Biomimetic synthesis of a peptide antibiotic, gratisin

Makoto Tamaki,*[a] Seiji Komiya,[a] Sadatoshi Akabori[a] and Ichiro Muramatsu[b]

[a] *Department of Chemistry, Faculty of Science, Toho University, Funabashi, Chiba 274, Japan*
[b] *Tokyo Bunka Junior College, Hon-cho, Nakano-ku, Tokyo 164, Japan*

Recently, we reported the direct formation of a peptide antibiotic, gramicidin S (GS), cyclo(-D-Phe-Pro-Val-Orn-Leu-)$_2$, by the dimerization–cyclization of pentapeptide active esters, D-Phe-Pro-Val-Orn-Leu-ONSu (-ONSu: succinimide ester), having the sequence identical with that of the linear precursor pentapeptide in the biosynthesis of GS and no protecting group on the side-chain of the Orn residue. This biomimetic approach has been extended to the synthesis of a peptide antibiotic, gratisin (GR), cyclo(-D-Phe-Pro-D-Tyr-Val-Orn-Leu-)$_2$, isolated from *Bacillus brevis* Y-33. In the cyclization of six hexapeptide succinimide esters having a Val, Orn, Leu, D-Phe, Pro or D-Tyr residue at each C-terminus, only H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu gave semi-GR and GR in yields of 31 and 8%, respectively. Other hexapeptide esters did not give semi-GR and GR. In both biomimetic syntheses of GS and GR, the amino acid sequences having a Leu residue at the C-terminus are essential. In addition, a change in the concentration of peptide and the polarity of reaction solvents influenced greatly the yields of cyclic monomer (semi-GR) and cyclic dimer (GR). However, the yield of GR by dimerization–cyclization of hexapeptide active ester was lower when compared with the direct formation of GS (38%). The difference may result from differences in the chain length and the configurations of amino acid residues around the Pro residue.

Introduction

In synthetic studies of gramicidin S (GS),[1,2] cyclo(-D-Phe-Pro-Val-Orn-Leu-)$_2$, and its analogues by dimerization–cyclization of pentapeptide precursors having protecting groups on the side-chains of the Orn residue, it was pointed out that the mode of cyclization in the chemical synthesis of GS is significantly different from that of its biosynthesis,[3] in which the C-terminal Leu residue of the precursor is fastened onto the GS synthetase.[4] 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 (-ONSu: succinimide ester) were lower than those from precursors having a D-Phe, Pro, Val or Orn(Z) residue at the C-terminus.[3]

[Val-Orn-Leu-D-Phe-Pro-D-Tyr / D-Tyr-Pro-D-Phe-Leu-Orn-Val] gratisin (GR)

[Val-Orn-Leu-D-Phe-Pro / Pro-D-Phe-Leu-Orn-Val] gramicidin S (GS)

Primary structures of GR and GS

Recently, we reported the direct formation of GS by the dimerization–cyclization of pentapeptide active esters having no protecting group on the side-chain of the Orn residue.[5] Among the five succinimide esters having a Val, Orn, Leu, D-Phe or Pro residue at each C-terminus, only H-D-Phe-Pro-Val-Orn-Leu-ONSu, having a sequence identical with that of the linear precursor pentapeptide in the biosynthesis of GS,[4] gave semi-GS (cyclic monomer) and GS (cyclic dimer) in yields of 15 and 38%, respectively. Other pentapeptide active esters did not give GS. It can be concluded from studies on the cyclization mode of H-D-Phe-Pro-Val-Orn-Leu-ONSu that, both in biological and in chemical syntheses, the formation of the cyclic peptides is subject to similar regiospecific control, although the conditions of their cyclizations are different, especially in biosynthesis, the enzyme probably giving a steric environment more favourable for the formation of GS.

In the present study, this biomimetic approach has been extended to the synthesis of a peptide antibiotic, gratisin (GR), cyclo(-D-Phe-Pro-D-Tyr-Val-Orn-Leu-)$_2$, which has a structure analogous to that of GS.[6,7] To investigate the formation of GR by the dimerization–cyclization of hexapeptide active esters having no protecting group on the side-chains of Orn and D-Tyr residues, the cyclizations of six linear hexapeptide-ONSus **1**–**6** having a Val, Orn, Leu, D-Phe, Pro or D-Tyr residue at each C-terminus were examined. In addition, Z-D-Phe-Pro-D-Tyr(BzlCl_2)-Val-Orn-Leu-ONSu, H-(-D-Phe-Pro-D-Tyr-Val-Orn-Leu-)$_2$-ONSu, and three hexapeptide-ONSus **7**–**9**, which possess D-Phe-Pro-Tyr, Phe-Pro-D-Tyr and Phe-Pro-Tyr sequences at the N-terminus, respectively, were cyclized, in order to investigate the cyclization mode of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu.

