Studies of Peptide Antibiotics. XXXV.[1] Synthesis of Gramicidin S by a Fragment Solid-Phase Method

Kazuki SATO, Hayao ABE,* Tetsuo KATO, and Nobuo IZUMIYA

Laboratory of Biochemistry, Faculty of Science 33, Kyushu University, Higashi-ku, Fukuoka 812

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In order to examine the usefulness of a fragment condensation procedure in solid-phase peptide synthesis, gramicidin S (GS) was synthesized by three different procedures such as stepwise elongation, fragment condensation with dipeptide fragments, and fragment condensation with tripeptide fragments. The two fragment condensation procedures gave intermediate peptide derivatives in high yields and in high purity. Such differences in the purity of the intermediate peptides disappeared in the final product, GS dihydrochloride. The GS synthesized by the three procedures exhibited the same antibacterial activities as those of natural GS.

A large number of peptides have been synthesized by a solid-phase method.[2] This technique has obvious merits to simplify and accelerate peptide synthesis.[3] However, a crucial defect in this method is unavoidable accumulation of contaminating by-products, and this makes purification of desired products very difficult.[4] Application of a fragment condensation procedure to the solid-phase synthesis is undoubtedly an attractive approach for the improvement in this solid-phase synthesis.[5] Although several biologically active peptides have been prepared by the solid-phase fragment condensation procedure,[6] various aspects of this procedure remain insufficiently explored. Furthermore, there is scarcely any experiment on the comparison between the stepwise elongation procedure and the fragment condensation procedure in the solid-phase synthesis of a biologically active peptide as a target compound.

This paper deals with the solid-phase synthesis of gramicidin S (GS) by various procedures and compares the properties of the synthesized peptides. The solid-phase synthesis of the open-chain linear decapeptide corresponding to the sequence of GS was carried out with three procedures as follows: procedure **a**, stepwise elongation using Boc-amino acids;[7] procedure **b**, fragment condensation with Boc-dipeptide fragments except for Boc-D-phenylalanine at N-terminus; and procedure **c**, fragment condensation with Boc-tripeptide fragments. The syntheses of the peptide fragments are outlined in Fig. 1.

The peptide fragments were prepared by the active ester method with HONSu[8] in order to prevent racemization and to simplify synthetic procedures. Each coupling reaction proceeded quickly in a good yield, unreacted active ester being removed as a water-soluble amide by the addition of 1-(2-aminoethyl)piperazine.[9] Each peptide fragment obtained was carefully recrys-

