Biomimetic Synthesis of Gramicidin S. Direct Formation of the Antibiotic from Pentapeptide Active Esters Having No Protecting Group on the Side Chain of the Orn Residue

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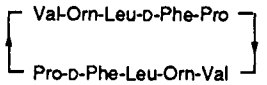
Abstract: The direct formation of gramicidin S (GS) by the dimerization–cyclization of pentapeptide active esters having no protecting group on the side chain of the Orn residue was examined. Among the five succinimide esters (-ONSu) carrying the Val, Orn, Leu, D-Phe, or Pro residue at each C-terminus, only H-D-Phe-Pro-Val-Orn-Leu-ONSu, having the sequence identical with that of the linear precursor pentapeptide in the biosynthesis of GS, gave semi-GS (cyclic monomer) and GS (cyclic dimer) in yields of 15 and 38%, respectively. Other pentapeptide esters did not give GS. The process of the cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu is proposed as follows. In the intramolecular reaction, the active ester of the Leu residue couples slowly with the α-amino group of the D-Phe residue to give the semi-GS but not with the δ-amino group of the Orn residue. In the GS formation, the active ester dimerizes to a decapeptide active ester, which takes the GS-like β-pleated sheet conformation and cyclizes to afford GS.

Introduction

Gramicidin S (GS)[1] is an antibiotic cyclodecapeptide consisting of two identical pentapeptide sequences.[2] In 1957, Schwyzer



and Sieber reported that the cyclization of the H-Val-Orn(Tos)-Leu-D-Phe-Pro-*p*-nitrophenyl ester yielded the cyclic decapeptide (the ditosyl derivative of GS) as a main product but not the cyclic pentapeptide.[3] Since then, various analogs of GS have been synthesized by this dimerization–cyclization methods.[4-7] From these studies, it was pointed out that the mode of cyclization in the chemical synthesis of GS is significantly different from that of the biosynthesis, in which the C-terminal Leu residue of the precursor is fastened onto the GS synthetase.[4,8,9] For example, the yields of the cyclic dimer (the di-Z derivative of GS) in the reaction of H-D-Phe-Pro-Val-Orn(Z)-Leu-N_3 and -ONSu were lower than those from precursors having a D-Phe, Pro, Val, or Orn(Z) residue at the C-terminus.[8]

In this paper, we report the cyclization of various linear pentapeptide-ONSus having no protecting group on the side chain of the Orn residue.

Results and Discussion

Five pentapeptide-ONSus (**1–5**) having no protecting group on the side chain of the Orn residue were cyclized in pyridine for 1 day at 25 °C (concentration of peptides in pyridine: 3×10^{-3} M). Purification of the main products in the reaction mixture

Val-Orn-Leu-D-Phe-Pro-ONSu (**1**)

Orn-Leu-D-Phe-Pro-Val-ONSu (**2**)

Leu-D-Phe-Pro-Val-Orn-ONSu (**3**)

D-Phe-Pro-Val-Orn-Leu-ONSu (**4**)

Pro-Val-Orn-Leu-D-Phe-ONSu (**5**)

was performed by gel filtration using Sephadex LH-20 and semipreparative high-performance liquid chromatography (HPLC). The primary structure of the product was deduced by amino acid analyses and fast-atom bombardment (FAB) mass spectra and confirmed by a direct comparison with authentic samples synthesized according to conventional methods.

The main products in each reaction are shown in Table I. Active esters **1**, **2**, **3**, and **5** mainly gave the cyclic compounds containing the amide bond formed between the ester group of the C-terminal residue and the δ-amino group of the Orn residue. Peptide **2** gave also a small amount of semi-GS by coupling between the ester group of the Val residue and the α-amino group of the Orn residue, but peptides **1**, **3**, and **5** did not give semi-GS. Further, these peptides did not yield any amount of GS. These results indicate that each ester group at the C-terminus of peptides **1**, **2**, **3**, and **5** favorably reacts with the δ-amino group of the Orn residue in the same molecule rather than with the α-amino group at the N-terminus of the same or different molecule. Recently, similar formation of the lactam ring was found in the biosynthesis of GS. That is, J. Vater et al. reported that D-Phe-Pro-Val-cycloOrn was produced by GS synthetase in omission of Leu

