Antimicrobially active cycloundecapeptides related to gramicidin S having a novel turn structure with *cis* D-Phe-Pro peptide bond†

Makoto Tamaki,*[a] Ichiro Sasaki,[a] Manabu Kokuno,[a] Mitsuno Shindo,[b] Masahiro Kimura[b] and Yoshiki Uchida[b]

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Antimicrobially active cycloundecapeptides related to gramicidin S, cyclo(-Val[1]-Orn[2]-Leu[3]-X[4]-D-Phe[5]-Pro[6]-Val[7]-Orn[8]- Leu[9]-D-Phe[10]-Pro[11]-) (X= Leu (**1**), Ala (**2**), Orn (**3**), Lys (**4**) and Arg (**5**)), were synthesized. From the CD and NMR studies, **1–5** possess antiparallel β-sheet conformation linked by a type II' β-turn around D-Phe[10]-Pro[11] and a novel turn structure around X[4]-D-Phe[5]-Pro[6] sequence with *cis* D-Phe-Pro peptide bond. The structural modifications at position 4 of **1–5** are beneficial to identification of novel antibiotic candidates without hemolytic activity.

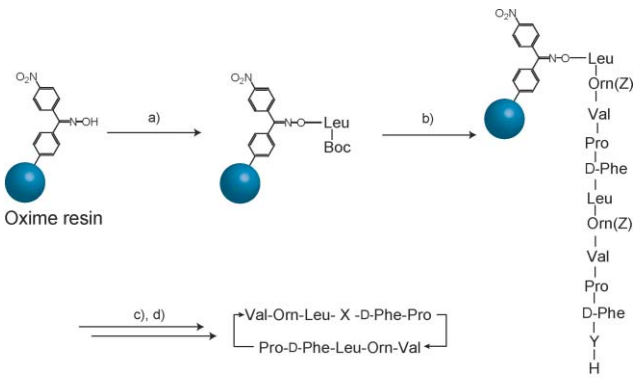
Introduction

Gramicidin S (GS), cyclo(-Val[1]-Orn[2]-Leu[3]-D-Phe[4]-Pro[5]-Val[6]-Orn[7]-Leu[8]-D-Phe[9]-Pro[10]-),[1–3] is a potent cyclopeptide antibiotic isolated from *Bacillus brevis*. Its secondary structure has been established as an antiparallel β-sheet conformation with C_2 symmetry and amphiphilicity linked by two type II' β-turns around two D-Phe-Pro sequences.[4,5] It has been proposed that the principal modes of antibiotic actions result from an interaction of GS with the cell membrane of the target microorganisms. GS then adopts an antiparallel β-sheet conformation with C_2 symmetry and amphiphilicity, which disrupts the cell membrane.[6] So far, no resistance has been found toward the antibiotic, presumably because such resistance would requires a significant alteration of the lipid composition of the cell membrane.[7] In view of widespread antibiotic resistance that has become a serious threat to public health,[8] amphiphilic antibiotics are attractive targets for drug discovery. However, GS has high hemolytic activity, preventing its direct use in combating the microbial resistance.[9] To find candidates with high antimicrobial and low hemolytic activities, many GS analogs of various ring sizes have been designed and synthesized.[2,3,10,11] However, syntheses of antimicrobially active analogues of GS having a disordered symmetry from C_2 have almost never been reported, because the stable, amphiphilic β-sheet structure of GS with C_2 symmetry is considered essential for its strong antibacterial activity.[2,3,10,11] In the present account, we designed and synthesized five novel GS analogues, cyclo(-Val[1]-Orn[2]-Leu[3]-X[4]-D-Phe[5]-Pro[6]-Val[7]-Orn[8]-Leu[9]-D-Phe[10]-Pro[11]-) (X= Leu (**1**), Ala (**2**), Orn (**3**), Lys (**4**), Arg (**5**)), in order to investigate the role of amphiphilic β-sheet structure with C_2 symmetry for the strong antibiotic activity of GS. The antibiotics **1–5**, which have one added X residue between Leu[3] and D-Phe[4] of GS, possess a disordered symmetry from C_2 in its primary structure.

Results and discussion

Synthesis

In the syntheses of **1–5** (Scheme 1), a protected linear precursor oxime, H–Y-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-oxime (Y = Leu, Ala, Orn(Z), Lys(Z) and Arg(NO_2), *Z*- = benzyloxycarbonyl-), was prepared by using Boc-solid phase peptide synthesis on oxime resin (loading of oxime group: 0.35 mmol g^{-1} resins).[12–14] The cyclization-cleavage of H–Y-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-oxime from the resin was performed in 1,4-dioxane with 2 equiv. of triethylamine and acetic acid for 1 day at room temperature to give cyclo[-Val-Orn(Z)-Leu-Y-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-]. The removal of all the masking groups by 25% HBr in acetic acid produced **1–4**. On the other hand, the masking groups of the protected precursor of **5** were removed by hydrogenolysis. Total yields of **1–5** from H-Leu-oxime resin are 34-56%. The purity and identity assessment of **1–5** were confirmed by thin-layer chromatography, high performance liquid chromatography



a) Boc-Leu-OH (3eq.), DCC (3eq.) in DCM 24 H b) Boc-amino acid (3eq.), BOP(3eq.), HOBt (3eq.), NEt3 (6.5eq.) in DMF 45min Deprotection 25% TFA / DCM 30min c) NEt3 (2eq.), AcOH (2eq.) in 1,4-dioxane 24 H d) 25% HBr/AcOH

Scheme 1 Syntheses of **1–5**. Y= Leu, Ala, Orn(Z), Lys(Z) and Arg(NO_2) X= Leu, Ala, Orn, Lys and Arg.

[a]*Department of Chemistry, Toho University, Funabashi, Chiba, 274-8510, Japan. E-mail: tamaki@chem.sci.toho-u.ac.jp; Fax: +81-47-472-1855; Tel: +81-47-472-4323*
[b]*Department of Food Science and Nutrition, Osaka Shoin Women's University, Higashi-Osaka, Osaka, 577-8550, Japan; Fax: +81-6-6723-8348; Tel: +81-6-6723-8181(ext335)*

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(HPLC), fast-atom bombardment (FAB) mass spectrometry and NMR spectra.