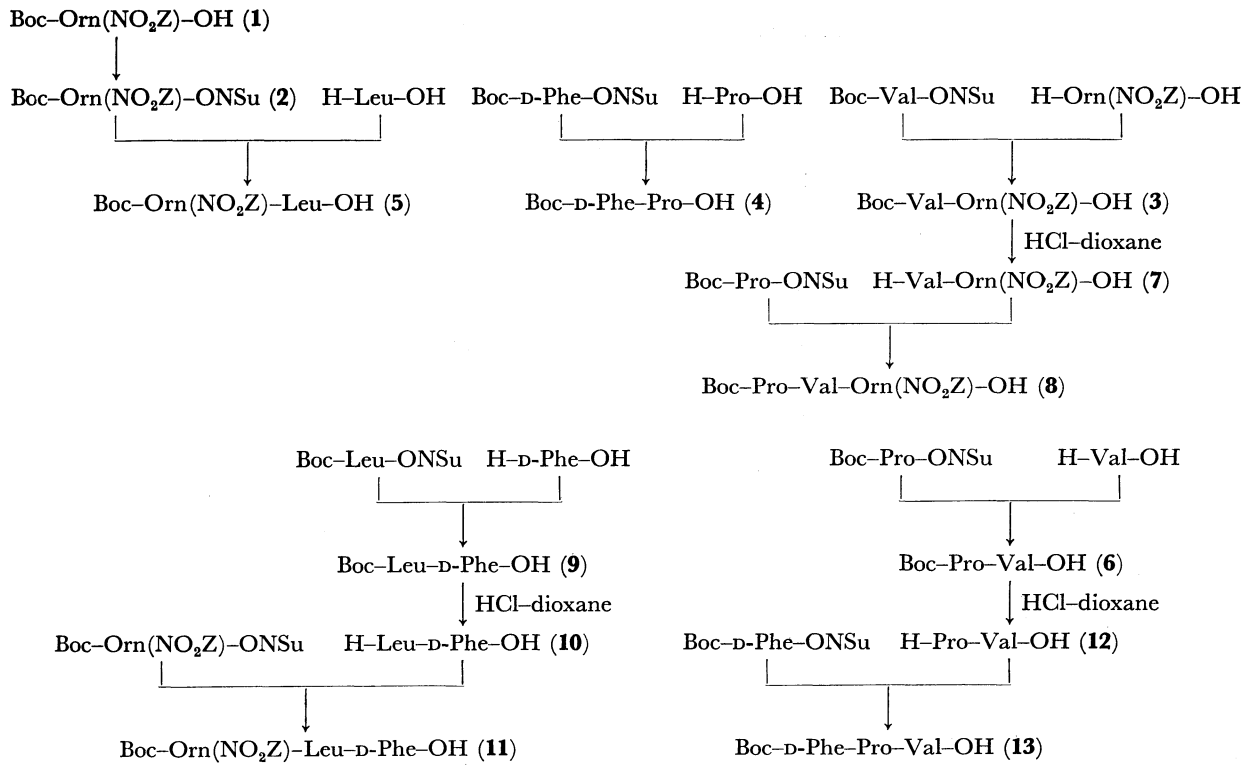

Fig. 1. Synthesis of peptide fragments.

* Present address: Institute of Biological Science, Mitsui-Toatsu Chemicals, Inc., Mobara 297.

tallized to remove the possible contamination of Boc-amino acid derived from the hydrolysis of the active ester during the coupling reaction in an aqueous solution. The δ-amino group of ornithine was protected by NO_2Z group.[10] Since this group showed extremely intense absorption at 268 nm, the purity of the synthetic intermediate peptides was ascertained spectrophotometrically by measuring the absorption of the group.[11]

Boc–Leu–resin
↓ 1. 1 M HCl–AcOH
↓ 2. 10% TEA–DMF
↓ 3. procedure **a**; Boc-amino acid and DCC
procedure **b**; Boc-dipeptide, HOBt, and DCC
procedure **c**; Boc-tripeptide, HOBt, and DCC
↓
Boc–(D-Phe–Pro–Val–Orn(NO_2Z)–Leu)$_2$–resin (**14a—c**)
↓ $N_2H_4 \cdot H_2O$–DMF
Boc–(D-Phe–Pro–Val–Orn(NO_2Z)–Leu)$_2$–N_2H_3 (**16a—c**)
↓ 0.1 M HCl–HCOOH
H–(D-Phe–Pro–Val–Orn(NO_2Z)–Leu)$_2$–$N_2H_3 \cdot 2HCl$
↓ $NaNO_2$, HCl (**17a—c**)
(H–(D-Phe–Pro–Val–Orn(NO_2Z)–Leu)$_2$–$N_3 \cdot HCl$)
↓ pyridine
cyclo(–(D-Phe–Pro–Val–Orn(NO_2Z)–Leu)$_2$–) (**18a—c**)
↓ H_2/Pd, HCl
cyclo(–(D-Phe–Pro–Val–Orn–Leu)$_2$–)$\cdot 2HCl$ (**19a—c**)

Fig. 2. Synthesis of GS by three procedures.

TABLE 1. SCHEDULE FOR SOLID-PHASE SYNTHESIS[a]

Step	Reagent	Volume (ml)	Times of repetition	Time (min)
1	AcOH wash	20	3	5
2	1 M HCl in AcOH	20	1	30
3	AcOH wash	20	3	5
4	EtOH wash	20	3	5
5	DMF wash	13	3	5
6	10% TEA in DMF	13	1	10
7	DMF wash	13	3	5
8	CH_2Cl_2 wash	12	3	5
9	Boc-amino acid in CH_2Cl_2	7	1	10
10	DCC in CH_2Cl_2	5	1	240[b]
11	CH_2Cl_2 wash	12	3	5
12	EtOH wash	20	3	5

a) Schedule in procedure **a** is described. b) 480 min for Val and Pro.

The solid-phase synthesis of GS is outlined in Fig. 2. The schedule for each coupling reaction is shown in Table 1. As a coupling reagent, DCC (four equivalents) was used in procedure **a**, and DCC and HOBt[12] (four equivalents, each) were used in procedures **b** and in **c**. The progress of each coupling reaction was followed by chloride titration[13] and by the Kaiser test.[14] The results of the chloride titration were shown in Fig. 3. An aliquot of the peptidyl resin was withdrawn, hydrolyzed, and subjected to amino acid analysis after fourth, fifth, and ninth coupling steps in

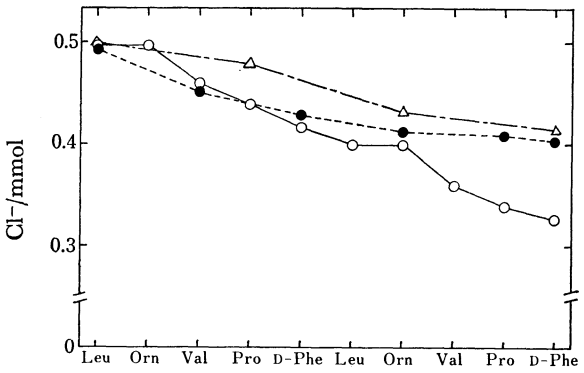

Fig. 3. Titration pattern of chloride ion in the course of synthesis of protected decapeptidyl-resin (**14**) by precedure **a** (○——○), by procedure **b** (●——●), and by procedure **c** (△-·-·-△). Final value was determined by a portion of the peptidyl-resin.

procedure **a** and after each coupling step in procedure **b** and **c**. The yield of each coupling reaction was calculated on the basis of the results of amino acid analysis as shown in Fig. 4.

