	0.63	0.56		
						F:	66:10	7.28	11.75				
f)	*cyclo*(-D-Phe-Val-Orn-Leu-D-Phe-Pro-)$_2$·2HCl	81	254—257 (dec.)	−90.8 (EtOH c 0.2)	$C_{78}H_{110}N_{14}O_{12}$ ·2HCl·6H_2O	C:	57.94	7.73	12.13			0.73	0.73
						F:	58.09	7.41	12.14				
	Gratisin peptide **VII**												
a)	Boc-Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl$_2$)-Pro-OBzl	63	111—115	−16.7	$C_{66}H_{81}N_7O_{12}Cl_2$ ·1/2H_2O	C:	63.70	6.64	7.88	0.88	0.54		
						F:	63.68	6.61	7.82				
b)	Boc-Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl$_2$)-Pro-NHNH$_2$	98	112—118	−16.9	$C_{59}H_{77}N_9O_{11}Cl_2$	C:	61.13	6.70	10.87	0.64	0.54		
						F:	60.92	7.01	10.98				
c)	Boc-Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl$_2$)-Pro-OH	92	135—139	−11.3	$C_{59}H_{75}N_7O_{12}Cl_2$	C:	61.88	6.60	8.56	0.50	0.50		
						F:	61.87	6.68	8.45				
d)	Boc-[Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl$_2$)-Pro-]$_2$-OH	20	145—148	−12.6 (c 0.5)	$C_{113}H_{140}N_{14}O_{21}Cl_4$ ·H_2O	C:	61.97	6.53	9.02	0.55	0.53		
						F:	61.58	6.53	9.39				
e)	*cyclo*[-Val-Orn(Z)-Leu-D-Phe-D-Tyr(BzlCl$_2$)-Pro-]$_2$	62	151—153	−16.6 (c 0.3)	$C_{108}H_{130}N_{14}O_{18}Cl_4$ ·H_2O	C:	62.72	6.24	9.48	0.70	0.58		
						F:	62.46	6.41	9.60				
f)	*cyclo*(-Val-Orn-Leu-D-Phe-D-Tyr-Pro-)$_2$·2HCl	83	231—235 (dec.)	−102.7 (EtOH c 0.2)	$C_{78}H_{110}N_{14}O_{14}$ ·2HCl·8H_2O	C:	55.60	7.65	11.64			0.68	0.78
						F:	55.36	7.14	11.54				
	Gratisin peptide **VIII**												
a)	Boc-D-Phe-D-Phe-Val-Orn(Z)-Leu-Pro-OBzl	52	207—208	−29.6	$C_{59}H_{77}N_7O_{11}$	C:	66.83	7.32	9.25	0.84	0.57		
						F:	66.67	7.43	9.26				
b)	Boc-D-Phe-D-Phe-Val-Orn(Z)-Leu-Pro-NHNH$_2$	85	174—176	−23.3	$C_{52}H_{73}N_9O_{10}$	C:	63.46	7.48	12.81	0.53	0.52		
						F:	63.21	7.45	12.65				
c)	Boc-D-Phe-D-Phe-Val-Orn(Z)-Leu-Pro-OH	75	156—162	−25.0	$C_{52}H_{71}N_7O_{11}$	C:	64.38	7.38	10.10	0.55	0.53		
						F:	64.16	7.48	10.20				
d)	Boc-[D-Phe-D-Phe-Val-Orn(Z)-Leu-Pro-]$_2$-OH	57	177—185	−18.6	$C_{99}H_{132}N_{14}O_{19}$ ·H_2O	C:	64.57	7.34	10.65	0.45	0.67		
						F:	64.11	7.17	10.64				
e)	*cyclo*[-D-Phe-D-Phe-Val-Orn(Z)-Leu-Pro-]$_2$	57	275—277 (dec.)	+55.5 (c 0.2)	$C_{94}H_{122}N_{14}O_{16}$ ·H_2O	C:	65.51	7.26	11.38	0.72	0.60		
						F:	65.28	7.19	11.25				
f)	*cyclo*(-D-Phe-D-Phe-Val-Orn-Leu-Pro-)$_2$·2HCl	78	258—260 (dec.)	−9.7 (EtOH c 0.2)	$C_{78}H_{110}N_{14}O_{12}$ ·2HCl·5H_2O	C:	58.55	7.69	12.26			0.66	0.81
						F:	58.90	7.21	11.85				