Structural evaluation

To investigate the secondary structure of **1–5**, the NMR spectra in DMSO-d_6 and CD spectra in methanol were measured. The NMR spectra were measured by ^{1}H NMR (400 MHz) in DMSO-d_6 (peptide concentration *ca.* 2.5 and 15 mM). All protons were assigned by means of H–H COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy) and ROESY (rotating frame nuclear Overhauser enhancement spectroscopy). In ^{1}H NMR spectra of **1–5**, no distinct conformation isomer was found. In a concentration of *ca.* 2.5 mM, the chemical shifts and temperature coefficients of $^{\alpha}$NH of amino acid residues of **1–5** show no significant concentration dependence, indicating that **1–5** are monomeric over the entire concentration range. In the ^{1}H NMR spectrum of **1**, the influence of the introduction of Leu residue between Leu^3 and D-Phe^4 residues of GS gave mainly the chemical shifts of the Leu^3, D-Phe^5 and Pro^6 residues of **1**. However, the chemical shifts of the Val^1, Orn^2, Val^7, Orn^8, Leu^9, D-Phe^{10} and Pro^{11} residues of **1** were similar to those of GS. These results suggested that the introduction of the added Leu residue between Leu and D-Phe residues greatly influence the conformation of Leu^3-Leu^4-D-Phe^5-Pro^6- sequence. On the other hand, the conformational change has less effect on the conformation around Val^7-Orn^8-Leu^9-D-Phe^{10}-Pro^{11}-Val^1-Orn^2 sequence of **1**. First, in order to establish secondary structure of **1** in DMSO-d_6, the temperature coefficient (Fig. 1), the vicinal spin–spin coupling constant of the amide protons (Fig. 2) and the chemical shift perturbation of the $^{\alpha}$H (Fig. 3) were examined. Low values of temperature coefficient were observed for four $^{\alpha}$NH of Val^{1,7} and Leu^{3,9} residues. In contrast, the values observed for $^{\alpha}$NH of Orn^{2,8}, Leu^4 and D-Phe^{5,10} residues were high. These results suggested that $^{\alpha}$NHs of Val^1, Leu^3, Val^7 and Leu^9 residues are shielded from the solvent and involved in four stable intramolecular hydrogen bonds, while the $^{\alpha}$NHs of Orn^2, Leu^4, D-Phe^5, Orn^8 and D-Phe^{10} residues are exposed to the solvent. The large $J_{NH\text{-}\alpha CH}$ values observed for Val^1, Orn^2, Leu^3, Val^7, Orn^8 and Leu^9 residues are strongly indicative of an extended β-sheet conformation.[15,16] (Fig. 2) The small $J_{NH\text{-}\alpha CH}$ values observed for Leu^4 and D-Phe^{10} residues suggested the presences of these amino acid residues in the turn regions.[15,16] On the other hand, D-Phe^5 residue showed the medium $J_{NH\text{-}\alpha CH}$ value (6.2 Hz). The chemical shift perturbations[17] of the $^{\alpha}$H of individual amino acid residues were shown in Fig. 3. The perturbations of chemical shifts of amino acid residues in Val^1-Orn^2-Leu^3 and Val^7-Orn^8-Leu^9 sequences are >0.1 ppm. The D-Phe^{10} and Pro^{11} residues are illustrated by a negative value. The values agreed well with those of GS. (Fig. 3) On the other hand, the Leu^4 and D-Phe^5 residues showed largely negative values, and Pro^6 residue a largely positive value in compared with those of GS. The results suggested that Val^7-Orn^8-Leu^9-D-Phe^{10}-Pro^{11}-Val^1-Orn^2-Leu^3 sequence in **1** has a similar β-sheet conformation to that of GS sequences, while the conformation around Leu^4-D-Phe^5-Pro^6 sequence is different from that of GS. Next, for detailed analysis, the spatial ROE correlations were measured. (Fig. 4) The results indicated that

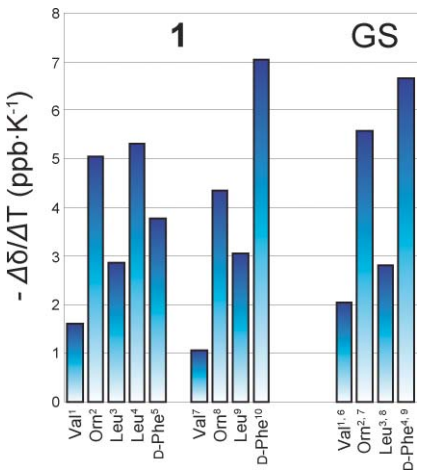

Fig. 1 Temperature dependences of amide protons in **1** and GS. Temperature coefficients ($-\Delta\delta/\Delta T$ (ppb·K^{-1})) of $^{\alpha}$NH of Val^1, Orn^2, Leu^3, Leu^4, D-Phe^5, Val^7, Orn^8, Leu^9 and D-Phe^{10} residues of **1** were 1.60, 5.05, 2.85, 5.31, 3.76, 1.06, 4.34, 3.00 and 7.04, respectively.

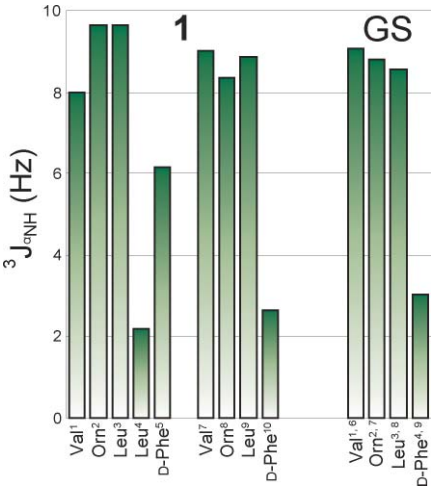

Fig. 2 $J_{NH\text{-}^{\alpha}CH}$ values of amide protons in **1** and GS. The $J_{NH\text{-}\alpha CH}$ values of Val^1, Orn^2, Leu^3, Leu^4, D-Phe^5, Val^7, Orn^8, Leu^9 and D-Phe^{10} residues of **1** were 8.0, 9.6, 9.6, 2.2, 6.2, 9.0, 8.4, 8.9, 2.6 Hz, respectively.

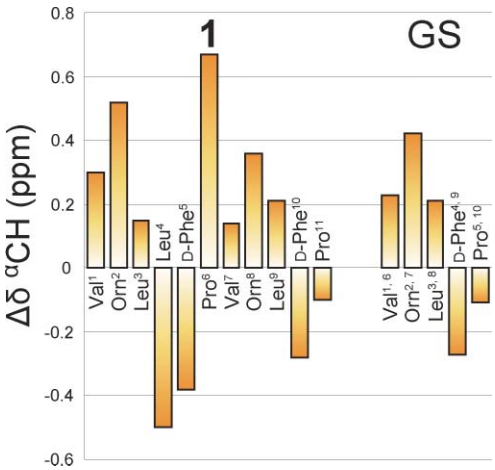

Fig. 3 Chemical shift perturbation of α protons in **1** and GS. Chemical shift perturbations ($\Delta\delta^{\alpha}$CH (ppm)) of Val^1, Orn^2, Leu^3, Leu^4, D-Phe^5, Val^7, Orn^8, Leu^9 and D-Phe^{10} residues of **1** were 0.30, 0.52, 0.15, –0.50, –0.38, 0.67, 0.14, 0.36, 0.21, –0.28, –0.10, respectively.

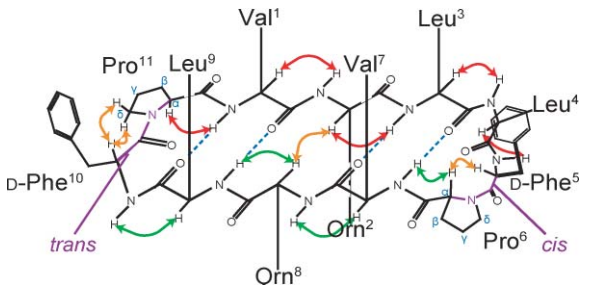

Fig. 4 Proposed secondary structure and spatial ROE crosspeaks of **1**. ROE spatial correlations between Pro[11] $^{\alpha}$CH and Val[1] $^{\alpha}$NH, Val[1] $^{\alpha}$CH and Orn[2] $^{\alpha}$NH, Orn[2] $^{\alpha}$CH and Leu[3] $^{\alpha}$NH, Leu[3] $^{\alpha}$CH and Leu[4] $^{\alpha}$NH, Leu[4] $^{\alpha}$CH and D-Phe[5] $^{\alpha}$NH, Pro[6] $^{\alpha}$CH and Val[7] $^{\alpha}$NH, Val[7] $^{\alpha}$CH and Orn[8] $^{\alpha}$NH, Orn[8] $^{\alpha}$CH and Leu[9] $^{\alpha}$NH, and Leu[9] $^{\alpha}$CH and D-Phe[10] $^{\alpha}$NH were observed.