H-Val-Orn-Leu-D-Phe-Pro-D-Tyr-ONSu	**1**
H-Orn-Leu-D-Phe-Pro-D-Tyr-Val-ONSu	**2**
H-Leu-D-Phe-Pro-D-Tyr-Val-Orn-ONSu	**3**
H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu	**4**
H-Pro-D-Tyr-Val-Orn-Leu-D-Phe-ONSu	**5**
H-D-Tyr-Val-Orn-Leu-D-Phe-Pro-ONSu	**6**
H-D-Phe-Pro-Tyr-Val-Orn-Leu-ONSu	**7**
H-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu	**8**
H-Phe-Pro-Tyr-Val-Orn-Leu-ONSu	**9**

Primary structures of hexapeptide active esters **1**–**9** related to GR

Results and discussion

The hexapeptide-ONSus **1**–**6** related to GR were synthesized using a solution-phase methodology. These active esters **1**–**6** were cyclized in pyridine for 1 day at 25 °C (concentration of peptide in pyridine: 3 mM). Purification of the main products in the reaction mixture was performed by gel filtration using Sephadex LH-20 and semipreparative high-performance liquid chromatography (HPLC). The primary structure of the products was elucidated by amino acid analyses and fast-atom bombardment (FAB) mass spectra, and was confirmed by a direct comparison with authentic samples synthesized according to conventional methods. The primary structures of the main products are shown in Table 1.[8]

Among the six active esters **1**–**6** having a Val, Orn, Leu,

Table 1 Primary structures of the cyclic products isolated from the cyclization mixture of linear hexapeptide active esters **1**–**6**

Active ester	Cyclic products	
	a	**b**
1	H-Val-Orn-Leu-D-Phe-Pro-D-Tyr	
2	H-Orn-Leu-D-Phe-Pro-D-Tyr-Val	
3	H-Leu-D-Phe-Pro-D-Tyr-Val-Orn	
4	D-Phe-Pro-D-Tyr-Val-Orn-Leu (semi-GR)	[D-Phe-Pro-D-Tyr-Val-Orn-Leu / Leu-Orn-Val-D-Tyr-Pro-D-Phe] (GR)
5	H-Pro-D-Tyr-Val-Orn-Leu-D-Phe	
6	H-D-Tyr-Val-Orn-Leu-D-Phe-Pro	

Table 2 Effect of peptide concentration on the cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4**[a,b]

Concentration of peptide in pyridine ($\times 10^{-3}$ M)	Ratio of cyclic products semi-GR : GR	Total yield (%) of semi-GR and GR
0.3	100 : 0	42
3	79 : 21	39
30	58 : 42	13

[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.

D-Phe, Pro or D-Tyr residue at each C-terminus, only peptide **4**, H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu, gave semi-GR (cyclic monomer) and GR (cyclic dimer) in yields of 31 and 8%, respectively. On the other hand, other hexapeptide active esters **1**–**3**, **5** and **6** did not give any amount of semi-GR and GR. Active esters **1**–**3**, **5** and **6** gave exclusively 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.

The present results are similar to those obtained on the cyclization of pentapeptide active esters related to GS having no protecting group on the side-chain of the Orn residue,[5] and indicate that for the direct formation of GR and GS by the dimerization–cyclization of hexa- and penta-peptide active esters having no protecting group on the side-chain of the Orn residue, the amino acid sequences having a Leu residue at the C-terminus, H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu and H-D-Phe-Pro-Val-Orn-Leu-ONSu, are essential. However, the yield of GR (8%) by cyclization–dimerization of peptide **4** is lower when compared with the direct formation of GS (38%). The difference may result from differences in the chain length and the configurations of amino acid residues around the Pro residue between each precursor active ester.

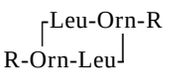
Next, the mode of the cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4** was investigated by several methods.

The effect of concentration of the hexapeptide active ester **4** on the cyclization yield was examined (concentrations of peptide in pyridine: 0.3, 3 and 30×10^{-3} M. Other conditions are similar to those described above) (Table 2). The proportion of semi-GR to GR in the product mixture depended on the concentration of the active ester in pyridine solution and indicates that formation of the cyclic dimer competes with that of the cyclic monomer.

The solvent effect on the cyclization of peptide **4** was investigated in various solvents [1,4-dioxane, benzene, $CHCl_3$, EtOH, dimethylformamide (DMF) and water]. The cyclization of the active ester in 1,4-dioxane gave semi-GR and GR in ~4 : 6 ratio, and its total yield was 25%. On the other hand, cyclization in water give mainly semi-GR in 20% yield and the production of GR was not detected by HPLC. Cyclizations in other solvents resulted in different ratios of semi-GR to GR. Thus, the polarity of the solvent affects the yield of GR.