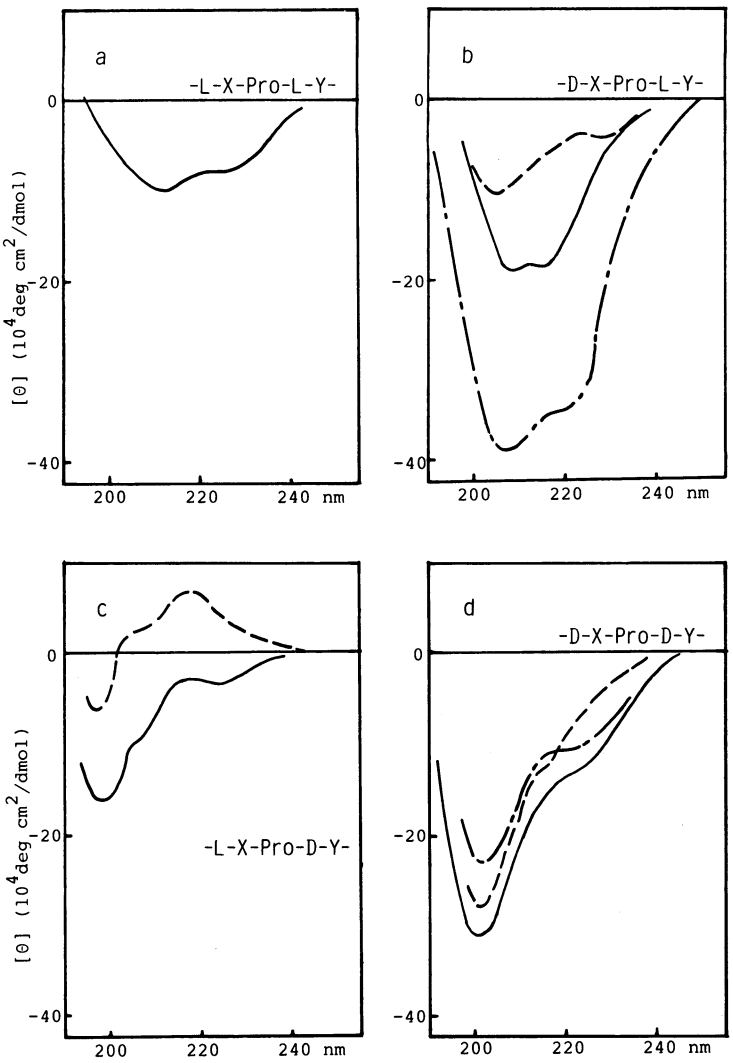


Fig. 3. CD spectra of GS and gratisin peptides **I**—**VIII** in aqueous solution. (a) **I**. *cyclo*(-Val-Orn-Leu-Phe-Pro-Tyr-)$_2$ ——; (b) **II**. *cyclo*(-Val-Orn-Leu-D-Phe-Pro-Tyr-)$_2$ ——, **VII**. *cyclo*(-Val-Orn-Leu-D-Phe-D-Tyr-Pro-)$_2$ ——, GS. *cyclo*(-Val-Orn-Leu-D-Phe-Pro-)$_2$ ----; (c) **III**. *cyclo*(-Val-Orn-Leu-Phe-Pro-D-Tyr-)$_2$ ——, **VIII**. *cyclo*(-Val-Orn-Leu-Pro-D-Phe-D-Phe-)$_2$ ——; (d) **IV**. *cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Tyr) ——, **V**. *cyclo*(-Val-Orn-Leu-D-Tyr-Pro-D-Phe-)$_2$ ——, **VI**. *cyclo*(-Val-Orn-Leu-D-Phe-Pro-D-Phe-)$_2$ ----.