NO_2Z NO_2Z
Boc—D-Phe—Pro—Val—Orn—Leu—D-Phe—Pro—Val—Orn—Leu—resin

Procedure a	98	97	87	100	96	94	96	95	100
Procedure b	98	97		100		92		95	
Procedure c	95			94			96		

Fig. 4. Coupling yield in each coupling step.

In procedure **a**, the coupling reaction of Boc-valine gave the unsatisfactory yield of 87%. In procedure **b**, the coupling of Boc–D-Phe–Pro–OH (**4**) with the tripeptidyl resin resulted in the low yield of 92%. The low yield in this step may be due to steric hindrance caused by the bulky side chain of the valine residue in the tripeptidly resin. The coupling reactions of the other three peptide fragments (**3**, **5**, **6**) and of the final Boc-D-phenylalanine gave good yields. In procedure **c**, all the coupling reactions gave excellent yields. Repetition of the coupling reaction was avoided in order to compare the reactivity of each reactant under the same reaction conditions. As shown in Fig. 4, the yield of each coupling reaction in procedure **a** was good except for valine; however, the nine consecutive coupling reactions caused lowering of the over-all coupling yield. The over-all yields calculated from the results of the amino acid analyses were 68, 83, and 86% in procedure **a**, **b**, and **c**, respectively.

After completion of the final coupling cycle, peptidyl resin (**14**) was dried under a reduced pressure and weighed. The yield estimated from the weight increase in procedure **a** agreed with those estimated from amino acid analysis and chloride titration. In procedures **b** and **c**, however, the yields estimated from the weight increases were lower than those estimated from the other methods. A possible reasoning of this disagreement

Table 2. Amino acid ratios of synthetic intermediates and synthetic gramicidin S

Compound	Procedure **a**					Procedure **b**					Procedure **c**				
	D-Phe	Pro	Val	Orn	Leu	D-Phe	Pro	Val	Orn	Leu	D-Phe	Pro	Val	Orn	Leu
14	0.85	0.88	0.92	1.00	1.00	0.91	0.92	0.93	0.95	1.00	0.92	0.94	0.95	0.98	1.00
15	0.86	0.85	0.97	1.14	1.00	0.96	1.00	0.97	1.01	1.00	0.96	1.02	0.95	0.98	1.00
16	0.93	1.01	1.07	1.16	1.00	1.03	1.00	1.01	1.00	1.00	0.97	1.00	0.98	1.00	1.00
18	0.97	1.01	0.95	0.99	1.00	1.01	1.01	1.02	1.01	1.00	1.01	1.00	0.99	1.02	1.00
19	0.90	0.90	0.98	0.95	1.00	1.02	0.98	0.97	1.04	1.00	0.96	0.99	1.00	1.00	1.00

is that frequent withdrawals of the peptidyl resin in the latter procedures caused much more loss of the resin than expected.

Hydrazinolysis of **14** was carried out as described previously,[11] and yielded a crude protected decapeptide hydrazide (**15**), this hydrazide being purified with recrystallization. The properties of **15** and purified hydrazide (**16**) were compared. Crude hydrazides **15a**, **15b**, and **15c** showed similar physical constants and chromatographic behavior. However, after recrystallization, purified hydrazides **16b** and **16c** showed some improvements in physical constants such as melting points and optical rotations and also in the results in amino acid analyses as shown in Table 2. The properties of **16a** were not changed significantly. Partially protected decapeptide hydrazide (**17**) was obtained quantitatively by the deprotection of Boc-group in **16** with hydrogen chloride in formic acid.[15] Thin-layer chromatography and paper electrophoresis of each of **17a—c** showed a main spot and another faint spot. All three hydrazides **17a**, **17b**, and **17c** revealed similar chromatographic behavior and similar results in measurements of the melting points and UV absorptions.

Cyclization of **17** by an azide method gave a protected cyclic decapeptide (**18**). Properties of **18a**, **18b**, and **18c** were compared, an acylated derivative (**18n**) of natural GS being used as a reference compound. There was no distinct differences in the patterns in thin-layer and Sephadex LH-20 column chromatography. However, products **18b** and **18c** showed better results in amino acid analyses and the optical rotations.

Hydrogenation of **18** gave synthetic GS·2HCl (**19**) as fine needles similar to the crystals of natural GS·2HCl. The synthetic and natural GS·2HCl showed the same results in thin-layer, paper, and carboxymethylcellulose column chromatography, paper electrophoresis, and measurements of the optical rotations and the melting points. The antibacterial activities of **19** also were the same as those of natural GS·2HCl.