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(1) Battersby, A. R.; Craig, L. C. *J. Am. Chem. Soc.* **1951**, *73*, 1887.
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(3) Schwyzer, R.; Sieber, P. *Helv. Chim. Acta* **1958**, *41*, 2186.
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Table I. Main Products of Cyclization of Pentapeptide Active Esters **1–5**

active esters	cyclic products a	cyclic products b
1	H-Val-Orn-Leu-D-Phe-Pro	
2	Orn-Leu-D-Phe-Pro-Val (semi-GS)	H-Orn-Leu-D-Phe-Pro-Val
3	H-Leu-D-Phe-Pro-Val-Orn	
4	D-Phe-Pro-Val-Orn-Leu (semi-GS)	[D-Phe-Pro-Val-Orn-Leu Leu-Orn-Val-Pro-D-Phe] (GS)
5	H-Pro-Val-Orn-Leu-D-Phe	

from the complete bioassay mixture and that the formation is favored by the nucleophilic characteristic of the δ-amino side chain.[10]

On the other hand, peptide **4**, H-D-Phe-Pro-Val-Orn-Leu-ONSu, gave semi-GS (cyclic monomer) and GS (cyclic dimer) in yields of 15 and 38%, respectively. The cyclic compound resulting from the reaction between the ester group of the Leu residue and the δ-amino group of the Orn residue was not found in the reaction mixture. Each of the main cyclization products from peptides **1**, **2**, **3**, and **5** has a six- or a >12-membered ring structure. If, in peptide **4**, the cyclization between the side-chain amino group and the C-terminus occurred, it would give a highly strained nine-membered ring. This should be one reason for the preferential formation of GS or semi-GS in the reaction of peptide **4**. The sequence of peptide **4** is interestingly identical with that of the precursor in the biosynthesis of natural GS.[9] The antibiotic spectrum of the cyclic dimer isolated was the same as that of natural GS, while all cyclic pentapeptides produced by the cyclization of linear pentapeptide active esters **1–5** found no antibiotic activity.

The mode of the cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu was investigated by several methods. Two analogous pentapeptide active esters **6** and **7** were used to investigate the effect of the length or the bulkiness of the side chains of the Orn and the Leu residues. The structures of the main products were

D-Phe-Pro-Val-Lys-Leu-ONSu (**6**)

D-Phe-Pro-Val-Orn-Ala-ONSu (**7**)

confirmed on HPLC analysis by direct comparison with authentic samples.[11] The ratios between the cyclic monomer and cyclic dimer in the cyclizations of peptides **6** and **7** were similar to that in the case of peptide **4**. That is, the conversion of the Orn and Leu residues into Lys and Ala residues, respectively, did not affect the reaction mode.

To further examine the reactivity between the δ-amino group of the Orn residue and the C-terminal ester group, benzyloxycarbonyl(Z)-D-Phe-Pro-Val-Orn-Leu-ONSu was left to stand in pyridine (concentration of the peptide: 3×10^{-3} M) at 45 °C for 1 day. The two cyclic products were isolated by gel filtration using Sephadex LH-20, followed by recrystalization. These products were identified as the cyclic monomer and cyclic dimer on the basis of molecular weight, which was determined by FAB mass spectrometry. The yields of cyclic monomer and dimer were 6 and 28%, respectively. This result indicates that the formation of Z-D-Phe-Pro-Val-Orn-Leu, having an amide bond between the δ-amino group of the Orn residue and the carboxyl group of the Leu residue, takes place slowly.

Table II. Effect of Peptide Concentration on the Cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu[a,b]

concentration of peptide in pyridine ($\times 10^{-3}$ M)	ratio of cyclic products semi-GS:GS	total yield (%) of semi-GS and GS
0.3	66:34	54
3	37:63	53
30	4:96	35

[a] The cyclization was carried out in pyridine at 25 °C for 1 day. [b] The ratio and yield of cyclic products were determined by HPLC analysis.

Table III. Effect of Reaction Temperature on the Cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu[a]

reaction temperature (°C)	ratio of cyclic products semi-GS:GS	total yield (%) of semi-GS and GS
0	28:72	48
25	37:63	53
50	55:45	40

[a] See the footnotes in Table II.

The effect of the concentration of the pentapeptide active ester on the cyclization yield was examined (Table II). The ratios of cyclic dimer (GS) to cyclic monomer (semi-GS) in products depended directly on the concentrations of the active esters in pyridine and indicated that the cyclic dimer formation competes with the cyclic monomer formation.

Next, the cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu was tried at 0, 25, and 50 °C for 1 day (concentration of the peptide in pyridine: 3×10^{-3} M) (Table III). With an increase in temperature, the yield of semi-GS increased and that of GS decreased. The higher temperature seems to result in the formation of a much greater amount of products bigger than the dimer and hence diminishes the yield of the dimer.