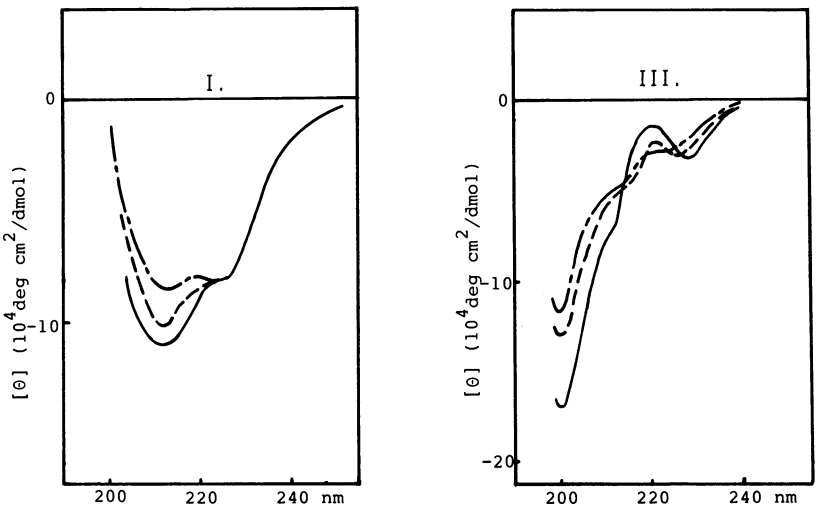


Fig. 4. CD spectra of gratisin peptides **I** and **III** at different temperature. 24 °C ——, 40 °C —— 60 °C —·—.

The shapes of CD spectra of active peptides **IV**, **VII**, **VIII**, and GS (Fig. 3) differ from each other, and no relationship between the pattern of the CD spectra in an aqueous solution and antibiotic activity could be found. These results indicate that these peptides have a different conformation in an aqueous solution. However, it is likely that when these peptides interact with a cell membrane of a target microorganisms, they adopt a GS-like conformation and exhibit antibiotic activities.

The CD spectroscopic study indicated that the partial sequence around a Pro residue is important for the conformation of these peptides. The significance of the sequence for conformation was similarly reported by Blout *et al.* on cyclic hexapeptides[24] and by Nagai *et al.* on linear tetrapeptides.[17]

The CD spectra of these synthetic peptides **I**—**VIII** in aqueous solutions were obtained at different temperatures (24, 40, and 60 °C) (Fig. 4). The CD spectra of peptides **II** and **IV**—**VIII** did not vary between 24 and 60 °C, indicating that the conformations of these peptides were stable within this temperature range. On the other hand, the ellipticities of peptides **I** and **III** decreased with a rise in temperature (Fig. 4). These results indicate that the conformations of peptides **I** and **III** with smaller activities are more labile than those of active peptides **II** and **IV**—**VIII**.

Experimental

All the melting points are uncorrected. CD spectra were obtained with a JASCO spectropolarimeter (model J-20 or J-500). The CD spectroscopy of gratisin peptides **I**—**VIII** was carried out using aqueous solutions of their dihydrochlorides. The molecular weights of the protected cyclododecapeptides were determined by a Hitachi-Perkin-Elmer vapor-pressure osmometer (model 115) using DMF as a solvent. Amino acid analyses were carried out using an ATTO MLC-703, after hydrolysis of the peptides in 6 M HCl at 110 °C for 24 h. 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). Yields, physical properties and analytical data of peptides **I**—**VIII** and the intermediary products are summarized in Table 3. The yield of each benzyl ester of Boc-hexapeptide was calculated on the basis of the amount of Pro-OBzl used as a starting material.