These results concerning the effects of concentration and solvent on the cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu are similar to those of the pentapeptide related to GS, H-D-Phe-Pro-Val-Orn-Leu-ONSu.[5]

Z-D-Phe-Pro-D-Tyr($BzlCl_2$)-Val-Orn-Leu-ONSu was cyclized in pyridine (concentration of the peptide: 3×10^{-3} M) at 45 °C for 1 day to examine the reactivity between the δ-amino group of the Orn residue and the C-terminal ester group. A cyclic product was isolated by gel filtration using Sephadex LH-20, followed by recrystallization. However, considerable amounts of the starting material were recovered unchanged from the reaction mixture. The cyclic product was identified as the cyclic dimer,



R = [Z-D-Phe-Pro-D-Tyr($BzlCl_2$)-Val]—

on the basis of its relative molecular mass, which was determined by FAB mass spectrometry. The yield of cyclic dimer was 7%. This result indicates that the formation of Z-D-Phe-Pro-D-Tyr($BzlCl_2$)-Val-Orn-Leu-, having a highly strained nine-membered ring, is very difficult. This should be one reason for the preferential formation of semi-GR or GR in the reaction of peptide **4**.

Recently, in a study of the contribution of the D-Phe-Pro-Val sequence in the direct formation of GS by the dimerization–cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu, it has been reported that the change in the configurations of the Phe and Val residues around the Pro residue greatly affected the cyclization.[9] That is, the active ester with a D-Phe-Pro-D-Val sequence produced exclusively [D-Val]-semi-GS in 58% yield. The active esters having Phe-Pro-D-Val and Phe-Pro-Val sequences did not yield any cyclic monomer or cyclic dimer. On the other hand, H-D-Phe-Pro-Val-Orn-Leu-ONSu gave semi-GS (cyclic monomer) and GS (cyclic dimer) in yields of 15 and 38%, respectively.

Next, in order to investigate the effect of configuration of Phe and Tyr residues on the cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4**, three hexapeptide-ONSus **7**–**9** related to GR, and which possess D-Phe-Pro-Tyr, Phe-Pro-D-Tyr and Phe-Pro-Tyr sequences at the N-terminus, respectively, were synthesized, and cyclized in pyridine for 1 day at 25 °C (concentration of peptide in pyridine: 3 mM). Peptide **7** having the D-Phe-Pro-Tyr sequence produced cyclic monomer ([L-Tyr]-semi-GR) and cyclic dimer ([L-Tyr]-GR) in yields of 57 and 15%, respectively, and this ratio of cyclic monomer to cyclic dimer is similar to that obtained from peptide **4** having the D-Phe-Pro-D-Tyr sequence. Peptide **8** having the Phe-Pro-D-Tyr sequence

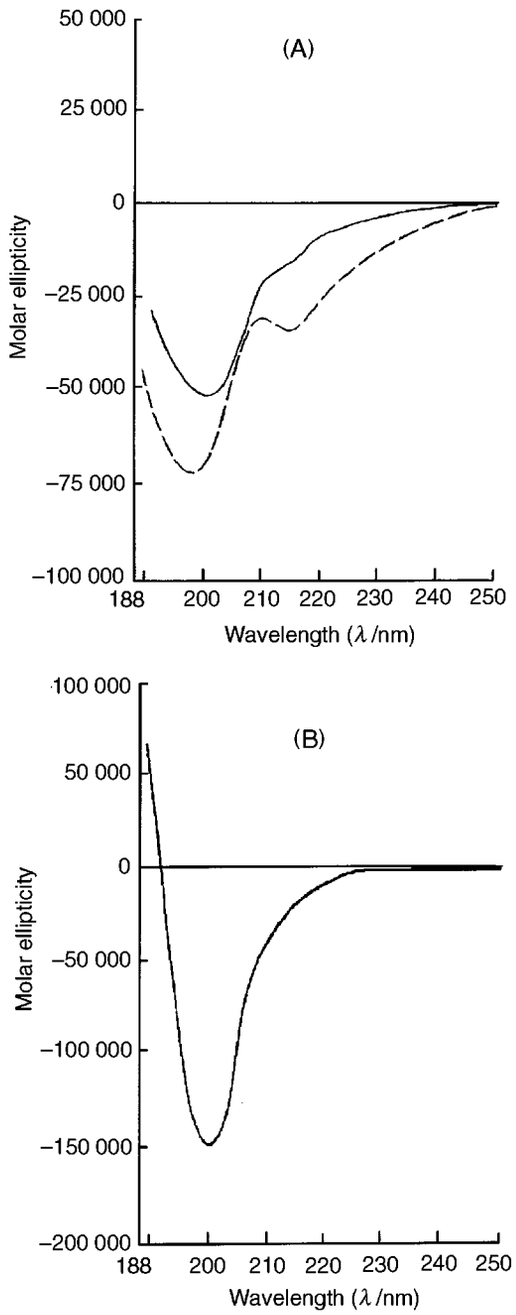


Fig. 1 CD spectra of (A) H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-OEt (——) and H-D-Phe-Pro-D-Val-Orn-Leu-OEt (– – –), and (B) semi-GR (——) in ethanol