The solvent effect on the cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu was investigated using 1,4-dioxane, carbon tetrachloride, benzene, chloroform, tetrahydrofuran, dichloromethane, pyridine, ethanol, methanol, dimethylformamide, dimethyl sulfoxide, and water. In the cyclization in water, sodium carbonate was used as a base. In other cases, triethylamine was used. The cyclizations of the active ester in carbon tetrachloride and benzene, although the mixture was not a clear solution, gave only GS as the cyclic product, and no other product was detected by HPLC analysis. On the other hand, the cyclizations in dimethylformamide, dimethyl sulfoxide, and water give mainly semi-GS but no GS. The cyclizations in other solvents yielded several products in addition to GS. Thus, the polarity of the solvent significantly affects the yield of GS.

Following the progress of the cyclization reactions of H-D-Phe-Pro-Val-Orn-Leu-ONSu and H-Val-Orn-Leu-D-Phe-Pro-ONSu at 25 °C (concentration of the peptide in pyridine: 3×10^{-3} M) by HPLC revealed that these cyclizations almost finish within 2 h. However, the cyclizations of GS precursors were generally performed for 1 day to complete the reaction.

The direct synthesis of GS in a preparative scale from a nonprotected active ester derived from 0.5 mmol of Boc-D-Phe-Pro-Val-Orn(Boc)-Leu-OH was attempted in benzene. GS·2HCl was isolated after gel filtration using Sephadex LH-20 column chromatography and recrystallization from ethanol–ether in 45% yield.[12]

(10) Vater, J.; Schlumbohm, W.; Palacz, Z.; Salnikow, J.; Gadow, A.; Kleinkauf, H. *Eur. J. Biochem.* **1987**, *163*, 297.

(11) [Lys$^{2,2'}$]-GS, [Lys^{2}]-semi-GS, [Ala$^{3,3'}$]-GS, and [Ala^{2}]-semi-GS were synthesized by a similar method described in the literatures: (a) Waki, M.; Abe, O.; Okawa, R.; Kato, T.; Makisumi, S.; Izumiya, N. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 2904. (b) Abe, O.; Izumiya, N. *Bull. Chem. Soc. Jpn.* **1970**, *43*, 1202.

(12) (a) The analytical data for this synthetic GS were shown as follows: mp 269–273 °C dec; $[\alpha]^{25}_{D}$ −266° (*c* 0.25, ethanol) (lit.[12b] mp 273–275 °C dec; $[\alpha]^{20}_{D}$ −270° (*c* 0.1, ethanol)). (b) Waki, M.; Izumiya, N. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 1687.

(13) The cyclic peptide H-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu·2HCl was synthesized by a similar method described in the literature: mp 215–216 °C dec; $[\alpha]^{25}_{D}$ −141° (*c* 0.4, ethanol). Tamaki, M.; Akabori, S.; Muramatsu, I. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 583.

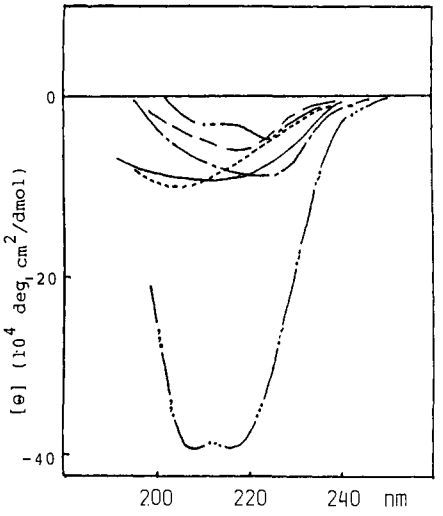

Figure 1. CD spectra of the main products in the cyclization of peptides **1–5** in water. H-Val-Orn-Leu-D-Phe-Pro, —; H-Orn-Leu-D-Phe-Pro-Val, – – –; H-Leu-D-Phe-Pro-Val-Orn, - - -; D-Phe-Pro-Val-Orn-Leu, —·—; H-Pro-Val-Orn-Leu-D-Phe, —··—; GS, —·—.

