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NOTE

# Design and syntheses of gramicidin S analogs, cyclo(-X-Leu-X-D-Phe-Pro-)$_2$ (X=His, Lys, Orn, Dab and Dap)

Makoto Tamaki[1], Kenta Fujinuma[1], Takuji Harada[1], Kazumasa Takanashi[1], Mitsuno Shindo[2], Masahiro Kimura[2] and Yoshiki Uchida[2]

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Gramicidin S (GS), cyclo(-Val$^{1,1'}$-Orn$^{2,2'}$-Leu$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-)$_2$,[1–3] is a potent cyclopeptide antibiotic isolated from *Bacillus brevis*. Its secondary structure has been established as an antiparallel β-sheet conformation with amphiphilicity.[4,5] The conformation is characteristically featured with the orientation of side chains in such a way that the charged Orn side chains are situated on one side of the molecule and the hydrophobic Val and Leu side chains are situated on the other side. The side-chain arrangement is apparently held together by a rigid conformation containing two D-Phe-Pro type II' β-turns. The antiparallel β-sheet conformation with amphiphilicity of GS has been considered to be essential for exhibiting its strong activity.[2,3] Recently, we found that [Orn$^{1,1',3,3'}$]-GS and [Lys$^{1,1',3,3'}$]-GS, which have GS-like antiparallel β-sheet conformation without amphiphilicity, possess 1/4 and 1/8 activities of GS against *Bacillus subtilis* NBRC 3513 and *Bacillus megaterium* ATCC 19213, respectively, but no activities against Gram-negative microorganisms and human blood cells.[6] The results suggested that the amphiphilic structure of GS is not necessary for exhibiting the antibiotic activity against Gram-positive bacteria, but important for exhibiting the activities against Gram-negative bacteria and sheep blood cell. In order to investigate further the role of the β-sheet conformation with amphiphilicity of GS for the antibiotic activity, we synthesized novel GS analogs, cyclo(-X-Leu-X-D-Phe-Pro-)$_2$ (X=His (**1**), Lys (**2**), Orn (**3**),[7] Dab (**4**) and Dap (**5**)) (Figure 1) and examined their antibiotic and hemolytic activities. GS analogs **1–5** have a primary structure with amphiphilicity, in which hydrophilic amino acid residues replace hydrophobic Val and Leu residues of GS, and hydrophobic Leu residues replace hydrophilic Orn residues of GS.

In the syntheses of **1–5**, a protected linear precursor oxime, H-D-Phe-Pro-Y-Leu-Y-D-Phe-Pro-Y-Leu-Y-oxime on resin (Y=His(3-Bom), Lys(Z), Orn(Z), Dab(Z) and Dap(Z)) (Bom-=benzyloxymethyl-, Z-=benzyloxycarbonyl-), was prepared by using Boc (Boc=*t*-Boc-) solid phase peptide synthesis on oxime resin (loading of oxime group: 0.35 mmol g$^{-1}$ resins) (Scheme 1).[8] Y residue (Y=His(3-Bom), Lys(Z), Orn(Z), Dab(Z) and Dap(Z)) as a C-terminal amino acid residue was used based on the propensity of the biosynthetic precursor of GS to form a conformation highly favorable for head–tail cyclization.[2,3] The cyclization-cleavage of H-D-Phe-Pro-Y-Leu-Y-D-Phe-Pro-Y-Leu-Y-oxime from the resin was performed in 1,4-dioxane with 2 equiv of triethylamine and AcOH for 1 day at room temperature to give cyclo(-Y-Leu-Y-D-Phe-Pro-)$_2$. The removal of all the masking groups by 25% HBr in AcOH produced **1–5** (Scheme 1). Total yields of **1–5** from H-Leu-oxime resin are 34–56%. The purity and identity assessment of **1–5** were confirmed by TLC, HPLC, and FAB-MS.

The antibiotic activities and hemolytic activities of **1–5** and GS are summarized in Table 1. **1** with weak basic imidazole side chains showed very little activities against any bacteria or sheep blood cells. The results indicated that the presence of weak basic imidazole side chains is not effective for the interaction with both bacterial membrane and sheep cell membrane. On the other hand, **2–5** with basic amino side chains showed antibiotic activities. **2** with four ε-amino side chains and **3** with four δ-amino side chains showed low antibiotic activities against Gram-positive microorganisms. **2** showed no activities against all Gram-negative bacteria tested. **3** showed the same activities to that of GS against *Pseudomonas aeruginosa* NBRC 3080, but no activity against *Escherichia coli* NBRC 12734. On the other hand, **4** with four γ-amino side chains and **5** with four β-amino side chains showed antibiotic activities against both Gram-positive and Gram-negative bacteria. **4** showed 1/4 activity of GS against *Bacillus subtilis* NBRC 3513, *Bacillus megaterium* ATCC 19213 and *Staphylococcus aureus* NBRC 12732, and 1/8 activity of GS against *Staphylococcus epidermidis* NBRC 12933. In addition, the activities of **4** are two times higher than that of GS against *Pseudomonas aeruginosa* NBRC 3080 and *Escherichia coli* NBRC 12734. The antibiotic activities of **5**