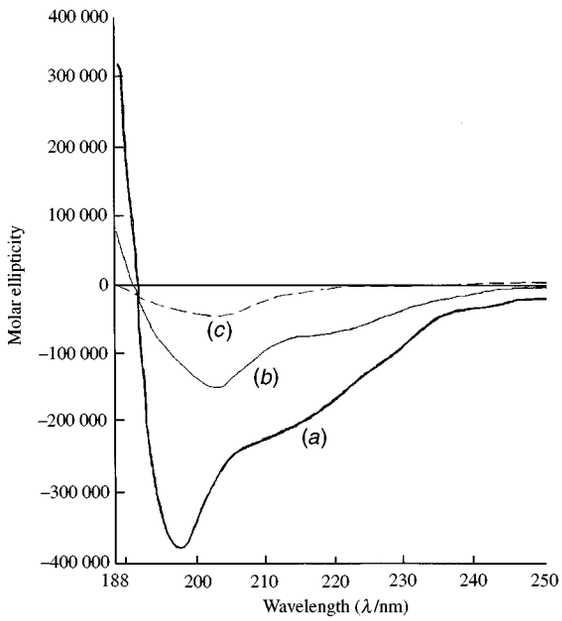


Fig. 2 CD spectra of (*a*) GR (——), (*b*) H-(D-Phe-Pro-D-Tyr-Val-Orn-Leu)$_2$-OEt (——), and (*c*) H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-OEt (– – –) in ethanol

gave cyclic monomer ([L-Phe]-semi-GR) in 32% yield, but the production of cyclic dimer was not observed. On the other hand, peptide **9** with the Phe-Pro-Tyr sequence did not produce the corresponding cyclic monomer or cyclic dimer. These results indicate that the amino acid sequences having a D-Phe residue at the N-terminus are essential for the production of cyclic dimer, and also that the configuration of the Tyr residue following the Pro residue contributes to the cyclization mode of these precursors.

The cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4** and H-(D-Phe-Pro-D-Tyr-Val-Orn-Leu)$_2$-ONSu in ethanol gave cyclic products similar to those formed in pyridine. That is, the modes of its cyclization in both solvents seem to be similar. To investigate the cyclization mode of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4**, circular dichroism (CD) spectra of ethyl esters corresponding to H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu and H-(D-Phe-Pro-D-Tyr-Val-Orn-Leu)$_2$-ONSu, and semi-GR and GR were measured in ethanol (Figs. 1 and 2). The CD spectrum of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-OEt [Fig. 1 (A)] showed a shoulder near 217 nm, and a negative trough at 202 nm, and its features are similar to that of H-D-Phe-Pro-D-Val-Orn-Leu-OEt, which the corresponding active ester, H-D-Phe-Pro-D-Val-Orn-Leu-ONSu, produced exclusively [D-Val]-semi-GS in 58% yield.[9] In addition, from CD and NMR studies of pentapeptide precursors related to GS, it was pointed out that the negative band or shoulder near 217 nm reflects mainly the N-terminus conformation in those molecules.[9] The present study suggests that the N-terminal part of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-OEt has an ordered conformation similar to that of H-D-Phe-Pro-D-Val-Orn-Leu-OEt, which makes it suitable for the intramolecular cyclization. This should be one reason for the preferential formation of semi-GR compared with that of GR in the cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4**. On the other hand, the CD spectrum of semi-GR showed a curve that slopes steadily without any trough at 200–220 nm [Fig. 1 (B)], suggesting that semi-GR has a disordered conformation in ethanol. Thus, no relationship between the CD spectra of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-OEt and semi-GR produced mainly by the cyclization of its active ester was found. The CD spectrum of H-(D-Phe-Pro-D-Tyr-Val-Orn-Leu)$_2$-OEt showed a shoulder near 220 nm and a trough near 200 nm, and its features resembled those of GR. In addition, cyclization of H-(D-Phe-Pro-D-Tyr-Val-Orn-Leu)$_2$-ONSu under conditions similar to those in the case of the hexapeptide active ester gave GR in 42% yield, but no H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-D-Phe-Pro-D-Tyr-Val-Orn-Leu. These results indicate that the dodecapeptide active ester formed by the intermolecular reaction of two molecules of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4** has a conformation in pyridine similar to that of GR, and cyclizes preferentially to afford the antibiotic.