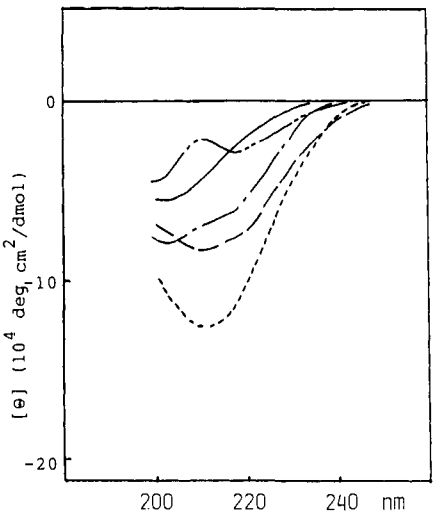

Figure 2. CD spectra of pentapeptide ethyl esters related to peptides **1–5** in ethanol. H-Val-Orn-Leu-D-Phe-Pro-OEt, —; H-Orn-Leu-D-Phe-Pro-Val-OEt, – – –; H-Leu-D-Phe-Pro-Val-Orn-OMe, - - -; H-D-Phe-Pro-Val-Orn-Leu-OEt, —·—; H-Pro-Val-Orn-Leu-D-Phe-OEt, —··—.

Next, the cyclizations of peptides **1–5** and H-(D-Phe-Pro-Val-Orn-Leu)$_2$-ONSu were performed in ethanol. They gave cyclic products similar to those formed in pyridine, although the total yields were lower than those in pyridine. That is, the modes of the cyclizations of peptides **1–5** in both solvents seem to be similar.

To investigate the mode of the cyclization of peptides **1–5**, CD spectra of the main products in aqueous solution and the spectra of the ethyl esters of these five peptides in ethanol solution were measured (Figures 1 and 2). The spectra of pentapeptide ethyl esters having the Val, Orn, Leu, or D-Phe residues at the C-terminus showed one or two negative troughs in the wavelength region 200–220 nm. In particular, Peptides **2** and **3**, which contain the β-turn-forming -D-Phe-Pro- sequence inside the molecules (at the 3–4 and 2–3 positions, respectively), show comparatively deep troughs. On the other hand, the ethyl ester having the Pro residue at the C-terminus showed a simple curve which decreased steadily with no trough at 200–220 nm. These profiles suggest that the pentapeptide ethyl esters with the Val, Orn, Leu, and D-Phe residues at the C-terminus have a somewhat ordered

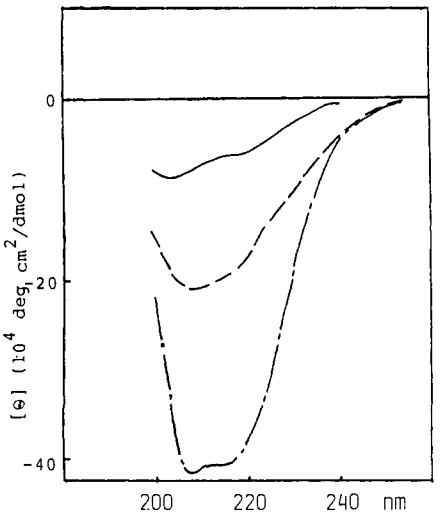

Figure 3. CD spectra of H-D-Phe-Pro-Val-Orn-Leu-OEt (—), H-(D-Phe-Pro-Val-Orn-Leu)$_2$-OEt (– – –), and GS (—·—) in ethanol.

conformation in ethanol, while the ethyl ester with the Pro residue at the C-terminus has an unordered conformation. CD spectra of these pentapeptide ethyl esters resembled those of the corresponding H-pentapeptide-OHs[15] possessing the Z-group on the side chain of the Orn residue, suggesting that the conformations of active esters **1–5** are similar to those of the corresponding H-pentapeptide-OHs. Recently, on studying of the syntheses of Z-semi-GS and diZ-GS by one-pot cyclization of H-pentapeptide-OHs having the Z-group on the side chain of the Orn residue, we found a good correlation between the CD spectra of these precursors in ethanol solution and the products of their cyclizations.[15] However, in the present studies, no correlation between the CD spectra of the pentapeptide ethyl esters and the main products in the cyclization of active esters **1–5** was found. This failure of the correlation may be ascribed to the competitive coupling between the δ-amino group of the Orn residue and the ester group at C-terminus accompanied in the cyclization of active esters **1–5** by no protecting group on the side chain of the Orn residue.