*Boc-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-OBzl (**Ia**).* DCC (2.06 g, 10 mmol) was added to a solution of Boc-Phe (2.65 g, 10 mmol), HOBt (1.48 g, 11 mmol), Pro-OBzl·HCl (2.42 g, 10 mmol) and TEA (1.39 ml) in $CHCl_3$ (50 ml) at 0 °C. This solution was stirred for 2 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 substances were 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 residue was dissolved in 4 M HCl/dioxane (20 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 with 10% Na_2CO_3 and water under cooling with an ice bath. To this solution were added Boc-Leu (2.4 g, 10 mmol), HOBt (1.48 g, 11 mmol) and DCC (2.06 g, 10 mmol) at 0 °C. The same procedure as described above was repeated for this reaction mixture. Further, Boc-Orn(Z), Boc-Val, and Boc-Tyr(Bzl) were successively coupled by the same method. All 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 (1.2×60 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, 5.49 g (47% from Pro-OBzl).

Compounds **IIa**—**VIIIa** were prepared in a similar manner. In the synthesis of **IVa**—**VIIIa**, WSCD·HCl was used instead of DCC as the coupling reagent.

*Boc-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-NHNH$_2$ (**Ib**).* A solution of **Ia** (2.3 g, 2.1 mmol) and hydrazine hydrate (2 ml) in DMF (15 ml) was allowed to stand for 5 d at room temperature. The solution was concentrated, and then water was added to the residue. The resulting solid product was collected by filtration; yield, 1.5 g (71%).

Compounds **IIb**—**VIIIb** were prepared in a similar manner.

*Boc-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-OH (**Ic**).* In a solution of **Ia** (1.75 g, 1.5 mmol) in MeOH (24 ml) and dioxane (12 ml), 2 M NaOH (1.5 ml) was added. The solution was then stirred for 12 h at room temperature. After the addition of water (10 ml), the solution was concentrated *in vacuo* at a low temperature, and then water (50 ml) was added to the residue. The resulting solid product was collected by filtration, washed with water and dried. The product was recrystallized from MeOH–ether; yield 1.43 g (88%).

Compounds **IIIc**, **VIIc**, and **VIIIc** were prepared in a similar manner.

*Boc-[Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-]$_2$-OH (**Id**).* Compound **Ic** (0.86 g, 0.8 mmol) was dissolved in TFA (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 (50 ml) was added to the residue, and the resulting solid, H-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-OH·TFA, was collected by filtration. To a solution of **Ib** (0.87 g, 0.8 mmol) in DMF (10 ml) were added 4.4 M HCl/dioxane (0.54 ml, 2.4 mmol) and isopentyl nitrite (0.12 ml, 0.9 mmol) at −40 °C. After stirring at −20 °C for 20 min, TEA (0.34 ml, 2.4 mmol) was added at −40 °C. This mixture was combined with a solution of the total amount of H-Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-OH·TFA prepared above and NMM (0.09 ml) in DMF (10 ml). The mixture was stirred at 0 °C for 3 d and concentrated. A solution 10% citric acid (50 ml) was added to the residue and the resulting product was collected by filtration, washed with water and dried. The product was purified by gel filtration on a Sephadex LH-20 column (2.5×120 cm) using DMF as a solvent. The fractions containing the desired product were then combined and concentrated. The product was recrystallized from hot EtOH; yield 1.1 g (67%).