On the basis of the above results, the mode of cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4** is proposed as follows. In the intramolecular reaction, the C-terminal ester reacts with the α-amino group of the D-Phe residue to give the semi-GR but does not react with the δ-amino group of the Orn residue. The cyclization is more favourable for the formation of semi-GR than for GR. On the other hand, in the intermolecular reaction, the dodecapeptide active ester formed by the coupling of two molecules of hexapeptide active ester cyclize to afford GR. Thus, the formation mechanism of GR by dimerization–cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-

Table 3 Physical properties and analytical data for intermediary products

No.	Compound	Mp (T/°C)	$[\alpha]_D^{25}$ (c 1.0, MeOH)	Formula	Found (%) (required) C	H	N
1	Boc-Val-Orn(Z)-Leu-D-Phe-Pro-D-Tyr($BzlCl_2$)-OBzl	85–88	−51.2	$C_{66}H_{81}Cl_2N_7O_{12}\cdot 2H_2O$	62.52 (62.35)	6.76 (6.74)	7.43 (7.71)
2	Boc-Orn(Z)-Leu-D-Phe-Pro-D-Tyr($BzlCl_2$)-Val-OBzl	89–91	−40.8	$C_{66}H_{81}Cl_2N_7O_{12}\cdot 0.5H_2O$	63.77 (63.71)	6.43 (6.64)	7.99 (7.88)
3	Boc-Leu-D-Phe-Pro-D-Tyr($BzlCl_2$)-Val-Orn(Z)-OBzl	104–105	−60.8	$C_{66}H_{81}Cl_2N_7O_{12}\cdot 0.5H_2O$	63.53 (63.71)	6.38 (6.64)	7.79 (7.88)
4	Boc-D-Phe-Pro-D-Tyr($BzlCl_2$)-Val-Orn(Z)-Leu-OBzl	137–140	−23.9	$C_{66}H_{81}Cl_2N_7O_{12}\cdot 0.5H_2O$	63.67 (63.71)	6.44 (6.64)	7.96 (7.88)
5	Boc-Pro-D-Tyr($BzlCl_2$)-Val-Orn(Z)-Leu-D-Phe-OBzl	153–154	−25.4	$C_{66}H_{81}Cl_2N_7O_{12}\cdot H_2O$	63.60 (63.25)	6.46 (6.67)	7.79 (7.88)
6	Boc-D-Tyr($BzlCl_2$)-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl	173–174	−42.5	$C_{66}H_{81}Cl_2N_7O_{12}\cdot 0.5H_2O$	63.86 (63.71)	6.75 (6.64)	7.77 (7.88)
7	Boc-[D-Phe-Pro-D-Tyr($BzlCl_2$)-Val-Orn(Z)-Leu]$_2$-OBzl	124–127	−37.5	$C_{120}H_{146}Cl_4N_{14}O_{21}\cdot 1.5H_2O$	63.13 (62.96)	6.74 (6.56)	8.42 (8.56)
8	Boc-Val-Orn-Leu-D-Phe-Pro-D-Tyr-OH	210–212	−77.1	$C_{44}H_{65}N_7O_{10}\cdot H_2O$	60.71 (60.74)	7.94 (7.76)	11.23 (11.27)
9	Boc-Orn-Leu-D-Phe-Pro-D-Tyr-Val-OH	200–201 (decomp.)	−64.2	$C_{44}H_{65}N_7O_{10}\cdot H_2O$	61.02 (60.74)	7.67 (7.76)	11.48 (11.27)
10	Boc-Leu-D-Phe-Pro-D-Tyr-Val-Orn-OH	160–162 (decomp.)	−46.5	$C_{44}H_{65}N_7O_{10}$	62.23 (62.02)	7.71 (7.69)	11.22 (11.51)
11	Boc-D-Phe-Pro-D-Tyr-Val-Orn-Leu-OH	167–169 (decomp.)	−40.2	$C_{44}H_{65}N_7O_{10}$	62.09 (62.02)	7.93 (7.69)	11.36 (11.51)
12	Boc-Pro-D-Tyr-Val-Orn-Leu-D-Phe-OH	192–195 (decomp.)	−4.1	$C_{44}H_{65}N_7O_{10}\cdot H_2O$	60.50 (60.74)	7.60 (7.76)	11.40 (11.27)
13	Boc-D-Tyr-Val-Orn-Leu-D-Phe-Pro-OH	192–196 (decomp.)	−62.1	$C_{44}H_{65}N_7O_{10}$	62.06 (62.02)	7.33 (7.69)	11.54 (11.51)
14	Boc-(D-Phe-Pro-D-Tyr-Val-Orn-Leu)$_2$-OH	198–202 (decomp.)	−45.2 (c 0.5)	$C_{83}H_{120}N_{14}O_{17}\cdot 9H_2O$	57.13 (57.03)	7.81 (7.96)	11.30 (11.22)
15	Boc-Val-Orn(Boc)-Leu-D-Phe-Pro-D-Tyr-OH	144–148 (decomp.)	−66.7	$C_{49}H_{73}N_7O_{12}\cdot 0.5H_2O$	61.64 (61.23)	8.01 (7.76)	9.81 (10.20)
16	Boc-Orn(Boc)-Leu-D-Phe-Pro-D-Tyr-Val-OH	113–117 (decomp.)	−34.9	$C_{49}H_{73}N_7O_{12}\cdot H_2O$	60.66 (60.66)	7.52 (7.79)	10.01 (10.11)
17	Boc-Leu-D-Phe-Pro-D-Tyr-Val-Orn(Boc)-OH	169–172 (decomp.)	−52.9	$C_{49}H_{73}N_7O_{12}\cdot H_2O$	60.65 (60.66)	8.01 (7.79)	9.99 (10.11)
18	Boc-D-Phe-Pro-D-Tyr-Val-Orn(Boc)-Leu-OH	134–138 (decomp.)	−24.0	$C_{49}H_{73}N_7O_{12}\cdot 0.5H_2O$	60.98 (61.23)	7.77 (7.76)	9.90 (10.20)
19	Boc-Pro-D-Tyr-Val-Orn(Boc)-Leu-D-Phe-OH	235–238 (decomp.)	−36.3 (c 0.2, DMF)	$C_{49}H_{73}N_7O_{12}\cdot 0.5H_2O$	61.37 (61.23)	7.82 (7.76)	9.92 (10.20)
20	Boc-D-Tyr-Val-Orn(Boc)-Leu-D-Phe-Pro-OH	164–166 (decomp.)	−45.6	$C_{49}H_{73}N_7O_{12}\cdot 0.5H_2O$	61.43 (61.23)	7.61 (7.76)	9.98 (10.20)
21	Z-D-Phe-Pro-Tyr($BzlCl_2$)-Val-Orn(Boc)-Leu-OH	109–113	−19.1	$C_{59}H_{75}Cl_2N_7O_{12}$	61.58 (61.88)	6.67 (6.60)	8.54 (8.56)
22	Boc-[D-Phe-Pro-D-Tyr-Val-Orn(Boc)-Leu]$_2$-OH	154–158	−35.3	$C_{93}H_{136}N_{14}O_{21}\cdot 4H_2O$	60.09 (60.11)	8.02 (7.81)	10.46 (10.55)
23	Boc-D-Phe-Pro-Tyr-Val-Orn(Boc)-Leu-OH	145–148	−57.1	$C_{49}H_{73}N_7O_{12}\cdot 0.5H_2O$	61.39 (61.23)	7.74 (7.76)	10.41 (10.20)
24	Boc-Phe-Pro-D-Tyr-Val-Orn(Boc)-Leu-OH	128–130	−13.3	$C_{49}H_{73}N_7O_{12}\cdot 0.5H_2O$	61.38 (61.23)	7.67 (7.76)	10.38 (10.20)
25	Boc-Phe-Pro-Tyr-Val-Orn(Boc)-Leu-OH	141–144	−39.8	$C_{49}H_{73}N_7O_{12}\cdot 1.5H_2O$	60.18 (60.10)	7.88 (7.82)	9.99 (10.01)
26	H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-OEt·2HCl[a]	158–162	−60.4 (c 0.9)	$C_{41}H_{63}Cl_2N_7O_8\cdot H_2O$	56.67 (56.68)	7.43 (7.31)	11.38 (11.28)
27	H-(D-Phe-Pro-D-Tyr-Val-Orn-Leu)$_2$-OEt·3HCl[a]	204–206	−70.2	$C_{80}H_{119}Cl_3N_{14}O_{15}\cdot 7H_2O$	54.97 (54.93)	7.59 (7.66)	10.89 (11.21)