The purities of the compounds obtained from the three different procedures turned to nearly the same in the final stage of the synthesis. A small amount of impurities seemed to be removed in the course of repeated chemical reactions and recrystallizations. Minute differences in purity, observed in **15** and **16**, were disappeared in **19**. Thus, the results of this solid-phase synthesis of GS indicated that the fragment condensation procedure had advantage of the ease of purification, the advantage being shown in the purification of **15**. In the usual fragment condensation, peptide fragments which placed glycine or proline at C-terminus have been employed to avoid racemization.[16] The results in this synthesis revealed that the use of DCC–HOBt as coupling reagents gave satisfactory results in coupling yield and in prevention of racemization, when applied to the solid-phase fragment condensation.

Experimental

All the melting points are uncorrected. The ratio in parentheses after a solvent system was indicated by vol. TLC was carried out on silica gel G (Merck) with the following solvent systems: R_f^1, $CHCl_3$–MeOH (5:1); R_f^2, 1-BuOH–AcOH–pyridine–H_2O (4:1:1:2). Paper chromatography was carried out on Toyo Roshi No. 52 paper; R_f^3, the same solvent as R_f^2 for TLC. PEP was carried out on the same paper under conditions of 500 V/30 cm with the solvent system; HCOOH–AcOH–MeOH–H_2O (1:3:6:10) at pH 1.8, the mobilities of samples being reported as the ratio to that of lysine (R_{Lys}). UV spectra were determined on a Hitachi-Perkin-Elmer spectrophotometer, Model 139. Optical rotations were measured on a Yanagimoto polarimeter, OR-20. Amino acid analyses were performed on a Hitachi amino acid analyzer, KLA-3B, after hydrolysis in a mixture of 6 M HCl and propionic acid (1:1) at 110 °C for 24 h.[17] Molecular weight was determined with a Hitachi Osmometer, Model 115, DMF being used as a solvent.

*Boc–Orn(NO_2Z)–OH·DCHA (**1**·DCHA).* To a solution of δ-NO_2Z-ornithine[11] (12.4 g, 40 mmol) and TEA (16.8 ml, 120 mmol) in water (40 ml) was added Boc–N_3 (8.22 g, 60 mmol) in dioxane (50 ml). The reaction mixture was treated according to the procedure of Sakakibara *et al.*,[18] and the product was recrystallized from MeOH–ether–petroleum ether; yield, 20.24 g (85%); mp 89—90 °C; $[\alpha]_D^{20}$+13.6° (*c* 1, MeOH); UV_{max} (EtOH) 268 nm (ε 1.01×10^4).

Found: C, 60.76; H, 8.53; N, 9.16%. Calcd for $C_{30}H_{48}O_8N_4$: C, 60.79; H, 8.16; N, 9.45%.

*Boc–Orn(NO_2Z)–ONSu (**2**).* Compound **1** (8.23 g, 20 mmol) which was derived from **1**·**DCHA** by the usual procedure was converted to the corresponding HONSu ester according to the procedure of Anderson *et al.*[19] The product was recrystallized from EtOAc–ether; yield, 5.56 g (55%); mp 87—88 °C; $[\alpha]_D^{20}$—4.4° (*c* 1, $CHCl_3$).

Found: C, 51.80; H, 5.78; N, 10.87%. Calcd for $C_{22}H_{28}O_{10}N_4$: C, 51.96; H, 5.55; N, 11.02%.

*Boc–Val–Orn(NO_2Z)–OH (**3**).* To a solution of δ-NO_2Z-ornithine (3.74 g, 12 mmol) and TEA (1.68 ml, 12 mmol) in water (25 ml) was added a solution of Boc–Val–ONSu (3.50 g, 10 mmol) in dioxane (25 ml) at room temperature. The reaction mixture was stirred overnight, and a few drops of 1-(2-aminoethyl)piperazine was added to the solution. After being stirred for 30 min, dioxane was evaporated *in vacuo* and the solution was acidified with 10% citric acid under cooling. Separated oil was extracted with EtOAc, and the organic layer was washed with water and dried (Na_2SO_4). The solution was evaporated to leave an oil, which was crystallized by the addition of ether. The product was recrystallized from

MeOH–ether; yield, 4.89 g (96%); mp 86—88°C; $[\alpha]_D^{20}$ −9.3° (*c* 1, MeOH); R_f^1 0.52.

Found: C, 54.55; H, 6.92; N, 10.72%. Calcd for $C_{23}H_{34}O_9N_4$: C, 54.11; H, 6.71; N, 10.98%.

Other protected peptide fragments (**4**—**6**, **8**, **9**, **11**, **13**), were prepared by a manner similar to that described for **3**.

Boc-D-Phe-Pro-OH (4). Yield, 81%; mp 171—173 °C; $[\alpha]_D^{20}$ −46.9° (*c* 1, MeOH); R_f^1 0.54.