The CD spectrum of H-(D-Phe-Pro-Val-Orn-Leu)$_2$-OEt was deeper than that of D-Phe-Pro-Val-Orn-Leu-OEt, and its features resemble those of GS (Figure 3). In addition, the cyclization of H-(D-Phe-Pro-Val-Orn-Leu)$_2$-ONSu under conditions similar to those in the case the reaction of pentapeptide active ester gave GS in a yield of 65% and no H-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu.[13] These results suggest that the decapeptide-ONSus have a β-pleated sheet conformation in pyridine similar to that of GS, and the resulting proximity of the α-amino group of the D-Phe residue to the carbonyl group of the active ester brings about the preferential formation of GS.

On the basis of the above results, the mode of cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu is proposed as follows. In the intramolecular reaction, the C-terminal ester reacts slowly with the α-amino group of the D-Phe residue to give the semi-GS but does not react with the δ-amino group of the Orn residue. On the other hand, as the result of the intermolecular reaction, the decapeptide active ester is formed by the coupling of two molecules of H-D-Phe-Pro-Val-Orn-Leu-ONSu. It then takes the GS-like β-pleated sheet conformation and cyclizes to afford GS. In nonpolar solvents (benzene or carbon tetrachloride), the pentapeptide active ester probably reacts in the solid phase in the latter process and gives GS with great facility.

In the present studies, the penta- and decapeptide precursors having the D-Phe residue at the N-terminus gave cyclic peptides

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Table IV. Physical Properties and Analytical Data for Intermediary Products[a,b,c]

no.	compound	mp (°C)	$[\alpha]_D^{25}$ (*c* 1, DMF)	formula (MW)[d]
1	Boc-Val-Orn(Boc)-Leu-D-Phe-Pro-OH	141–144	−40.2 (*c* 0.5)	$C_{40}H_{64}O_{10}N_6 \cdot H_2O$ (807.0)
2	Boc-Orn(Boc)-Leu-D-Phe-Pro-Val-OH	106–110	−52.2	$C_{40}H_{64}O_{10}N_6 \cdot H_2O$ (807.0)
3	Boc-Leu-D-Phe-Pro-Val-Orn(Boc)-OH	108–113	−46.5	$C_{40}H_{64}O_{10}N_6 \cdot H_2O$ (807.0)
4	Boc-D-Phe-Pro-Val-Orn(Boc)-Leu-OH	107–112	−54.4	$C_{40}H_{64}O_{10}N_6 \cdot 0.5H_2O$ (798.0)
5	Boc-Pro-Val-Orn(Boc)-Leu-D-Phe-OH	220 dec	−31.4	$C_{40}H_{64}O_{10}N_6 \cdot H_2O$ (807.0)
6	Boc-D-Phe-Pro-Val-Lys(Boc)-Leu-OH	108–111	−48.6	$C_{41}H_{66}O_{10}N_6 \cdot 1.5H_2O$ (830.0)
7	Boc-D-Phe-Pro-Val-Orn(Boc)-Ala-OH	98–102	−48.0	$C_{37}H_{58}O_{10}N_6 \cdot 0.5H_2O$ (755.9)
8	Boc-(D-Phe-Pro-Val-Orn(Boc)-Leu)$_2$-OH	153–156	−51.6	$C_{75}H_{118}O_{17}N_{12} \cdot 4H_2O$ (1531.9)
9	Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl	176–180	−36.3	$C_{50}H_{68}O_{10}N_6$[e] (913.1)
10	Boc-Val-Orn-Leu-D-Phe-Pro	228–230 dec	−101.5 (*c* 0.5)	$C_{35}H_{54}O_7N_6 \cdot H_2O$ (688.9)
11	Z-Orn(Boc)-Leu-D-Phe-Pro-Val-OH	86–90	−42.9 (*c* 0.6)	$C_{43}H_{62}O_{10}N_6 \cdot 1.5H_2O$ (850.0)
12	Z-Orn-Leu-D-Phe-Pro-Val	137–141	−172.0 (*c* 0.3)	$C_{38}H_{52}O_7N_6 \cdot 0.5H_2O$ (713.9)
13	Z-Leu-D-Phe-Pro-Val-Orn(Boc)-OH	92–102	−35.5 (*c* 0.5)	$C_{43}H_{62}O_{10}N_6 \cdot H_2O$ (841.0)
14	Z-Leu-D-Phe-Pro-Val-Orn	119–123	−44.4 (*c* 0.3)	$C_{38}H_{52}O_7N_6 \cdot 2.5H_2O$ (749.9)
15	Z-D-Phe-Pro-Val-Orn(Boc)-Leu-OH	102–105	−46.8	$C_{43}H_{62}O_{10}N_6 \cdot H_2O$ (841.0)
16	Z-Pro-Val-Orn(Boc)-Leu-D-Phe-OH	207–209 dec	−27.2	$C_{43}H_{62}O_{10}N_6 \cdot H_2O$ (841.0)
17	Z-Pro-Val-Orn-Leu-D-Phe	302–305 dec	−11.4 (*c* 0.4, DMSO)	$C_{38}H_{52}O_7N_6 \cdot H_2O$ (722.9)
18	Z-D-Phe-Pro-Val-Orn(Boc)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-OH	120–123	−61.2 (*c* 0.5)	$C_{81}H_{114}O_{17}N_{12} \cdot 3.5H_2O$ (1590.9)
19	Z-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-Val-Orn(Z)-Leu	135–138	−59.4 (*c* 0.5)	$C_{76}H_{104}O_{14}N_{12} \cdot 3H_2O$ (1463.8)