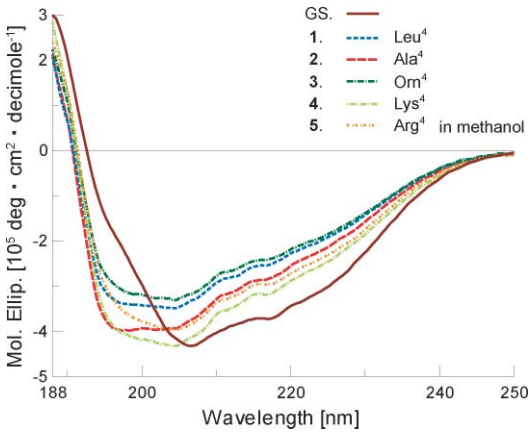

Fig. 5 CD spectra of **1–5** and GS in methanol.

these amide bonds in **1** are *trans* conformation. In addition, ROE spatial correlations between D-Phe[5] $^{\alpha}$CH and Pro[6] $^{\alpha}$CH, and D-Phe[10] $^{\alpha}$CH and Pro[11] $^{\delta}CH_2$ were observed. The results indicated that D-Phe[5]-Pro[6] amide bond is *cis* conformation, while D-Phe[10]-Pro[11] amide bond is *trans* conformation. The observation of ROE between Orn[2] $^{\alpha}$CH and Orn[8] $^{\alpha}$CH in 1 indicated that Orn[2] $^{\alpha}$CH and Orn[8] $^{\alpha}$CH may orient closely toward the inside of the β-sheet ring structure of **1**. In addition, the ROE spatial correlation was observed between Orn[8] $^{\delta}NH_3^+$ and Pro[11] $^{\alpha}$CH. From X-ray analyses[4,5] and NMR analyses[18] of GS, it was reported that, in addition to the four intramolecular hydrogen bonds between Val and Leu residues in this antibiotic, there are one or two intramolecular hydrogen bonds between Orn[8] NH_3^+ and D-Phe C=O. The NMR data indicated that Val[7]-Orn[8]-Leu[9]-D-Phe[10]-Pro[11]-Val[1]-Orn[2]-Leu[3] sequence in **1** has a GS-like antiparallel β-sheet structure with a type II' β-turn around D-Phe[10]-Pro[11]. Further detailed analyses were performed about the conformation of Leu[4]-D-Phe[5]-Pro[6] sequence. From ROE data, Leu[4]-D-Phe[5]-Pro[6] sequence has a *trans-cis* peptide bond backbone. From an inspection of the Corey–Pauling–Koltun molecular models, using the Karplus formula,[19] we assigned values of $\theta = 64°$ and 138° to the dihedral angles θ of $^{\alpha}$NH-$^{\alpha}$CH of Leu[4] and D-Phe[5], respectively. As a result, Leu[4] C=O preceding D-Phe[5] residue orient in close proximity to Pro[6] $^{\alpha}$H, and the $^{\alpha}$H of Pro[6] residue ($\delta = 5.11$) in **1** shift fairly downfield (0.78 ppm) in compared with that of GS ($\delta = 4.33$). The splitting signal of D-Phe[5] $^{\beta}CH_2$ was two multiplets, indicating that they are non-equivalent and fixed in certain arrangement. The $^{\alpha}$NH proton resonance of D-Phe[5] residue ($\delta = 8.67$) fairly shifted upfield (–0.40 ppm) in comparison with that of GS ($\delta = 9.07$), while $^{\beta}CH_2$, $^{\gamma}CH_2$ and $^{\delta}CH_2$ of Pro[6] residue showed a single multiplet peak. The results indicated that the aromatic ring of D-Phe[5] residue is closer to $^{\alpha}$NH of D-Phe[5] residue, and farther from Pro pyrrolidine ring. Similar results were obtained from the NMR studies of **2–5**. On the basis of the present NMR studies, it is proposed that **1–5** possesses a antiparallel β-sheet conformation with amphiphilic character, which is helding by a type II' β-turn around D-Phe[10]-Pro[11] and a novel turn structure around X[4]-D-Phe[5]-Pro[6] sequence with *cis* D-Phe[5]-Pro[6] amide bond. (Fig. 4)

CD spectra of **1–5** and GS were measured in methanol, in order to investigate the structure–activity relationship of **1–5**. (Fig. 5) CD spectra of **1–5** were observed a curve similar to each other. The results indicated that the conformations of **1–5** in methanol are similar to each other. The CD spectra of **1–5** in the 215-225 nm region showed a pattern similar to that of GS. On the other hand, in the 190–210 nm region, two troughs at 198 and 205 nm were observed in the spectra of **1–5**, while a trough at 206 nm was observed in the spectrum of GS. From conformation studies of GS by using model compounds,[20,21] it is clear that the negative band near 206 nm and the shoulder at ~220 nm of GS are attributable to a combined trough of the type II' β-turn and the β-sheet structure, respectively. The present results indicated that CD spectra of **1–5** are attributable to a combined trough of the type II' β-turn around D-Phe-Pro sequence, a novel turn around X-D-Phe-Pro sequence and the antiparallel β-sheet structure composited by two Val-Orn-Leu sequence, respectively. Thus, a good correlation was found between NMR and CD data.

Biological activity

The antibiotic activities of **1–5** and GS are summarized in Table 1. The difference of antibiotic activities among **1–5** reflects the characters of side chains of the introduced amino acid residues at position 4, because the antibiotics have similar conformations to each other. **1–5** showed the same level of antimicrobial activity as GS against all Gram-positive microorganisms tested. **1–5** showed less activity against Gram-negative *Pseudomonas aeruginosa* NBRC3080. On the other hand, **3–5** with the basic side chains showed the same activity as that of GS toward Gram-negative *Escherichia coli* NBRC12734. The results indicated that perhaps the β-sheet structure with C_2 symmetry was not necessary for the strong antibiotic activity of GS analogues. In addition,

Table 1 Antibiotic activities of **1–5** and GS[a]

	A[b]	B[b]	C[b]	D[b]	E[b]
1	3.13	3.13	3.13	50	>100
2	3.13	3.13	3.13	>100	>100
3	6.25	3.13	3.13	25	100
4	3.13	3.13	3.13	25	100
5	6.25	6.25	6.25	25	100
GS	3.13	3.13	3.13	25	25

[a] Minimum inhibitory concentration(MIC) value in μg mL^{-1} was determined by a medium dilution method with 10^6 organisms per millilitre. Antibiotic activity test were performed four times for each peptide. [b] Gram-positive. [c]Gram-negative. A: *Bacillus subtilis* NBRC 3513, B: *Bacillus megaterium* ATCC 19213, C: *Staphylococcus aureus* NBRC 12732, D: *Escherichia coli* NBRC 12734, E: *Pseudomonas aeruginosa* NBRC 3080

it is interesting to note that **3–5**, which have one added basic amino acid residue, showed some activity against different Gram-negative microorganisms. The hemolytic activities against sheep erythrocytes of **1–5** and GS are summarized in Fig. 6. The hemolytic activity of **1** was higher than that of GS. The activity of **2** at 100 μM was 52% of GS. On the other hand, the introduction of Orn, Lys and Arg residues at position 4 results in considerable decrease of the hemolytic activity compared with that of GS. Thus, we found that the antibiotics **3–5**, which have cationic amino acid residues between Leu[3] and D-Phe[4] residues of GS, possess differential interaction against the prokaryotic membrane and eukaryotic membrane.