Found: C, 62.90; H, 7.30; N, 7.75%. Calcd for $C_{19}H_{26}O_5N_2$: C, 62.96; H, 7.23; N, 7.73%.

Boc-Orn(NO$_2$Z)-Leu-OH (5). Yield, 82%; mp 73—75 °C; $[\alpha]_D^{20}$ −10.2° (*c* 1, MeOH); R_f^1 0.66.

Found: C, 55.10; H, 7.12; N, 10.42%. Calcd for $C_{24}H_{36}O_9N_4$: C, 54.95; H, 7.12; N, 10.42%.

Boc-Pro-Val-OH·DCHA (6·DCHA). Yield, 63%; mp 126—127 °C; $[\alpha]_D^{20}$ −40.4° (*c* 1, MeOH); R_f^1 0.61.

Found: C, 65.39; H, 10.08; N, 8.35%. Calcd for $C_{27}H_{49}O_5N_3$: C, 65.43; H, 9.98; N, 8.84%.

Boc-Pro-Val-Orn(NO$_2$Z)-OH (8). Yield, 69%; mp 159—161 °C; $[\alpha]_D^{20}$ −48.2° (*c* 1, MeOH); R_f^1 0.51.

Found: C, 54.96; H, 6.72; N, 11.58%. Calcd for $C_{28}H_{41}O_{10}N_5$: C, 55.34; H, 6.80; N, 11.53%.

Boc-Leu-D-Phe-OH·DCHA (9·DCHA). Yield, 82%; mp 115—116 °C; $[\alpha]_D^{20}$ −46.3° (*c* 1, MeOH); R_f^1 0.67.

Found: C, 68.73; H, 9.73; N, 7.46%. Calcd for $C_{32}H_{53}O_5N_3$: C, 68.66; H, 9.54; N, 7.51%.

Boc-Orn(NO$_2$Z)-Leu-D-Phe-OH (11). Yield, 80%; mp 90—91 °C; $[\alpha]_D^{20}$ −24.1° (*c* 1, MeOH); R_f^1 0.58.

Found: C, 58.91; H, 6.88; N, 10.23%. Calcd for $C_{33}H_{45}O_{10}N_5$: C, 59.00; H, 6.75; N, 10.43%.

Boc-D-Phe-Pro-Val-OH (13). Yield, 54%; mp 172—173 °C; $[\alpha]_D^{20}$ −78.8° (*c* 1, MeOH); R_f^1 0.61.

Found: C, 62.37; H, 7.69; N, 9.14%. Calcd for $C_{24}H_{35}O_6N_3$: C, 62.45; H, 7.64; N, 9.11%.

H-Val-Orn(NO$_2$Z)-OH·HCl (7·HCl). Compound **3** (2.55 g, 5 mmol) was dissolved in 2.6 M hydrogen chloride in dioxane (39 ml). The solution was allowed to stand for 1 h at room temperature and then evaporated. Resulting oil was solidified by the addition of ether. The product was recrystallized from EtOH–ether; yield, 1.93 g (86%); mp 159—161 °C; $[\alpha]_D^{20}$ +10.3° (*c* 1, 6 M HCl); R_f^2 0.80.

Found: C, 48.98; H, 6.53; N, 11.79%. Calcd for $C_{18}H_{27}O_7N_4Cl$: C, 48.37; H, 6.09; N, 12.54%.

H-Leu-D-Phe-OH·HCl (10·HCl). Compound **9** was treated as described for **7**; yield, 83%; mp 200—202 °C; $[\alpha]_D^{20}$ +37.0° (c 1, 6 M HCl); R_f^2 0.78.

H-Pro-Val-OH (12). Compound **6** (1.57 g, 5 mmol) was treated as described for **7**. The oily residue (**12·HCl**) was dissolved in water (50 ml) and the solution was neutrallized with TEA (0.7 ml, 5 mmol). The addition of EtOH (50 ml) gave crystals, which were recrystallized from water–EtOH; yield, 0.88 g (82%); mp 242—243 °C (dec); $[\alpha]_D^{20}$ −70.2° (*c* 1, 6 M HCl); R_f^2 0.56.

Found: C, 55.80; H, 8.62; N, 12.98%. Calcd for $C_{10}H_{18}O_3N_2$: C, 56.05: H, 8.47; N, 13.08%.

Boc-(D-Phe-Pro-Val-Orn(NO$_2$Z)-Leu)$_2$-resin (14a—c).

14a: Boc-Leu-resin[20] (1.88 g, containing 0.5 mmol of Boc-leucine), which was prepared from chloromethylated copolystyrene divinylbenzene (2%) resin (0.65 mmol of Cl/g), was placed in a reaction vessel and swollen with AcOH (15 ml) overnight; this starting material was prepared similarly for **14b** and **14c**. Elongation of peptide chain was carried out according to the schedule shown in Table 1. The coupling time was generally 4 h and exceptionally 8 h for Boc-valine and for Boc-proline. After completion of the final coupling cycle, the resin was washed with DMF, EtOH, AcOH, and CH_2Cl_2 successively, and dried *in vacuo*. Weight increase, 473 mg (68%).