[a] Compounds **1–8** were linear precursors used in the cyclization of penta- and decapeptide-ONSus having no protecting group on the δ-amino group of Orn residue. [b] Compounds **9–17** were intermediary products of authentic samples synthesized by a conventional method. [c] Compounds **18** and **19** were intermediary products of H-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu. [d] The results of elemental analysis agreed with calculated values within ±0.3% except for those for **9**. [e] Calcd for $C_{50}H_{68}O_{10}N_6$: C, 65.77; H, 7.51; N, 9.20. Anal. Found: C, 65.62; H, 7.85; N, 9.35.

in substantial quantities. A similar result was reported by Brady et al. on the cyclization of linear hexapeptides containing a D-amino acid residue.[14] The presence of the D-Phe residue at the N-terminus in deca- and pentapeptide precursors might have an important significance in the cyclization.

In *Bacillus brevis*,[9] GS is produced via the dimerization of a pentapeptide fragment (-D-Phe-Pro-Val-Orn-Leu-) and the ensuing cyclization of the resulting decapeptide on the GS synthtase. As described above, the cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu affords GS just as in the biosynthesis, but the other pentapeptide active ester having a different C-terminal residue does not. It is of interest that, both in biological and in chemical syntheses, the formations of the cyclic peptides are subjected to a similar regiospecific control, although the conditions of their cyclizations are different from each other and, especially in biosynthesis, the enzyme probably gives a steric environment more favorable for the formation of GS.

Experimental Section

Amino acid analysis of each hydrolysate of the peptides was carried out with a Hitachi 835 amino acid analyzer. Molecular weights of the cyclic products were determined by using FAB mass spectrometry on a JEOL JMS-D300 mass spectrometer (in Asahi Chemical Industry Company). CD spectra were measured by using a JASCO spectropolarimeter (Model J-500) with a 0.1-mm cell at room temperature. HPLC was performed on an LC-800 series instrument (Jasco, Japan) consisting of an 880 intelligent HPLC pump, a 875-UV intelligent UV/vis detector, an 860-CO column oven, a Model 7125 syringe loading sample injector (Rheodyne, Cotati, CA, USA), and a Finepak SIL C18 column (4.6 × 250 mm, 10 μm particle size, Jasco, Japan). Chromatography was carried out by a linear gradient of 65–95% methanol/5% $NaClO_{4aq}$[16] over 60 min with a flow rate of 1 mL/min at 30 °C. The column eluent was monitored at 220 nm. The microorganisms employed in the assays were *Staphylococcus aureus* ATCC 6538, *Streptomyces pyogenes* N. Y. 5, *Corynebacterium diphtheriae* P. W. 8, *Micrococcus pyogenes* ATCC 10240, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NIHJ-JC2, and *Proteus vulgaris* OX 19. Minimum inhibitory concentrations (in μg mL^{-1}) of the compounds were determined by an agar dilution method with 10^6 organisms per milliliter.