Compounds **IIId**, **VIId**, and **VIIId** were prepared in a similar manner. In the removal of the Boc group of compounds **VIIc** and **VIIIc**, 4 M HCl/dioxane was used instead of TFA.

cyclo[-*Tyr(Bzl)-Val-Orn(Z)-Leu-Phe-Pro-*]$_2$ (**Ie**). *Active-Ester Method.* To a solution of **Id** (300 mg, 0.14 mmol) in DMF (10 ml) was added HOSu (32 mg, 0.28 mmol) and WSCD·HCl (53 mg, 0.28 mmol) at 0 °C, and then the mixture was stirred for 7 h at room temperature. The solvent was evaporated and then water was added to the residue. The resulting solid product was collected by filtra-

tion, washed with water and dried. It was dissolved in TFA (5 ml) containing a few drops of anisole at 0 °C. The mixture was stirred for 30 min at room temperature and then concentrated. The residue, trifluoroacetate of dodecapeptide active ester, was triturated with ether, collected by filtration and dissolved in DMF (10 ml). The solution was added, dropwise, to pyridine (150 ml) at 60 °C. After stirring for 3 h at 60 °C and overnight at room temperature, the solvent was evaporated, and then water was added to the residue. The resulting solid product was collected by filtration, washed with water and dried. The crude product was purified by chromatography on a silica-gel column (1.2× 30 cm) using a solvent system of $CHCl_3$–MeOH (50:1). The fractions containing the desired product were combined and concentrated. The product was recrystallized from EtOH–ether; yield, 83 mg (29%).

Compounds **IIIe**, **VIIe**, and **VIIIe** were prepared in a similar manner.

A Trial by an Azide Method. The cyclization of H-[Phe-Pro-Tyr(Bzl)-Val-Orn(Z)-Leu-]$_2$-$NHNH_2$ with an azide method was attempted by a procedure (described later) used in the preparation of compound **IIe**. In the purification of the product by column chromatography on silica-gel, a small amount of ninhydrin negative substance was isolated. The analytical results of the product are as follows: amino acid ratios in acid hydrolyzate; Phe 1.10, Pro 0.96, Tyr 0.86, Val 1.01, Orn 1.01, Leu 0.56. Elemental analysis: C, 66.61; H, 7.10; N, 10.81%. These results are not in agreement with the values expected for the desired product, *cyclo*[-Phe-Pro-Tyr(Bzl)-Val-Orn(Z)-Leu-]$_2$, but coincide with the following value calculated for the tentative urea-type structure described earlier: amino acid ratios; Phe 1, Pro 1, Tyr 1, Val 1, Orn 1, Leu 0.5. Calcd for $C_{108}H_{135}N_{15}O_{18}\cdot H_2O$: C, 66.54; H, 7.08; N, 10.77%.

cyclo(*-Tyr-Val-Orn-Leu-Phe-Pro-*)$_2\cdot$*2HCl* (***If***). Compound **Ie** (50 mg, 0.026 mmol) was dissolved in a mixture of 90% MeOH (30 ml), dioxane (5 ml), and 1 M HCl (0.1 ml). It was hydrogenated in the presence of palladium black for 15 h at room temperature. After removal of the catalyst, the filtrate was concentrated *in vacuo*. The product was recrystallized from EtOH-ether; yield, 38 mg (94%).

Gratisin peptides, **IIIf**, **VIIf**, and **VIIIf** were prepared in a similar manner.

*Boc-[Tyr(Bzl)-Val-Orn(Z)-Leu-*D*-Phe-Pro-]$_2$-OBzl* (***IIc***). Compound **IIa** (1.17 g, 1 mmol) was dissolved in 4 M HCl/dioxane (20 ml) containing anisole (0.5 ml) at 0 °C. After stirring at room temperature for 30 min, the solution was concentrated *in vacuo*. Ether (50 ml) was added to the residue, and the resulting solid, H-Tyr(Bzl)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl·HCl, was collected by filtration. To a solution of **IIb** (1.1 g, 1 mmol) in DMF (10 ml) were added 8.83 M HCl/dioxane (0.34 ml) and isopentyl nitrite (0.15 ml, 1 mmol) at −40 °C. After stirring at −20 °C for 15 min, the solution was cooled again to −40 °C and neutralized with TEA (0.42 ml, 3 mmol). This solution was combined with a chilled solution of H-Tyr(Bzl)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl·HCl mentioned above and NMM (0.11 ml, 1 mmol) in DMF (10 ml). The reaction mixture was stirred at 0 °C for 3 d, and then concentrated. The residue was dissolved in AcOEt (150 ml), and the solution was washed with 5% citric acid, water, 5% Na_2CO_3 and water, and dried over sodium sulfate. After concentrating the solution, the residue was purified by gel filtration on a Sephadex LH-20 column (2.5×120 cm) using DMF as solvent, and the fractions containing the desired product were combined and concentrated. Further purification was performed by chromatography on a silica-gel column (1.2×30 cm) using a solvent system of $CHCl_3$–MeOH (50:1), following by recrystallization from hot EtOH; yield, 1.23 g (60%).