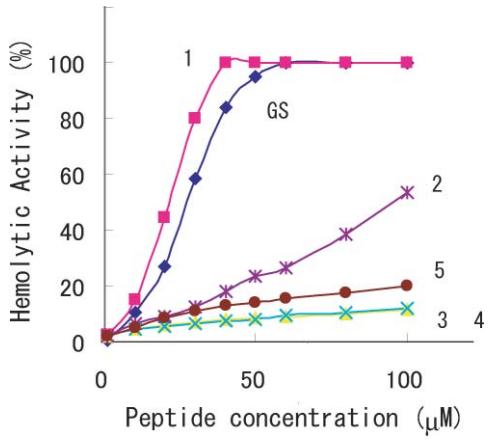

Fig. 6 Dose dependence curves of hemolysis (%) against sheep erythrocytes induced by **1–5** and GS. The experiments were carried out three times for each peptide.

Conclusions

In conclusion, we found that **1–5** have a novel antiparallel β-sheet conformation held in by a type II' β-turn around D-Phe-Pro and a novel turn structure around X-D-Phe-Pro sequence with *cis* D-Phe-Pro amide bond, and the structural modifications at position 4 of **1–5** are beneficial to identification of novel antibiotic candidates with lower hemolytic activity. Our findings should be helpful in finding drug candidates with high antimicrobial and low hemolytic activities that are capable of combating microbial resistance. Currently, we are investigating the design and synthesis of other antimicrobially active analogues of GS having a disordered symmetry from C_2 on the basis of the present studies in order to find new types of drug candidates with high antimicrobial and low hemolytic activities.

Experimental

Chemistry

Melting points were measured on Mel-Temp II melting point apparatus (Laboratory Devices, MA, USA) and are uncorrected. Unless otherwise noted, all materials were obtained from commercial supplies and used without further purification. Low-resolution mass spectra (LR-MS) were obtained by using FAB mass spectrometry on a JEOL600H mass spectrometer (JEOL LTD., Tokyo, Japan). HPLC analyses were achieved using analytical reverse phase HPLC system (JASCO LTD., Tokyo, Japan), equipped with an 880 intelligent HPLC pump, an 875-UV intelligent UV/Vis detector, an 860-CD column oven, and a TSK-Gel C18 column (4.6 × 150 mm, 10 μm particle size, TOSOH Co., Tokyo, Japan). Chromatographies were carried out by a flow rate of 1 ml min^{-1} at 30 °C and monitored at 220 nm. Elution solvents used methanol-0.1% trifluoroacetic acid (TFA) aq (70 : 30) for **1**, methanol-0.1% TFAaq (65 : 35) for **2** and methanol-0.1% TFAaq (55 : 45) for **3–5**. Analytical thin-layer chromatography was carried out on Merck silica-gel F_{254} plates with the following solvent systems (v/v): R_f^1, n-BuOH : pyridine : AcOH : H_2O (4 : 1 : 1 : 2); R_f^2, n-BuOH : AcOH : H_2O (4 : 1 : 1).

Preparation of H-Leu-oxime resin

Oxime resin (4-nitrobenzophenone oxime resin) was prepared according to the literature.[22,23] To the oxime resin (1.0 g) was added the solution Boc-Leu-OH (1.0 mmol) and DCC (1.0 mmol) in dichloromethane (15 ml) in a manual SPS vessel. The mixture was shaken for 15 h at room temperature. The solvent was filtrered off and the resin was washed with dichloromethane (15 ml, 2 times), dichloromethane–ethanol (1 : 1, v/v) (15 ml, 4 times), and dichloromethane (15 ml, 2 times), and then dried in vacuo. An aliquot of Boc-Leu resin was withdrawn and subjected to the picrate assay to estimate the substitution level (loading of oxime group: 0.35 mmol g^{-1} resins). Boc-Leu-oxime resin (514 mg, 0.18 mmol) obtained was treated with 25% TFA in dichloromethane for 30 min to remove Boc group, and then the resin was washed with dichloromethane (8 ml, 2 times), isopropyl alcohol (8 ml, 1 time), and dichloromethane (8 ml, 2 times). H-Leu-oxime resin obtained was used to the syntheses of **1–5**.

***Cyclo*(-Val-Orn-Leu-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)·2HBr (1).** Preparation of Boc-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-oxime on resin was performed by the stepwise elongation from H-Leu-oxime resin (0.18 mmol) using Boc-amino acid (0.54 mmol), benzotriazole-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP) (239 mg, 0.54 mmol), 1-hydroxybenzotriazole (HOBt) (73.0 mg, 0.54 mmol) and triethylamine (Et_3N) (0.16 ml, 1.17 mmol) in DMF (8 ml). Boc-undecapeptide-oxime resin obtained was treated with 25% TFA in dichloromethane for 30 min to remove Boc group, and then the resin was washed with dichloromethane (8 ml, 2 times), isopropyl alcohol (8 ml, 1 time), and dichloromethane (8 ml, 2 times). The formation of the cyclic peptide by the cyclization-cleavage of H-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-oxime on resin was performed in 1,4-dioxane (8 ml) with Et_3N (0.05 ml, 0.36 mmol) and acetic acid (0.02 ml, 0.36 mmol) for 1 day at room temperature. After the reaction mixture was evaporated, the residues obtained were purified by gel filtration on a Sephadex LH-20 (1.5 × 120 cm) using methanol as the eluting solvent, followed by reprecipitation with MeOH-ether to give cyclo[-Val-Orn(Z)-Leu-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-]. Next, the removal of Z groups of the protected cyclic peptide was performed by 25% HBr in acetic acid for 1 h. The addition of ether to the reaction mixture afforded a precipitate, which was then filtered and washed with ether. The residues obtained were purified by gel filtration on a Sephadex LH-20 (1.5 × 120 cm) using methanol as the eluting solvent, followed by reprecipitation with methanol-ether to give **1**.