Syntheses of *tert*-Butoxycarbonyl(Boc)-penta- and decapeptides. Boc-penta- and -decapeptides, in which the δ-amino group of the Orn residue and the N-terminal amino group were protected by the Boc group, were prepared by a conventional method. In the synthesis of Boc-D-Phe-Pro-Val-Orn(Boc)-Leu-OH as an example, Boc-D-Phe-Pro-Val-OBzl and Z-Orn(Boc)-Leu-OEt were prepared by stepwise elongation using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (WSCD·HCl) and 1-hydroxybenzotriazole (HOBt). Boc-D-Phe-Pro-Val-OBzl was converted into the corresponding acid by saponification. The Z-group of Z-Orn(Boc)-Leu-OEt was removed by hydrogenolysis, and then the resulting ester was coupled with Boc-D-Phe-Pro-Val-OH to give Boc-D-Phe-Pro-Val-Orn(Boc)-Leu-OEt. This pentapeptide was saponified to afford Boc-D-Phe-Pro-Val-Orn(Boc)-Leu-OH. Other Boc-penta- and -decapeptides were synthesized in a similar manner. The peptides were characterized by elemental analyses, thin-layer chromatography (TLC), HPLC, and amino acid analyses of their hydrolysates. The physical properties and analytical data of these peptides are shown in Table IV.

Reaction of Penta- and Decapeptide-ONSus. Boc-pentapeptides (50–100 mg) were converted into the corresponding succinimide esters using N-hydroxysuccinimide (HONSu) and WSCD·HCl. Boc-pentapeptide-ONSus were treated with trifluoroacetic acid (TFA) to remove all Boc groups. Pentapeptide-ONSu trifluoroacetates were dissolved in small amounts of dimethylformamide, and the solutions were added dropwise into pyridine at 25 °C (usual concentration of the active esters was 3×10^{-3} M). After the mixture was stirred for 1 day at 25 °C, the solvent was evaporated. The residues were dissolved in methanol and analyzed by HPLC.

Reaction of linear decapeptide-ONSu and analysis of the product were carried out by a similar method.

Purification of Cyclic Peptides as the Reaction Products. Main products from the reaction mixtures of pentapeptide-ONSus, in which Val, Orn, D-Phe, and Pro residues occupy the C-terminus, were purified by gel filtration on a Sephadex LH-20 column (1.2 × 150 cm), using methanol as the eluting solvent, and by reprecipitation from methanol–ether. Semi-GS was similarly purified from the cyclization products of H-D-Phe-Pro-Val-Orn-Leu-ONSu. GS was purified by semipreparative HPLC on a Finepak SIL C18 column (7.6 × 250 mm, 10 μm particle size, Jasco) using the solvent system of methanol–5% $NaClO_4$ (4:1), followed by gel filtration on the Sephadex LH-20 column (1.2 × 150 cm), using methanol as solvent. The cyclic peptides were characterized by TLC, HPLC, FAB mass spectra, and amino acid analyses of their hydrolysates. The physical properties and analytical data for these peptides are shown in Table V.

Determination of the Free Amino Group in Cyclic Peptides. Cyclic peptides isolated from reaction mixtures of pentapeptide active esters **1–5** were treated with 2,4-dinitrofluorobenzene. The resulting dinitrophenyl cyclic peptides were hydrolyzed in 6 N HCl for 24 h at 110 °C. The free amino group of the peptides was confirmed by comparing the results of the amino acid analyses of the hydrolysates of both the DNP-treated peptide and the nontreated peptide.

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Table V. Physical Properties and Analytical Data for Cyclic Peptides

no.	compound	mp (°C) dec	$[\alpha]_D^{25}$ (EtOH)	formula (MW)[b]	MS (FAB) (M + H$^+$)
1	H-Val-Orn-Leu-D-Phe-Pro·HCl	284–285	−135 (*c* 0.3, 50% $EtOH_{aq}$)	$C_{30}H_{46}O_5N_6$·HCl·1.5H_2O (634.2)	571
2	H-Orn-Leu-D-Phe-Pro-Val·HCl	210–215	−190 (*c* 0.4)	$C_{30}H_{46}O_5N_6$·HCl·3.0H_2O (661.2)	571
3	H-Leu-D-Phe-Pro-Val-Orn·HCl	201–203	−114 (*c* 0.5)	$C_{30}H_{46}O_5N_6$·HCl·3.0H_2O (661.2)	571
4	cyclo(D-Phe-Pro-Val-Orn-Leu)$_2$·2HCl[a]	273–275	−266 (*c* 0.3)	$C_{60}H_{92}O_{10}N_{12}$·2HCl·3.5H_2O (1277.4)	1141
5	cyclo(D-Phe-Pro-Val-Orn-Leu)·HCl	220–221	−153 (*c* 0.2)	$C_{30}H_{46}O_5N_6$·HCl·3.0H_2O (661.2)	571
6	H-Pro-Val-Orn-Leu-D-Phe·HCl	280	−70 (*c* 0.4, H_2O)	$C_{30}H_{46}O_5N_6$·HCl·2.5H_2O (652.2)	571

[a] mp 273–275 °C dec; $[\alpha]_D^{20}$ −270° (*c* 0.1, EtOH) in the literature.[12] [b] The elemental analysis agreed with calculated values within ±0.3%.