Compounds **IVc**, **Vc**, and **VIc** were prepared in a similar manner. But the gel filtration on a Sephadex LH-20 column was not required for purification.

*Boc-[Tyr(Bzl)-Val-Orn(Z)-Leu-*D*-Phe-Pro-]$_2$-$NHNH_2$* (***IId***). Compound **IIc** (1.1 g, 0.5 mmol) was treated with hydrazine hydrate (1.5 ml) as described for the preparation of **Ib**. The product was purified by gel filtration as described in the preparation of **Id**; yield, 0.96 g (94%).

Compound **IVd**, **Vd**, and **VId** were prepared in a similar manner.

cyclo*[-Tyr(Bzl)-Val-Orn(Z)-Leu-*D*-Phe-Pro-]$_2$* (***IIe***). Compound **IId** (500 mg, 0.24 mmol) was dissolved in 4 M HCl/dioxane (15 ml) containing anisole (0.5 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 hydrochloride of the dodecapeptide hydrazide in DMF (10 ml) was added 8.8 M HCl/dioxane (0.09 ml) and isopentyl nitrite (0.036 ml, 0.27 mmol) at −40 °C. After 15 min, the mixture was poured, dropwise, into pyridine (200 ml) at 0 °C. After stirring for 3 d at 0 °C, the solution was concentrated. Addition of water to the residue afforded a precipitate, which was filtrated and washed with water. Purification was performed by chromatography on a silica-gel coulmn (1.2×30 cm) using a solvent system of $CHCl_3$–MeOH (50:1), followed by recrystallized from hot EtOH; yield 198 mg (43%).

Compounds **IVe**, **Ve**, and **VIe** were prepared in a similar manner. In removal for Boc group of compounds **IVd** and **Vd**, TFA was used instead of 4 M HCl/dioxane.

cyclo*(-Tyr-Val-Orn-Leu-*D*-Phe-Pro-)$_2\cdot$2HCl* (***IIf***). Compound **IIe** (100 mg, 0.052 mmol) was dissolved in 90% MeOH (20 ml), and then 1 M HCl (0.15 ml) was added to the solution. The mixture was hydrogenated in the presence of palladium black for 15 h. After removing 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 EtOH as solvent, and by reprecipitation from EtOH–ether; yield, 72 mg (90%).

Compounds **IVf**, **Vf**, and **VIf** were prepared in a similar manner. But, in this case, the gel-filtration purification procedure was not required.

Cellulose Plate Electrophoresis. Electrophoresis of the products was carried out with a cellulose (Avicel) plate and with a solvent system of HCOOH–AcOH–MeOH–H_2O (1:3:6:10 v/v, pH 1.3) for 2 h at 500 V/20 cm. Each of the peptides **I**—**VIII** revealed a single spot, the mobility being the same as that of GS.

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References

1) G. G. Zharikova, S. P. Myaskovskaya, and A. B. Silaev, *Vestn. Mosk. Univ., Biol. Pochovoved.*, **27**, 110 (1972).

2) S. P. Myaskovskaya, G. G. Zharikova, and A. B. Silaev, *Vestn. Mosk. Univ., Biol. Pochovoved.*, **28**, 123 (1973).