White powder. Yield 86.6 mg (0.061 mmol, 34% from Boc-Leu-oxime resin (514 mg, 0.18 mmol)). Mp. 252-253 °C. (mp 276–278 (dec.) in the literature[24]) LR-FAB-MS (matrix: *m*-NBA (*m*-nitro benzyl alcohol)) calcd for $C_{66}H_{104}N_{13}O_{11}$ $[M+H]^+$=1255, Found *m/z* 1255 ($[M+H]^+$, 40.7%), 1277 ($[M+Na]^+$, 5.8%). R_f^1 0.82; R_f^2 0.57. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.11 (d, 1H, $^{\alpha}$NH D-Phe^{10}, $^3J_{\alpha NH}$ = 2.6 Hz), 8.87 (d, 1H, $^{\alpha}$NH Orn^8, $^3J_{\alpha NH}$ = 8.4 Hz), 8.67 (d, 1H, $^{\alpha}$NH D-Phe^5, $^3J_{\alpha NH}$ = 6.2 Hz), 8.59 (d, 1H, $^{\delta}$NH Orn^2, $^3J_{\alpha NH}$ = 9.6 Hz), 8.56 (d, 1H, $^{\alpha}$NH Leu^3, $^3J_{\alpha NH}$ = 9.6 Hz), 8.28 (d, 1H, $^{\alpha}$NH Leu^9, $^3J_{\alpha NH}$ = 9.6 Hz), 8.09 (m, 2H, $^{\delta}$NH Orn^2), 8.03 (d, 1H, $^{\alpha}$NH Leu^4, $^3J_{\alpha NH}$ = 2.2 Hz), 7.95 (m, 2H, $^{\delta}$NH Orn^8), 7.40 (d, 1H, $^{\alpha}$NH Val^7, $^3J_{\alpha NH}$ = 9.0 Hz), 7.34–7.10 (m, 5H, H_{ar} D-Phe^5, m, 5H, H_{ar} D-Phe^{10}), 7.28 (d, 1H, $^{\alpha}$NH Val^1, $^3J_{\alpha NH}$ = 8.0 Hz), 5.11 (m, 1H, $^{\alpha}$H Pro^6), 4.88 (m, 1H, $^{\alpha}$H Orn^2), 4.72 (m, 1H, $^{\alpha}$H Orn^8), 4.59 (m, 1H, $^{\alpha}$H Leu^9), 4.53 (m, 1H, $^{\alpha}$H Leu^3), 4.48 (m, 1H, $^{\alpha}$H Val^1), 4.38 (m, 1H, $^{\alpha}$H D-Phe^{10}), 4.34 (m, 1H, $^{\alpha}$H Pro^{11}), 4.32 (m, 1H, $^{\alpha}$H Val^7), 4.28 (m, 1H, $^{\alpha}$H D-Phe^5), 3.88 (m, 1H, $^{\alpha}$H Leu^4), 3.58 (m, 1H, $^{\delta}$H Pro^{11}), 3.50 (m, 1H, $^{\delta}$H Pro^6), 3.34 (m, 1H, $^{\delta}$H Pro^6), 3.12 (m, 1H $^{\beta}$H D-Phe^5), 3.00 (m, 1H $^{\beta}$H D-Phe^{10}), 2.93 (m, 1H $^{\delta}$H Orn^8), 2.87 (m, 1H $^{\beta}$H D-Phe^{10}), 2.76 (m, 1H $^{\delta}$H Orn^8), 2.68 (m, 2H $^{\delta}$H Orn^2), 2.67 (m, 1H $^{\beta}$H D-Phe^5), 2.52 (m, 1H, $^{\delta}$H Pro^{11}), 2.12 (m, 2H $^{\beta}$H Pro^6), 2.09 (m, 1H $^{\beta}$H Val^1), 1.99 (m, 1H $^{\beta}$H Pro^{11}), 1.82 (m, 1H $^{\beta}$H Orn^8), 1.77 (m, 1H, $^{\gamma}$H Leu^4, m, 1H $^{\beta}$H Val^7), 1.73 (m, 2H, $^{\gamma}$H Pro^6, m, 1H, $^{\gamma}$H Orn^8), 1.66 (m, 1H $^{\beta}$H Orn^8), 1.62 (m, 2H, $^{\gamma}$H Pro^{11}), 1.59 (m, 2H, $^{\gamma}$H Orn^2), 1.57 (m, 2H, $^{\beta}$H Orn^2, m, 1H, $^{\gamma}$H Leu^3), 1.55 (m, 2H, $^{\beta}$H Leu^3, m, 1H, $^{\gamma}$H Orn^8), 1.51 (m, 1H, $^{\beta}$H Leu^4, m, 1H, $^{\beta}$H Pro^{11}), 1.48 (m, 1H, $^{\gamma}$H Leu^9), 1.44 (m, 1H, $^{\beta}$H Leu^9), 1.35 (m, 1H, $^{\beta}$H Leu^4), 1.21 (m, 1H, $^{\beta}$H Leu^9), 0.89 (m, 6H $^{\delta}$H Leu^4), 0.82 (m, 6H $^{\gamma}$H Val^1, m, 6H $^{\gamma}$H Val^7, m, 6H $^{\delta}$H Leu^9), 0.81 (m, 6H $^{\delta}$H Leu^3).

2–5 were synthesized from Boc-Leu-oxime resin (514 mg, 0.18 mmol) by using a similar manner to that of **1**. The masking groups of the protected precursor of **5** were removed by hydrogenolysis.

***Cyclo*(-Val-Orn-Leu-Ala-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)·2HBr (2).** White powder. Yield 84.1 mg (0.061 mmol, 34% from Boc-Leu-oxime resin (514 mg, 0.18 mmol)). Mp. 234–237 °C. LR-FAB-MS (matrix: *m*-NBA) calcd for $C_{63}H_{98}N_{13}O_{11}$ $[M+H]^+$=1213, Found *m/z* 1213 ($[M+H]^+$, 46.7%), 1235 ($[M+Na]^+$, 3.5%). R_f^1 0.68; R_f^2 0.48. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (d, 1H, $^{\alpha}$NH D-Phe^{10}, $^3J_{\alpha NH}$ = 2.8 Hz), 8.87 (d, 1H, $^{\alpha}$NH Orn^8, $^3J_{\alpha NH}$ = 8.5 Hz), 8.66 (d, 1H, $^{\alpha}$NH Orn^2, $^3J_{\alpha NH}$ = 9.5 Hz), 8.60 (d, 1H, $^{\delta}$NH D-Phe^5, $^3J_{\alpha NH}$ = 6.5 Hz), 8.49 (d, 1H, $^{\alpha}$NH Leu^3, $^3J_{\alpha NH}$ = 9.3 Hz), 8.31 (d, 1H, $^{\alpha}$NH Ala^4, $^3J_{\alpha NH}$ = 2.2 Hz), 8.27 (d, 1H, $^{\alpha}$NH Leu^9, $^3J_{\alpha NH}$ = 9.2 Hz), 7.67 (m, 2H, $^{\delta}$NH Orn^2, m, 2H, $^{\delta}$NH Orn^8), 7.45 (d, 1H, $^{\alpha}$NH Val^7, $^3J_{\alpha NH}$ = 9.3 Hz), 7.32–7.10 (m, 5H, H_{ar} D-Phe^5, m, 5H, H_{ar} D-Phe^{10}), 7.24 (d, 1H, $^{\alpha}$NH Val^1, $^3J_{\alpha NH}$ = 8.4 Hz), 5.09 (m, 1H, $^{\alpha}$H Pro^6), 4.88 (m, 1H, $^{\alpha}$H Orn^2), 4.70 (m, 1H, $^{\alpha}$H Orn^8), 4.59 (m, 1H, $^{\alpha}$H Leu^9), 4.53 (m, 1H, $^{\alpha}$H Leu^3), 4.47 (m, 1H, $^{\alpha}$H Val^1), 4.38 (m, 1H, $^{\alpha}$H D-Phe^{10}), 4.30 (m, 1H, $^{\alpha}$H Pro^{11}), 4.29 (m, 1H, $^{\alpha}$H Val^7), 4.27 (m, 1H, $^{\alpha}$H D-Phe^5), 3.88 (m, 1H, $^{\alpha}$H Ala^4), 3.62 (m, 1H, $^{\delta}$H Pro^{11}), 3.42 (m, 2H, $^{\delta}$H Pro^6), 3.12 (m, 1H $^{\beta}$H D-Phe^5), 2.98 (m, 1H $^{\beta}$H D-Phe^{10}), 2.86 (m, 1H $^{\beta}$H D-Phe^{10}), 2.71 (m, 2H $^{\delta}$H Orn^2, m, 2H $^{\delta}$H Orn^8), 2.68 (m, 1H $^{\beta}$H D-Phe^5), 2.46 (m, 1H, $^{\delta}$H Pro^{11}), 2.10 (m, 1H, $^{\beta}$H Val^1, m, 2H, $^{\beta}$H Pro^6), 1.97 (m, 1H $^{\beta}$H Pro^{11}), 1.88 (m, 1H $^{\beta}$H Orn^8), 1.77 (m, 2H, $^{\gamma}$H Pro^6, m, 1H $^{\beta}$H Val^7, m, 1H, $^{\beta}$H Orn^8), 1.60 (m, 1H $^{\beta}$H Orn^2, m, 2H, $^{\gamma}$H Orn^8, m,2H, $^{\gamma}$H Pro^{11}), 1.55 (m, 1H, $^{\beta}$H Leu^3, m, 1H, $^{\gamma}$H Leu^3), 1.48 (m, 1H, $^{\beta}$H Orn^2, m, 1H, $^{\beta}$H Leu^3, m, 1H, $^{\beta}$H Pro^{11}), 1.45 (m, 2H, $^{\gamma}$H Orn^2, m, 1H, $^{\beta}$H Leu^9), 1.39 (m, 1H, $^{\gamma}$H Leu^9), 1.22 (m, 3H, $^{\beta}$H Ala^4, m, 1H, $^{\beta}$H Leu^9), 0.84 (m, 6H $^{\delta}$H Leu^9), 0.81 (m, 6H $^{\gamma}$H Val^1, m, 6H $^{\gamma}$H Val^7), 0.78 (m, 6H $^{\delta}$H Leu^3).