14b: Instead of Boc-amino acid in procedure **a**, the Boc-dipeptide fragment and HOBt (four equivalents, each) were added. After the addition of DCC (four equivalents), the vessel was shaken for 24 h. Weight increase, 542 mg (78%).

14c: The Boc-tripeptide fragment, HOBt, and DCC (four equivalents, each) were added, and the reaction time was 48 h. Weight increase, 508 mg (73%).

Boc-(D-Phe-Pro-Val-Orn(NO$_2$Z)-Leu)$_2$-N$_2$H$_3$ (16a—c).

16a: To a suspension of **14a** (1.19 g, 0.18 mmol) in DMF (13 ml) was added 100% hydrazine hydrate (0.35 ml, 7.2 mmol). The mixture was shaken for 3 days at room temperature, the resin was removed by filtration and washed with DMF (13 ml×3), and the combined filtrate was evaporated *in vacuo*. The residue was treated with water (100 ml) to give yellowish powder; yield of crude hydrazide (**15a**), 273 mg (94%); mp 141—148 °C; $[\alpha]_D^{20}$ −103° (*c* 0.5, MeOH); purity estimated from absorption at 268 nm, 98%; R_f^1 0.76 with a faint tailing strip; R_f^2 0.93. Compound **15a** (200 mg) was dissolved in hot MeOH (20 ml), an insoluble material in a small amount being filtered off. The filtrate was evaporated to 4 ml and white precipitate of purified hydrazide (**16a**) was obtained by the addition of ether; yield, 178 mg (89%); mp 150—151 °C; $[\alpha]_D^{20}$ −119° (*c* 0.5, MeOH); purity at 268 nm, 96%.

Found: C, 58.98; H, 7.12; N, 13.82%. Calcd for $C_{79}H_{110}O_{20}N_{16}\cdot H_2O$: C, 58.50; H, 6.96; N, 13.82%.

16b: Compound **14b** (1.06 g, 0.18 mmol) was treated as described for **15a**; yield of crude hydrazide (**15b**), 286 mg (98%); mp 136—143 °C; $[\alpha]_D^{20}$ −117° (*c* 0.5, MeOH); purity at 268 nm, 96%; R_f^1 0.76 with a faint tailing strip; R_f^2 0.93. Compound **15b** (200 mg) was treated as described for **16a**; yield of **16b**, 188 mg (94%); mp 152 °C; $[\alpha]_D^{20}$ −145° (*c* 0.5, MeOH); purity at 168 nm, 98% (Found: C, 58.06; H, 6.96; N, 13.62%).

16c: Compound **14c** (1.14 g, 0.18 mmol) was treated as described for **15a**; yield of crude hydrazide (**15c**), 285 mg (98%); mp 134—140 °C; $[\alpha]_D^{20}$ −125° (*c* 0.5, MeOH); purity at 268 nm, 95%; R_f^1 0.76 with a faint tailing strip; R_f^2 0.93. Compound **15c** (200 mg) was treated as described for **16a**; yield **16c**, 184 mg (92%); mp 153 °C; $[\alpha]_D^{20}$ −135° (*c* 0.5, MeOH); purity at 268 nm, 97% (Found: C, 58.29; H, 6.96; N, 14.37 %).

H-(D-Phe-Pro-Val-Orn(NO$_2$Z)-Leu)$_2$-N$_2$H$_3$·2HCl(17a—c).

17a: A solution of **16a** (164 mg, 0.1 mmol) in 0.1 M hydrogen chloride in formic acid (2.2 ml) was allowed to stand for 8 h at room temperature and then evaporated. The oily residue was solidified by the addition of ether; yield, 160 mg (99%); mp 168—171 °C; R_f^1 0.49 with a faint tailing strip; R_f^2 0.79; R_{Lys} 0.53 with a faint tailing strip; purity at 268 nm, 96%.

17b: This was prepared from **16b** (164 mg, 0.1 mmol) as described for **17a**; yield, 161 mg (100%); mp 175 °C; R_f^1 0.49 with a faint tailing strip; R_f^2 0.79; R_{Lys} 0.53 with a faint tailing strip; purity at 268 nm, 95%.