3) G. G. Zharikova, A. P. Zarubina, D. M. Kherat, S. P. Myaskovskaya, and V. N. Maksimov, *Antibiotiki IKh*

Produtsenty., **1975**, 163.

4) Amino acid residues with no prefix are of L-configuration unless otherwise noted. The abbreviations for amino acids and peptides are in accordance with the rules of IUPAC-IUB Commission of Biochemical Nomenclature. Abbreviations used are as follows: Boc, *t*-butoxycarbonyl; Z, benzyloxycarbonyl; OBzl, benzyloxy; Bzl, benzyl; $BzlCl_2$, 2,6-dichlorobenzyl; 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.

5) M. Tamaki, M. Takimoto, S. Sofuku, and I. Muramatsu, *J. Antibiotics.*, **34**, 1227 (1981).

6) M. Tamaki, M. Takimoto, S. Sofuku, and I. Muramatsu, "Peptide Chemistry 1980" ed by K. Okawa, Protein Research Foundation, Osaka (1981), p. 203.

7) M. Tamaki, M. Takimoto, S. Sofuku, and I. Muramatsu, *J. Antibiotics.*, **36**, 751 (1983).

8) M. Tamaki, M. Takimoto, S. Sofuku, and I. Muramatsu, "Peptide Chemistry 1982" ed by S. Sakakibara, Protein Research Foundation, Osaka (1983), p. 111.

9) P. Y. Chou and G. D. Fasman, *J. Mol. Biol.*, **115**, 135 (1977).

10) M. Kuo and W. A. Gibbons, *Biochemistry.*, **18**, 5855 (1979).

11) D. W. Urry and R. Walter, *Pro. Nat. Acad. Sci. U.S.A.*, **68**, 956 (1971).

12) I. L. Karle, J. Karle, T. Wieland, W. Burgermeister, H. Faulstich, and B. Witkop, *Pro. Nat. Acad. Sci. U.S.A.*, **70**, 1836 (1973).

13) S. G. Laland, Φ. Frφyshov, C. Gilhuus-Moe, and T. L. Zimmer, *Nature (New Biology).*, **239**, 43 (1972).

14) F. Lipmann, *ACC. Chem. Res.*, **6**, 361 (1973).

15) K. D. Kopple, A. Go, T. J. Schamper, and C. S. Wilcox, *J. Am. Chem. Soc.*, **95**, 6090 (1973).

16) M. Kawai and U. Nagai, *Biopolymers.*, **17**, 1549 (1978).

17) K. Sato, M. Kawai, and U. Nagai, *Bull. Chem. Soc. Jpn.*, **56**, 1527 (1983).

18) H. Yonezawa, K. Okamoto, M. Kaneda, N. Tominaga, and N. Izumiya, "Peptide Chemistry 1982" ed by S. Sakakibara, Protein Research Foundation, Osaka (1983), p. 283.

19) R. Nagai, M. Waki, M. Kondo, H. Aoyagi, T. Kato, S. Makisumi, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **40**, 963 (1967).

20) S. Ando, H. Aoyagi, M. Waki, T. Kato, and N. Izumiya, "Peptide Chemistry 1981" ed by T. Shioiri, Protein Research Foundation, Osaka (1982), p. 85.

21) K. Sato and U. Nagai, "Peptide Chemistry 1980" ed by K. Okawa, Protein Research Foundation, Osaka (1981), p. 193.

22) S. N. Semenov, E. I. Mel'nik, L. G. Snezhikova, A. I. Miroshikov, and V. T. Ivanov, *Bioorg. Khim.*, **3**, 1055 (1977).

23) H. Takiguchi and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **47**, 221 (1974).

24) L. M. Gierasch, C. M. Deber, V. Madison, C. Niu, and E. R. Blout, *Biochemistry.*, **20**, 4730 (1981).