Table VI. Physical Properties and Analytical Data for Penta- and Decapeptide Ethyl Esters Related to GS

no.	compound	mp (°C)	$[\alpha]_D^{25}$ (*c* 0.5, DMF)	formula (MW)[a]
1	H-Val-Orn-Leu-D-Phe-Pro-OEt·2TFA	122–126	−17.5	$C_{32}H_{52}O_6N_6$·2TFA·1.5H_2O (871.9)
2	H-Orn-Leu-D-Phe-Pro-Val-OEt·2TFA	90–94	−30.0	$C_{32}H_{52}O_6N_6$·2TFA·1.5H_2O[b] (871.9)
3	H-Leu-D-Phe-Pro-Val-Orn-OMe·2TFA	175–177	−66.8	$C_{31}H_{50}O_6N_6$·2TFA·2H_2O[c] (866.9)
4	H-D-Phe-Pro-Val-Orn-Leu-OEt·2TFA	100–105	−59.7	$C_{32}H_{52}O_6N_6$·2TFA·1.5H_2O (871.9)
5	H-Pro-Val-Orn-Leu-D-Phe-OEt·2TFA	199–200 dec	−5.5	$C_{32}H_{52}O_6N_6$·2TFA·1.5H_2O (871.9)
6	H-(D-Phe-Pro-Val-Orn-Leu)$_2$-OEt·3TFA	153–156	−85.4	$C_{62}H_{98}O_{11}N_{12}$·3TFA·3H_2O (1583.7)

[a] The results of elemental analysis agreed with calculated values within ±0.3% except those for compounds **2** and **3**. [b] Anal. Calcd for $C_{32}H_{52}O_6N_6$·2TFA·1.5H_2O: C, 49.59; H, 6.59; N, 9.64. Found: C, 49.41; H, 6.29; N, 10.00. [c] Anal. Calcd for $C_{31}H_{50}O_6N_6$·2TFA·2H_2O: C, 48.50; H, 6.51; N, 9.69. Found: C, 48.85; H, 6.30; N, 9.57.

Syntheses of Cyclic Peptides as Authentic Samples. The preparations of Z-pentapeptide-OHs, in which the δ-amino group of the Orn residue is protected by a Boc group, were carried out by a method similar to that described for Boc-pentapeptide-OH. The Z-pentapeptide-OHs were converted to the corresponding succinimide esters and then treated by TFA to remove the Boc group on the Orn residue. These Z-pentapeptide-ONSus were cyclized by the same method as was used in the cyclization of pentapeptide-ONSu. The products were purified by semipreparative HPLC on a Finepak SIL C18 column (7.6 × 250 mm, 10 μm particle size, Jasco) using the solvent system of methanol–water (2:1), followed by reprecipitation from methanol–ether. These cyclic peptides were then hydrogenated in methanol containing 1 N HCl in the presence of palladium black for 20 h. The products were purified by recrystallization from methanol–ether. H-Val-Orn-Leu-D-Phe-Pro·HCl was synthesized by the one-pot cyclization of Boc-Val-Orn-Leu-D-Phe-Pro-OH using WSCD·HCl and HOBt, followed by the removal of the Boc-group by 4 N HCl–dioxane. Boc-Val-Orn-Leu-D-Phe-Pro-OH was prepared by hydrogenation of Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl, synthesized by stepwise elongation using WSCD·HCl and HOBt from Pro-OBzl. The products were characterized by TLC, HPLC, elemental analyses, amino acid analyses, and FAB mass spectra. The CD spectra of the authentic cyclic peptides were the same as those of the corresponding products from pentapeptides **1–5**. The analytical data for the intermediary products are shown in Table IV.

Syntheses of Penta- and Decapeptide Ethyl Esters. Penta- and decapeptide ethyl esters used in the measurements of CD spectra were prepared by the action of TFA from the corresponding Boc-penta- and -decapeptide ethyl esters. The analytical data for the esters are shown in Table VI.

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Supplementary Material Available: Details of the cyclization of the pentapeptide precursors to GS and CD spectra of these precursors; HPLC profiles of the crude products of these cyclizations; and tables of elemental analysis data (23 pages). Ordering information is given on any current masthead page.