***Cyclo*(-Val-Orn-Leu-Orn-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)·3HBr (3).** White powder. Yield 151 mg (0.10 mmol, 56% from Boc-Leu-oxime resin (514 mg, 0.18 mmol)). Mp. 232–234 °C. LR-FAB-MS (matrix: *m*-NBA) calcd for $C_{65}H_{102}N_{14}O_{11}$ $[M+H]^+$= 1256, Found *m/z* 1256 ($[M+H]^+$, 25.5%), 1278 ($[M+Na]^+$, 2.0%). R_f^1 0.69; R_f^2 0.42. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (d, 1H, $^{\alpha}$NH D-Phe^{10}, $^3J_{\alpha NH}$ = 2.6 Hz), 8.87 (d, 1H, $^{\alpha}$NH Orn^8, $^3J_{\alpha NH}$ = 8.6 Hz), 8.68 (d, 1H, $^{\alpha}$NH D-Phe^5, $^3J_{\alpha NH}$ = 6.2 Hz), 8.62 (d, 1H, $^{\delta}$NH Orn^2, $^3J_{\alpha NH}$ = 9.6 Hz), 8.53 (d, 1H, $^{\alpha}$NH Leu^3, $^3J_{\alpha NH}$ = 9.6 Hz), 8.25 (d, 1H, $^{\alpha}$NH Leu^9, $^3J_{\alpha NH}$ = 9.0 Hz), 8.22 (d, 1H, $^{\alpha}$NH Orn^4, $^3J_{\alpha NH}$ = 2.2 Hz), 7.78 (m, 2H, $^{\delta}$NH Orn^8), 7.74 (m, 2H, $^{\delta}$NH Orn^2), 7.66 (m, 2H, $^{\delta}$NH Orn^4), 7.43 (d, 1H, $^{\alpha}$NH Val^7, $^3J_{\alpha NH}$ = 9.2 Hz), 7.32–7.09 (m, 5H, H_{ar} D-Phe^5, m, 5H, H_{ar} D-Phe^{10}), 7.25 (d, 1H, $^{\alpha}$NH Val^1, $^3J_{\alpha NH}$ = 8.4 Hz), 5.10 (m, 1H, $^{\alpha}$H Pro^6), 4.88 (m, 1H, $^{\alpha}$H Orn^2), 4.72 (m, 1H, $^{\alpha}$H Orn^8), 4.56 (m, 1H, $^{\alpha}$H Leu^9), 4.54 (m, 1H, $^{\alpha}$H Leu^3), 4.47 (m, 1H, $^{\alpha}$H Val^1), 4.38 (m, 1H, $^{\alpha}$H D-Phe^{10}), 4.32 (m, 1H, $^{\alpha}$H Pro^{11}), 4.31 (m, 1H, $^{\alpha}$H Val^7), 4.28 (m, 1H, $^{\alpha}$H D-Phe^5), 3.88 (m, 1H, $^{\alpha}$H Orn^4), 3.59 (m, 1H, $^{\delta}$H Pro^{11}), 3.41 (m, 2H, $^{\delta}$H Pro^6), 3.15 (m, 1H $^{\beta}$H D-Phe^5), 3.00 (m, 1H $^{\beta}$H D-Phe^{10}), 2.89 (m, 1H $^{\beta}$H D-Phe^{10}), 2.84 (m, 2H $^{\delta}$H Orn^4), 2.78 (m, 2H $^{\delta}$H Orn^8), 2.71 (m, 2H $^{\delta}$H Orn^2), 2.68 (m, 1H $^{\beta}$H D-Phe^5), 2.49 (m, 1H, $^{\delta}$H Pro^{11}), 2.11 (m, 2H, $^{\beta}$H Pro^6), 2.08 (m, 1H, $^{\beta}$H Val^1), 1.93 (m, 1H $^{\beta}$H Pro^{11}), 1.86 (m, 1H, $^{\gamma}$H Pro^6), 1.80 (m, 1H $^{\beta}$H Orn^8), 1.77 (m, 1H $^{\beta}$H Val^7), 1.73 (m, 1H, $^{\beta}$H Orn^8), 1.71 (m, 1H, $^{\gamma}$H Pro^6), 1.64 (m, 1H $^{\beta}$H Orn^4), 1.61 (m, 1H, $^{\beta}$H Orn^2, m, 2H, $^{\gamma}$H Orn^8), 1.57 (m, 2H, $^{\gamma}$H Orn^4), 1.55 (m, 1H, $^{\gamma}$H Leu^3), 1.54 (m, 1H, $^{\beta}$H Orn^2), 1.51 (m, 2H, $^{\beta}$H Leu^3), 1.50 (m, 1H, $^{\gamma}$H Leu^9, m, 1H, $^{\beta}$H Pro^{11}, m, 2H, $^{\gamma}$H Pro^{11}), 1.48 (m, 2H, $^{\gamma}$H Orn^2), 1.44 (m, 1H, $^{\beta}$H Leu^9), 0.81 (m, 6H $^{\gamma}$H Val^1, m, 6H $^{\delta}$H Leu^3, m, 6H $^{\gamma}$H Val^7, m, 6H $^{\delta}$H Leu^9).