[a] Compounds in experiments 26 and 27 were hexa- and dodeca-peptide ethyl esters used in the measurement of CD spectra.

ONSu **4** is similar to that of GS by the cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu.

GS, which has a structure analogous to that of GR and was isolated from a strain of *Bacillus brevis* ATCC 9999, 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 synthetase.[4] Recently, we found that although the environment in the chemical synthesis of GS is significantly different from that of the biosynthesis, H-D-Phe-Pro-Val-Orn-Leu-ONSu, having a sequence identical with that of the linear precursor pentapeptide in the biosynthesis of GS, gave GS directly.[5] On the other hand, GR, which is obtained from *Bacillus brevis* Y-33, a mutant of the GS producer, is also produced on the synthetase, but its biosynthetic process has not yet been estimated.[6,10] As described above, the cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4** affords GR, but the other hexapeptide active ester having a different C-terminal residue does not. In addition, the mechanism of formation of GR by dimerization–cyclization of H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu **4** is similar to that in the cyclization of H-D-Phe-Pro-Val-Orn-Leu-ONSu. From the results of our investigation, it is quite possible that GR is produced *via* dimerization–cyclization of a hexapeptide fragment having a Leu residue at the C-terminus, -D-Phe-Pro-D-Tyr-Val-Orn-Leu-, on the synthetase in a similar way to that of the GS synthesis.