17c: This was prepared from **16c** (164 mg, 0.1 mmol) as described for **17a**; yield, 157 mg (97%); mp 175 °C; R_f^1 0.49 with a faint tailing strip; R_f^2 0.79; R_{Lys} 0.53 with a faint tailing strip; purity at 268 nm, 94%.

cyclo(-(D-*Phe-Pro-Val-Orn(NO$_2$Z)-Leu)$_2$-*) (18a—c).*

18a: To a solution of **17a** (144 mg, 0.09 mmol) in a mixture of AcOH (0.45 ml) and DMF (1.8 ml) were added 1 M HCl (0.1 ml) and 1 M $NaNO_2$ (0.1 ml) at −30 °C. After being left for 30 min, the reaction mixture was added into pyridine (37 ml) at 0 °C. After being stirred for 3 days at 5 °C, the

solution was evaporated and the residue was dissolved in a mixture (12 ml) of MeOH and water (10: 1). The solution was applied on columns (1.9×20 cm, each) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were washed with the same solvent (200 ml) and the combined effluent was evaporated to leave an oily residue, which was crystallized by the addition of ether. The product was recrystallized from MeOH–ether; yield, 63 mg (46%); mp 218—223 °C; $[\alpha]_D^{20}$ −230° (*c* 0.3, MeOH); purity at 268 nm, 96%; R_f^1 0.71; R_f^2 0.94.

Found: C, 59.67; H, 6.95; N, 12.64%; mol wt, 1509. Calcd for $C_{76}H_{102}O_{18}N_{14}\cdot 2H_2O$: C, 59.38; H, 6.90; N, 12.76%; mol wt, 1500.

18b: This was prepared from **17b** (144 mg, 0.09 mmol) as described for **18a**; yield, 64 mg (47%); mp 225—230 °C;$[\alpha]_D^{20}$ −242° (*c* 0.3, MeOH); purity at 268 nm, 101%; R_f^1 0.71; R_f^2 0.94; mol wt, 1504 (Found: C, 60.16; H, 6.94; N, 12.74 %).

18c: This was prepared from **17c** (144 mg, 0.09 mmol) as described for **18a**; yield, 48 mg (35%); mp 225—230 °C; $[\alpha]_D^{20}$ −243° (*c* 0.3, MeOH); purity at 268 nm, 101%; R_f^1 0.71; R_f^2 0.94; mol wt, 1507 (Found: C, 60.30; H, 6.97; N, 12.71%).

*Preparation of **18n** from Natural GS.* To a solution of natural GS (21 mg, 0.017 mmol) in pyridine (1.7 ml) was added *p*-nitrobenzyloxycarbonyl chloride[10] (0.36 g, 1.7 mmol) at −10 °C. After being stirred for 7 h at 0 °C, the solution was evaporated. The resulting residue afforded a yellowish precipitate (0.25 g) by the addition of water. The precipitate was dissolved in MeOH (5 ml), and the solution was applied on a column of Sephadex LH-20 (1.8×110 cm) and developed with MeOH. The fractions with the desired product were collected and evaporated, the resulting oily residue being crystalized by the addition of ether; yield, 23 mg (75 %); mp 245—247 °C; $[\alpha]_D^{20}$ −248° (*c* 0.3, MeOH); purity at 268 nm, 100%; R_f^1 0.71; R_f^2 0.94 (Found: C, 59.54; H, 6.96; N, 12.61%).

*Sephadex LH-20 Column Chromatography of **15a—c** and **18a—c**.*

15a: A portion (10 mg) of **15a** was applied on a column of Sephadex LH-20 (1.8×110 cm) using MeOH as an eluate. The chromatogram showed a single peak at 145 ml.

15b** and **15c: The chromatograms of **15b** and **15c** in the same conditions also showed the same patterns as that of **15a**.

18a: A portion (5 mg) of **18a** was applied on the same column as described for **15a**. The chromatogram showed the same single peak at 149 ml as that of the reference compound (**18n**).

18b** and **18c: The chromatograms of **18b** and **18c** in the same conditions also showed the same patterns as that of **18n**.

*cyclo(-(D-Phe–Pro–Val–Orn–Leu)$_2$-)·2HCl (**19a—c**).*

19a: Compound **18a** (30 mg, 0.02 mmol) dissolved in 0.01 M hydrogen chloride in MeOH (6 ml) was hydrogenated using palladium black as a catalyst. The filtrate was evaporated and the residual solid was recrystallized from EtOH–1 M HCl; yield, 18 mg (76%); mp 269—275 °C; $[\alpha]_D^{20}$ −260° (*c* 0.4, EtOH); R_f^2 0.83, R_f^3 0.80.

Found: C, 56.53; H, 7.79; N, 12.41%. Calcd for $C_{60}H_{92}O_{10}N_{12}\cdot 2HCl\cdot 4H_2O$: C, 56.01; H, 7.94; N, 13.07%.

19b: This was prepared from **18b** (30 mg, 0.02 mmol) as described for **19a**; yield, 15 mg (60%); mp 274—279 °C;$[\alpha]_D^{20}$ −252° (*c* 0.4, EtOH); R_f^2 0.83; R_f^3 0.80 (Found: C, 55.85; H, 7.63; N, 12.46%).