***Cyclo*(-Val-Orn-Leu-Lys-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)·3HBr (4).** White powder. Yield 95.2 mg (0.063 mmol, 35% from Boc-Leu-oxime resin (514 mg, 0.18 mmol)). Mp. 234–237 °C. LR-FAB-MS (matrix: *m*-NBA) calcd for $C_{66}H_{105}N_{14}O_{11}$ $[M+H]^+$= 1270, Found *m/z* 1270 ($[M+H]^+$, 13.6%). R_f^1 0.70; R_f^2 0.37. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.09 (d, 1H, $^{\alpha}$NH D-Phe^{10}, $^3J_{\alpha NH}$ = 3.3 Hz), 8.86 (d, 1H, $^{\alpha}$NH Orn^8, $^3J_{\alpha NH}$ = 8.9 Hz), 8.62 (d, 1H, $^{\alpha}$NH Orn^2, $^3J_{\alpha NH}$ = 9.5 Hz), 8.60 (d, 1H, $^{\delta}$NH D-Phe^5, $^3J_{\alpha NH}$ = 6.1 Hz), 8.51 (d, 1H, $^{\alpha}$NH Leu^3, $^3J_{\alpha NH}$ = 9.5 Hz), 8.24 (d, 1H, $^{\alpha}$NH Leu^9, $^3J_{\alpha NH}$ = 9.0 Hz), 8.13 (d, 1H, $^{\alpha}$NH Lys^4, $^3J_{\alpha NH}$ = 2.6 Hz), 7.69 (m, 2H, $^{\delta}$NH Orn^8), 7.68 (m, 2H, $^{\delta}$NH Orn^2, m, 2H, $^{\varepsilon}$NH Lys^4), 7.39 (d, 1H, $^{\alpha}$NH Val^7, $^3J_{\alpha NH}$ = 9.2 Hz), 7.34–7.10 (m, 5H, H_{ar} D-Phe^5, m, 5H, H_{ar} D-Phe^{10}), 7.24 (d, 1H, $^{\alpha}$NH Val^1, $^3J_{\alpha NH}$ = 8.8 Hz), 5.06 (m, 1H, $^{\alpha}$H Pro^6), 4.88 (m, 1H, $^{\alpha}$H Orn^2), 4.73 (m, 1H, $^{\alpha}$H Orn^8), 4.59 (m, 1H, $^{\alpha}$H Leu^9), 4.53 (m, 1H, $^{\alpha}$H Leu^3), 4.47 (m, 1H, $^{\alpha}$H Val^1), 4.38 (m, 1H, $^{\alpha}$H D-Phe^{10}), 4.32 (m, 1H, $^{\alpha}$H Val^7), 4.30 (m, 1H, $^{\alpha}$H Pro^{11}), 4.29 (m, 1H, $^{\alpha}$H D-Phe^5), 3.85 (m, 1H, $^{\alpha}$H Lys^4), 3.60 (m, 1H, $^{\delta}$H Pro^{11}), 3.41 (m, 2H, $^{\delta}$H Pro^6), 3.14 (m, 1H $^{\beta}$H D-Phe^5), 2.97 (m, 1H $^{\beta}$H D-Phe^{10}), 2.86 (m, 1H $^{\beta}$H D-Phe^{10}), 2.79 (m, 2H $^{\delta}$H Orn^8), 2.78 (m, 2H $^{\varepsilon}$H Lys^4), 2.70 (m, 2H $^{\delta}$H Orn^2), 2.68 (m, 1H $^{\beta}$H D-Phe^5), 2.50 (m, 1H, $^{\delta}$H Pro^{11}), 2.09 (m, 1H, $^{\beta}$H Val^1, m, 2H, $^{\beta}$H Pro^6), 1.96 (m, 1H $^{\beta}$H Pro^{11}), 1.82 (m, 1H, $^{\beta}$H Orn^8), 1.77 (m, 1H $^{\beta}$H Val^7, m, 1H $^{\beta}$H Orn^8), 1.69 (m, 2H, $^{\gamma}$H Pro^6), 1.59 (m, 1H, $^{\beta}$H Leu^3, m, 2H, $^{\gamma}$H Orn^8), 1.56

(m, 2H $^{\beta}$H Lys^{4}), 1.55 (m, 1H, $^{\beta}$H Leu^{3}), 1.52 (m, 1H, $^{\gamma}$H Leu^{3}), 1.50 (m, 1H, $^{\beta}$H Pro^{11}, m, 2H, $^{\gamma}$H Pro^{11}), 1.48 (m, 1H, $^{\beta}$H Orn^{2}, m, 1H, $^{\beta}$H Leu^{3}, m, 1H, $^{\gamma}$H Leu^{9}), 1.45 (m, 2H, $^{\gamma}$H Orn^{2}), 1.41 (m, 2H, $^{\gamma}$H Lys^{4}, m, 1H, $^{\beta}$H Leu^{9}), 1.30 (m, 2H, $^{\delta}$H Lys^{4}), 1.22 (m, 1H, $^{\beta}$H Leu^{9}), 0.80 (m, 6H $^{\gamma}$H Val^{1}, m, 6H $^{\delta}$H Leu^{3}, m, 6H $^{\gamma}$H Val^{7}, m, 6H $^{\delta}$H Leu^{9}).

***Cyclo*(-Val-Orn-Leu-Arg-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)·3HCl (5).** *Cyclo*[-Val-Orn(Z)-Leu-Arg(NO_2)-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-] was prepared in 85% yield from Boc-Leu-oxime resin (514 mg, 0.18 mmol) by using a similar method to that of **1**. The protected precursor of **5** (100 mg, 0.062 mmol) obtained was dissolved in methanol-1,4-dioxane (1 : 1) (2 ml), and then 1M HCl (0.1 ml) was added to the solution. The mixture was hydrogenated in the presence of 10% palladium carbon for 24 h. After removing the catalyst, the filtrate was concentrated *in vacuo*. The residues obtained were purified by gel filtration on a Sephadex LH-20 (1.5 × 120 cm) using methanol as the eluting solvent, followed by reprecipitation with methanol-ether to give **5**.