Table 4 Physical properties and analytical data for cyclic products[a]

No.	Compound	Mp (*T*/°C)	$[\alpha]_D^{25}$ (*c* 0.5, MeOH)	FAB-MS *m/z* (required)
1	H-Val-Orn-Leu-D-Phe-Pro-D-Tyr-·HCl	247–249 (decomp.)	−66.7 (*c* 0.2)	734.4235 ($M + H^+$, $C_{39}H_{56}N_7O_7$ requires *m/z*, 734.4194)
2	H-Orn-Leu-D-Phe-Pro-D-Tyr-Val-·HCl	165–167 (decomp.)	−36.9	734.4216 ($M + H^+$, $C_{39}H_{56}N_7O_7$ requires *m/z*, 734.4194)
3	H-Leu-D-Phe-Pro-D-Tyr-Val-Orn-·TFA	108–111 (decomp.)	−13.3	734.4235 ($M + H^+$, $C_{39}H_{56}N_7O_7$ requires *m/z*, 734.4194)
4	H-Pro-D-Tyr-Val-Orn-Leu-D-Phe-·TFA	258–260 (decomp.)	−36.3 (*c* 0.5, DMF)	734.4234 ($M + H^+$, $C_{39}H_{56}N_7O_7$ requires *m/z*, 734.4194)
5	H-D-Tyr-Val-Orn-Leu-D-Phe-Pro-·TFA	189–192	−109.2	734.4252 ($M + H^+$, $C_{39}H_{56}N_7O_7$ requires *m/z*, 734.4194)
6	H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-D-Phe-Pro-D-Tyr-Val-Orn-Leu-·2HCl[b]	219–222 (decomp.)	−35.3 (*c* 0.2)	1467 ($M + H^+$, $C_{78}H_{111}N_{14}O_{14}$ requires *m/z*, 1467)
7	H-D-Phe-Pro-D-Tyr-Val-Orn-Leu-Leu-Orn-Val-D-Tyr-Pro-D-Phe-H ·2HCl	198–200 (decomp.)	−53.4 (*c* 0.5, DMF)	1467 ($M + H^+$, $C_{78}H_{111}N_{14}O_{14}$ requires *m/z*, 1467)

[a] The results of amino acid analyses of these peptides agreed closely with the theoretical values. [b] The cyclic peptide **7** was synthesized by a similar method described in the literature (ref. 11).

Experimental

Mps were measured on an Ishii melting point apparatus and are uncorrected. Amino acid analysis of each hydrolysate of the peptides was carried out with an Hitachi 835 amino acid analyser. Relative molecular masses of the cyclic products were determined by using FAB mass spectrometry on a JEOL JMS-D-300 mass spectrometer (Asahi Chemical Industry Company). CD spectra were obtained with a JASCO spectropolarimeter (model J-720) using 0.1 mm cells at room temperature. CD spectra of semi-GR, GR and its ethyl esters corresponding to active esters **1**–**6** were measured in ethanol solutions at a concentration of 0.5 mM.

HPLC was performed on an LC-800 series instrument (JASCO, Japan) consisting of an 880 intelligent HPLC pump, an 875-UV intelligent UV/visible detector, an 860-CD 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 using a linear gradient of 50–80% MeOH–5% aq. $NaClO_4$ during 60 min with a flow rate of 1 ml min^{-1} at 30 °C. The column eluent was monitored at 220 nm. The microorganisms employed in the assays were *Staphylococcus aureus* MS353, *Streptomyces pyogenes* N.Y.5, *Corynebacterium diphtheriae* P.W.8, *Micrococcus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NIHJ-JC2 and *Proteus vulgaris* OX19. Minimum inhibitory concentrations (in μg ml^{-1}) of the compounds were determined by an agar dilution method with 10^6 organisms per millilitre.

Syntheses of Boc-hexa- and -dodeca-peptides

Method 1. Boc-hexapeptides related to active esters **1**–**6** and Boc-dodecapeptide, 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 liquid-phase methodologies. In the synthesis of Boc-Val-Orn(Boc)-Leu-Phe-Pro-Tyr-OH as an example, Boc-Phe-Pro-Tyr(Bzl)-Val-Orn(Z)-Leu-OBzl was prepared by stepwise elongation using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (WSCD·HCl) and *N*-hydroxy-1,2,3-benzotriazole (HOBt) from Leu-OBzl. The Z- and -Bzl groups of Boc-Val-Orn(Z)-Leu-Phe-Pro-Tyr(Bzl)-OBzl were removed by hydrogenolysis. Then the resulting Boc-hexapeptide-OH was treated with $(Boc)_2O$ to give Boc-Val-Orn(Boc)-Leu-Phe-Pro-Tyr-OH. Other Boc-hexapeptides were synthesized in a similar manner.