19c: This was prepared from **18c** (30mg, 0.02 mmol) as described for **19a**; yield, 16 mg (65%); mp 274—279 °C; $[\alpha]_D^{20}$ −253° (*c* 0.4, EtOH); R_f^2 0.83; R_f^3 0.80 (Found: C, 56.12; H, 7.65; N, 12.64%).

*Comparison of Synthetic GS·2HCl (**19a—c**) with Natural GS·2HCl.* Physical constants and R_f values of **19a—c** were shown above. Physical constants and R_f values of natural GS·2HCl; mp, 274—276 °C; $[\alpha]_D^{20}$ −258° (*c* 0.6, EtOH); R_f^2 0.83 R_f^3; 0.80. In addition to showing the same R_f values in paper and thin-layer chromatography, the natural and all three synthetic GS·2HCl revealed indistinguishable paper electrophoretic patterns and identical behavior in the carboxymethylcellulose column chromatography in the conditions described previously.[21]

Microbiological Assays. The minimum amount of the synthetic and natural GS·2HCl necessary for the complete inhibition of growth of several microorganisms was determined by a dilution method using a nutrient agar. All three synthetic GS·2HCl exhibited the same antibacterial activities as those of natural GS·2HCl. The minimum inhibitory concentration of the compounds were 2—4 μg/ml on *Staphylococcus aureus*, 4—7 μg/ml on *Bacillus subtilis*, and 50—100 μg/ml on *Escherichia coli*.

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References

1) Part XXXIV of this series: K. Okamoto, K. Nonaka, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **50**, 231 (1977).

2) A. Marglin and R. B. Merrifield, *Ann. Rev. Biochem.*, **39**, 841 (1970); G. R. Marchall and R. B. Merrifield, "Biochemical Aspects of Reactions on Solid Supports," Academic Press, New York, N. Y. (1971), pp. 111—169.

3) R. B. Merrifield, *Fed. Proc.*, **21**, 412 (1962); R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).

4) E. Bayer, H. Eckstein, K. Hagel, W. A. König, W. Brüning, H. Hagenmaier, and W. Parr, *J. Am. Chem. Soc.*, **92**, 1735 (1970).

5) F. Weygand and U. Ragnarsson, *Z. Naturforsch.*, **21b**, 1141 (1966).

6) B. F. Gisin, R. B. Merrifield, and D. C. Tosteson, *J. Am. Chem. Soc.*, **91**, 2691 (1969); H. Yajima and Y. Kiso, *Chem. Pharm. Bull.*, **22**, 1087 (1974); U. Ragnarsson, S. M. Karlsson, and U. Hamberg, *Int. J. Pept. Protein Res.*, **7**, 307 (1975); Protein Synthesis Group, Shanghai Institute of Biochemistry, Academia Sinica, *Scientia Sinica*, **18**, 745 (1975); R. E. Larsson, P. Melin, and U. Ragnarsson, *Int. J. Pept. Protein Res.*, **8**, 39 (1976).

7) Abbreviations according to IUPAC-IUB Commission, *J. Biol. Chem.*, **247**, 977 (1972), are used throughout. Additional abbreviations: DCC, dicyclohexylcarbodiimide: DMF, *N,N*-dimethylformamide; HOBt, 1-hydroxybenzotriazol; HONSu, *N*-hydroxysuccinimide; TEA, triethylamine; Boc, *t*-butyloxycarbonyl; NO_2Z, *p*-nitrobenzyloxycarbonyl; PEP, paper electrophoresis. Amino acid symbols except D-Phe denote the L-configuration.

8) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

9) E. Wünsch, G. Wendlberger, and A. Högel, *Chem. Ber.*, **104**, 2430 (1971).

10) F. H. Carpenter and D. T. Gish, *J. Am. Chem. Soc.*, **74**, 3818 (1952).

11) M. Ohno, K. Kuromizu, H. Ogawa, and N. Izumiya, *J. Am. Chem. Soc.*, **93**, 5251 (1971).

12) F. Weygand, D. Hoffmann, and E. Wünsch, *Z. Natur-*

forsch., **21b**, 426 (1966).
13) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," Freeman & Co., San Francisco (1969), p. 55.
14) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
15) W. Troll and R. K. Cannan, *J. Biol. Chem.*, **200**, 803 (1953).
16) H. Yajima, H. Kawatani, and H. Watanabe, *Chem. Pharm. Bull.*, **18**, 1333 (1970).
17) J. Scotchler, R. Lozier, and A. B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).
18) S. Sakakibara, H. Honda, K. Takeda, M. Miyoshi, T. Ohnishi, and K. Okumura, *Bull. Chem. Soc. Jpn.*, **42**, 809 (1969).
19) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957).
20) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," Freeman & Co., San Francisco (1969), p. 32.
21) H. Aoyagi, T. Kato, M. Waki, O. Abe, R. Okawa, S. Makisumi, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **42**, 782 (1969).