White powder. Yield 35 mg (0.025 mmol, 40% from the protected precursor of **5**). Mp. 241–243 °C. LR-FAB-MS (matrix: *m*-NBA) calcd for $C_{66}H_{105}N_{16}O_{11}$ [M+H]$^{+}$ = 1298, Found *m*/*z* 1298 ([M+H]$^{+}$, 17.5%), 1320 ([M+Na]$^{+}$, 1.4%). R_f[1] 0.68; R_f[2] 0.44. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.08 (d, 1H, $^{\alpha}$NH D-Phe^{10}, $^{3}J_{\alpha NH}$ = 2.7 Hz), 8.84 (d, 1H, $^{\alpha}$NH Orn^{8}, $^{3}J_{\alpha NH}$ = 8.3 Hz), 8.69 (d, 1H, $^{\alpha}$NH D-Phe^{5}, $^{3}J_{\alpha NH}$ = 6.0 Hz), 8.59 (d, 1H, $^{\delta}$NH Orn^{2}, $^{3}J_{\alpha NH}$ = 9.3 Hz), 8.51 (d, 1H, $^{\alpha}$NH Leu^{3}, $^{3}J_{\alpha NH}$ = 9.5 Hz), 8.29 (d, 1H, $^{\alpha}$NH Arg^{4}, $^{3}J_{\alpha NH}$ = 1.8 Hz), 8.25 (d, 1H, $^{\alpha}$NH Leu^{9}, $^{3}J_{\alpha NH}$ = 8.8 Hz), 8.04 (m, 2H, $^{\delta}$NH Orn^{2}), 7.95–7.90 (m, 1H, $^{\delta}$NH Arg^{4}, m, 3H, NH Arg^{4}, m, 2H, $^{\delta}$NH Orn^{8}), 7.46 (d, 1H, $^{\alpha}$NH Val^{7}, $^{3}J_{\alpha NH}$ = 8.3 Hz), 7.32–7.10 (m, 5H, H_{ar} D-Phe^{5}, m, 5H, H_{ar} D-Phe^{10}), 7.27 (d, 1H, $^{\alpha}$NH Val^{1}, $^{3}J_{\alpha NH}$ = 8.4 Hz), 5.11 (m, 1H, $^{\alpha}$H Pro^{6}), 4.88 (m, 1H, $^{\alpha}$H Orn^{2}), 4.73 (m, 1H, $^{\alpha}$H Orn^{8}), 4.59 (m, 1H, $^{\alpha}$H Leu^{9}), 4.56 (m, 1H, $^{\alpha}$H Leu^{3}), 4.47 (m, 1H, $^{\alpha}$H Val^{1}), 4.34 (m, 1H, $^{\alpha}$H D-Phe^{10}), 4.32 (m, 1H, $^{\alpha}$H Pro^{11}), 4.29 (m, 1H, $^{\alpha}$H D-Phe^{5}, m, 1H, $^{\alpha}$H Val^{7}), 3.90 (m, 1H, $^{\alpha}$H Arg^{4}), 3.59 (m, 1H, $^{\delta}$H Pro^{11}), 3.41 (m, 2H, $^{\delta}$H Pro^{6}), 3.15 (m, 2H, $^{\delta}$H Orn^{8}), 3.12 (m, 1H $^{\beta}$H D-Phe^{5}), 3.02 (m, 1H $^{\beta}$H D-Phe^{10}), 2.97 (m, 1H $^{\beta}$H D-Phe^{10}), 2.88 (m, 1H $^{\delta}$H Arg^{4}), 2.82 (m, 1H $^{\delta}$H Arg^{4}), 2.71 (m, 2H $^{\delta}$H Orn^{2}), 2.68 (m, 1H $^{\beta}$H D-Phe^{5}), 2.51 (m, 1H, $^{\delta}$H Pro^{11}), 2.13 (m, 2H, $^{\beta}$H Pro^{6}), 2.10 (m, 1H, $^{\beta}$H Val^{1}), 1.98 (m, 1H $^{\beta}$H Pro^{11}), 1.80 (m, 2H $^{\beta}$H Orn^{8}), 1.77 (m, 2H, $^{\gamma}$H Pro^{6}, m, 1H $^{\beta}$H Val^{7}), 1.68 (m, 1H, $^{\gamma}$H Arg^{4}), 1.66 (m, 1H, $^{\gamma}$H Arg^{4}), 1.63 (m, 2H, $^{\beta}$H Arg^{4}, m, 2H, $^{\gamma}$H Orn^{8}), 1.57 (m, 2H $^{\beta}$H Orn^{2}, m, 1H, $^{\gamma}$H Leu^{3}, m, 1H, $^{\gamma}$H Leu^{9}), 1.51 (m, 2H, $^{\beta}$H Leu^{3}, m, 2H, $^{\beta}$H Leu^{9}), 1.49 (m, 2H, $^{\gamma}$H Pro^{11}), 1.48 (m, 2H, $^{\gamma}$H Orn^{2}, m, 1H, $^{\beta}$H Pro^{11}), 0.84 (m, 6H $^{\gamma}$H Val^{7}), 0.81 (m, 6H $^{\gamma}$H Val^{1}, m, 6H $^{\delta}$H Leu^{3}, m, 6H $^{\delta}$H Leu^{9}).

CD spectra

The CD spectra were obtained by use of a JASCO spectra-polarimeter; model J-820 (JASCO LTD., Tokyo, Japan), using a 0.5-mm quartz cell at room temperature. The CD spectroscopy of **1–5** was carried out with a methanol solution at a concentration of 1.1–1.5 × 10^{-4} M.

NMR spectra

NMR spectra were measured in DMSO-d_6 at 30, 35, 40, 45 and 50 °C (peptide concentration *ca.* 2.5 and 15 mM) on a JEOL Ecp400 spectrometer (JEOL LTD., Tokyo, Japan) using standard pulse sequences and soft ware. COSY, TOCSY (HOHAHA), ROESY spectra with 1024 points in F2 and 256 points in F1 were recorded with a sweep width of 6000 Hz in the phase-sensitive mode using time-proportional phase incrementation. TOCSY and ROESY spectra were obtained with mixing times of 50 and 250 ms, respectively. Spin-lock fields for TOCSY and ROESY spectra were 6.9 and 3.2 kHz, respectively. A data was employed, and the radiation field was 3.2kHz.

Determination of antibiotic activity (MIC) of the cyclic peptides

Bacillus subtilis NBRC 3513, *Bacillus megaterium* ATCC 19213, *Staphylococcus aureus* NBRC 12732, *Escherichia coli* NBRC l2734 and *Pseudomonas aeruginosa* NBRC 3080 were grown overnight at 37 °C on nutrient agar medium and harvested in sterile saline. Densities of bacterial suspensions were determined at 600 nm, using a standard curve relating absorbance to number of colony forming units (CFU).

MICs of the synthetic peptides against several bacterial strains were assayed by the microplate dilution method as follows: 100 µl of serial dilution of the synthetic peptide was added to a mixture of 10 µl of bacterial suspension (approximately 10^{6} CFU/ml) and 90 µl of Mueller-Hinton broth (Difco Laboratories, NJ, USA) in each well of a flat-bottomed microplate (Corning Laboratory Sciences Company, NY, USA). The highest peptide concentration tested was 100 µg ml^{-1}. The plates were then incubated overnight at 37 °C for MIC evaluation. MIC was expressed as the lowest final concentration (µg/m1) at which no growth was observed. The experiments were performed four times for each peptide.

Determination of hemolytic activity (MHC) of the cyclic peptides

The hemolytic activity of the peptide was determined using sheep red blood cells (RBCs). Freshly collected sheep blood in Alsever's solution (preserved blood) was purchased from Nippon Bio-Test Laboratories Inc, and used as the source of the RBCs. The RBCs were isolated from preserved blood by centrifugation at 3000 rpm for 5 min at 4 °C and then washed three times with HEPES-NaOH buffered saline (HBS; 150 mM NaCl/5 mM HEPES-NaOH pH 7.4) just prior to the assay. The cyclic peptides were dissolved in water or DMSO to produce 1 mM cyclic peptide stock solution and stored –20 °C. Then, each concentration of cyclic peptide was prepared by dilution of the cyclic peptide stock solution in HBS.

To each micro tube added 200 µL of erythrocytes (1% hematocrit in HBS) and 100 µL of each concentration of cyclic peptide. The micro tubes were incubated at 37 °C for 30 min and centrifuged for 5 min at 3000 rpm and 4 °C. The supernatant of each micro tube transferred to a polystyrene microtiter plate, and the absorbance was measured at 540 nm by a microtiter plate reader. HBS (no peptide) and triton X-100 (added 2 µL of 10% triton X-100) were used as references. The hemolytic activity was calculated as follows:{Abs peptide–Abs HBS}/{Abs triton X-100–Abs HBS}×100. The experiments were carried out three times for each peptide.

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