Method 2. Boc-hexapeptides related to active esters **7**–**9** were prepared by liquid-phase methodologies. In the synthesis of Boc-D-Phe-Pro-Tyr-Val-Orn(Boc)-Leu-OH as an example, the coupling of Boc-D-Phe-Pro-Tyr(Bzl)-Val-OH and H-Orn(Boc)-Leu-OEt prepared by stepwise elongation was performed using WSCD·HCl and HOBt. The protected groups of Boc-D-Phe-Pro-Tyr(Bzl)-Val-Orn(Boc)-Leu-OEt were removed by saponification and hydrogenolysis. Other Boc-hexapeptides were synthesized in a similar manner.

The peptides were characterized by elemental analyses, TLC, HPLC, and amino acid analyses of their hydrolysates. The physical properties and analytical data of these peptides are shown in Table 3.

Reaction of hexa- and dodeca-peptide-ONSus

Boc-hexapeptide-OHs (50–100 mg) were converted into the corresponding succinimide esters by using HONSu and WSCD·HCl. Boc-hexapeptide-ONSus were treated with trifluoroacetic acid (TFA) to remove all Boc groups. Hexapeptide-ONSu trifluoroacetates were dissolved in small amounts of DMF, and the solutions were added dropwise to pyridine at 25 °C (concentration of the active esters was 3 mM). After the mixture had been stirred for 1 day at 25 °C the reaction mixtures were analysed by HPLC. Main products from the reaction mixtures of hexapeptide-ONSus were purified by gel filtration on a Sephadex LH-20 column (1.5 × 150 cm) using ethanol as the elution solvent, semipreparative HPLC, and by reprecipitation from methanol–diethyl ether. 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 Tables 4 and 5.

Determination of the free amino group in cyclic peptides

A cyclic peptide isolated from reaction mixtures of D-Phe-Pro-D-Tyr-Val-Orn-Leu-ONSu was treated with 2,4-dinitrofluorobenzene. The resulting dinitrophenyl cyclic peptide was hydrolysed in 6 M HCl for 24 h at 110 °C. The free amino group of the peptide was confirmed by comparing the results of the amino acid analyses of the hydrolysates of both the 2,4-dinitrophenol (DNP)-treated peptide and the non-treated peptide. The free amino group in other cyclic peptides was determined in a similar manner.

Syntheses of cyclic peptides as authentic samples

These cyclic peptides were synthesized by one-pot cyclization of the corresponding Boc-hexapeptide-OH (compounds **8**–**13** shown in Table 3) using WSCD·HCl and HOBt, followed by removal of the Boc-group by 4 M HCl–1,4-dioxane. Main

Table 5 Physical properties and analytical data for cyclic products[a]

No.	Compound	Mp (*T*/°C)	$[\alpha]_D^{25}$ (*c* 1.0, MeOH)	Formula	FAB-MS *m/z* ($M + H^+$)	Found (%) (required) C	H	N
8	cyclo(-D-Phe-Pro-D-Tyr-Val-Orn-Leu-)·HCl	226–230 (decomp.)	−38.5	$C_{39}H_{55}N_7O_7$·HCl	734	61.18 (60.88)	7.38 (7.33)	12.59 (12.73)
9	cyclo(-D-Phe-Pro-Tyr-Val-Orn-Leu-)·HCl	196–199	−86.7 (*c* 0.2)	$C_{39}H_{55}N_7O_7$·HCl·H_2O	734	59.20 (59.42)	7.60 (7.41)	12.42 (12.44)
10	cyclo(-Phe-Pro-D-Tyr-Val-Orn-Leu-)·HCl	228–231	−45.5	$C_{39}H_{55}N_7O_7$·HCl·H_2O	734	59.12 (59.42)	7.14 (7.41)	12.66 (12.44)
11	cyclo(-Phe-Pro-Tyr-Val-Orn-Leu-)·HCl	261–265 (decomp.)	−54.4	$C_{39}H_{55}N_7O_7$·HCl	734	60.89 (60.88)	7.42 (7.33)	12.62 (12.73)

[a] The results of amino acid analyses of these peptides agreed closely with the theoretical values.

products from the reaction mixtures were purified by gel filtration on a Sephadex LH-20 column (1.5 × 150 cm) using ethanol as the elution solvent and by reprecipitation from methanol–diethyl ether. The cyclic peptides were characterized by TLC, HPLC, FAB mass spectra, and amino acid analyses of their hydrolysates.

Syntheses of hexa- and dodeca-peptide ethyl esters

Hexa- and dodeca-peptide ethyl esters used in the measurement of CD spectra were prepared by the ethyl esterification of the corresponding Boc-hexa- and -dodeca-peptide-OH groups by using WSCD·HCl in ethanol and the removal of the Boc group by the action of 4 M HCl–1,4-dioxane. Analytical data for the esters are shown in Table 3.

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