[1]Department of Chemistry, Toho University, Funabashi, Japan and [2]Department of Health and Nutrition, Osaka Shoin Women's University, Higashi-Osaka, Japan
Correspondence: Professor Y Uchida, Department of Health and Nutrition, Osaka Shoin Women's University, Hishiyanishi 4-2-26, Higashi-Osaka, Osaka 577-8550, Japan.
E-mail: uchida.yoshiki@osaka-shoin.ac.jp
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are lower than that of **4**. Further, **1–5** showed much lower hemolytic potency (Table 1). The results indicated that the presences of four γ-amino side chains at 1, 1′, 3 and 3′ positions of **4** are effective for the interaction with the membrane of both Gram-positive and Gram-negative membrane, although not effective for the interaction with sheep cell membrane.

Next, CD spectra of **1–5** and GS were measured in methanol, to investigate the structure–activity relationship of **1–5** (Figure 2). In CD spectra of **1–4**, two troughs were observed near 206 and 220 nm, and its curves are similar to that of GS. However, the depths of two troughs at 206 and 220 nm of **1–4** are shallower than that of GS. From conformation studies of GS by using model compounds, [9,10] 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 suggested that the conformations of **1–4** and GS in methanol are similar to each other, but the conformations of **1–4** are more unstable than that of GS, as the depths of two troughs of **1–4** are shallower than that of GS. On the other hand, the two troughs of **4** were considerably deeper than those of **1–3**. It is interesting to note that among **1–4**, **4** possesses the most stable conformation and the highest antibiotic activity. On the other hand, in CD spectrum of **5**, two troughs were observed near 196 and 220 nm, suggesting that **5** adopts disordered conformation in comparison with those of **1–4**. Recently, Urakawa *et al*.[11] reported similar results in studies of cyclic heptapeptides related to polymyxin B.[11] That is, the replacement of Dab residues in cyclic heptapeptides of polymyxin B into Orn and Dap residues influences largely its conformation and antibiotic activity.

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

| | *MIC (μg ml$^{-1}$)* | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***A*** | ***B*** | ***C*** | ***D*** | ***E*** | ***F*** | ***G*** |
| **GS** | 3.13 | 3.13 | 3.13 | 3.13 | 25 | 25 | 100 |
| **1** | >50 | 50 | >50 | >50 | >50 | >50 | 2.5 |
| **2** | 50 | 25 | 50 | 50 | >100 | >100 | 4.5 |
| **3** | 25 | 25 | 100 | 25 | 25 | >100 | 4.1 |
| **4** | 12.5 | 12.5 | 25 | 12.5 | 12.5 | 12.5 | 4.8 |
| **5** | 25 | 12.5 | 50 | 25 | 50 | 50 | 0.4 |

Abbreviations: **A**, *Bacillus subtilis* NBRC 3513; **B**, *Bacillus megaterium* ATCC 19213; **C**, *Staphylococcus epidermidis* NBRC 12933; **D**, *Staphylococcus aureus* NBRC 12732; **E**, *Pseudomonas aeruginosa* NBRC 3080; **F**, *Escherichia coli* NBRC 12734; MIC, minimum inhibitory concentration.
[a]MIC value in μg ml$^{-1}$. MICs of the synthetic peptides against several bacterial strains were assayed by the microplate dilution method.
[b]**G** is hemolytic percentage of the peptides (40 μM) in buffer solution against sheep erythrocytes.

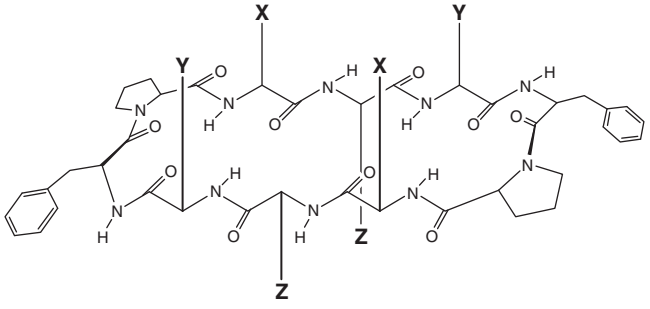



**Figure 1** Secondary structures of GS and **1–5**. GS: **X**=Val, **Y**=Leu, **Z**=Orn. **1**: **X**, **Y**=His, **Z**=Leu. **2**: **X**, **Y**=Lys, **Z**=Leu. **3**: **X**, **Y**=Orn, **Z**=Leu. **4**: **X**, **Y**=Dab, **Z**=Leu. **5**: **X**, **Y**=Dap, **Z**=Leu.

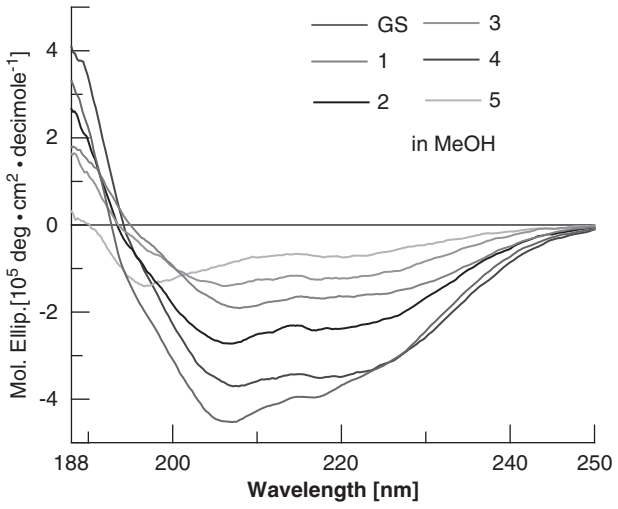
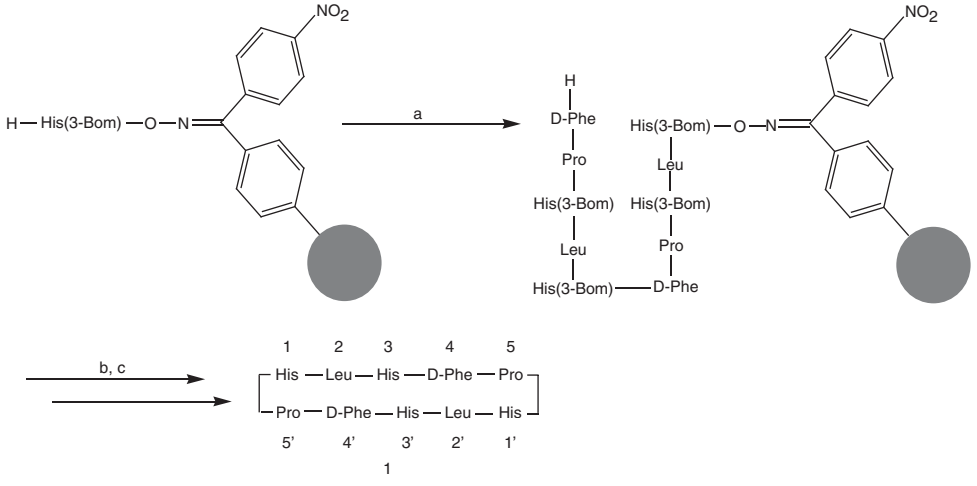



**Figure 2** CD spectra of **1–5** and Gramicidin S in methanol.




**Scheme 1** Synthesis of **1**. Other GS analogs **2–5** were synthesized by a similar method to that of **1**. Reagents and conditions; (a) Boc-amino acid (3 equiv), BOP (3 equiv), HOBt (3 equiv) and $NEt_3$ (6.5 equiv) in DMF for 90 min. Deprotection by 25% TFA/DCM for 30 min; (b) $NEt_3$ (2 equiv) and AcOH (2 equiv) in 1,4-dioxane for 24 h; (c) 25% HBr/AcOH.

In conclusion, we have found that among GS analogs **1**–**5**, **4**, cyclo (-Dab-Leu-Dab-D-Phe- Pro-)$_2$, has a stable GS-like β-sheet conformation and shows high antibiotic activities against all bacteria tested, and very low hemolytic potency against sheep blood cell. The results suggested that the presence of four γ-amino side chains at 1, 1′, 3 and 3′ positions of **4** is effective for the interaction with the membrane of both Gram-positive and Gram-negative bacteria, although not effective for the interaction with sheep cell membrane. 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 syntheses of other antimicrobially active analogs of GS with various basic amino acids on the basis of the present studies to find new types of drug candidates with high antimicrobial and low hemolytic activities.

## EXPERIMENTAL PROCEDURE

Melting points were measured on Mel-Temp II m.p. apparatus (Laboratory Devices, Cambridge, 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-MS on a JEOL600H mass spectrometer. Analytical TLC was carried out on Merck silica-gel $F_{254}$ plates with the following solvent systems (v/v): $R_f^1$, n-BuOH:AcOH:$H_2O$ (4:1:2); $R_f^2$, n-BuOH:pyridine:AcOH:$H_2O$ (4:1:1:2).

### *CYCLO*(-HIS-LEU-HIS-D-PHE-PRO-)$_2$ · 4HBR (1)

Preparation of H-[-D-Phe-Pro-His(3-Bom)-Leu-His(3-Bom)-]$_2$-oxime on resin was performed by using Boc-solid phase peptide synthesis from H-Leu-oxime resin (514 mg, 0.18 mmol). The formation of the cyclic peptide by the cyclization-cleavage of H-[-D-Phe-Pro-His(3-Bom)-Leu- His(3-Bom)-]$_2$-oxime on resin was performed in 1,4-dioxane with 2 equiv of triethylamine and AcOH for 1 day at room temperature. The removal of all masking groups of cyclo[-D-Phe-Pro-His(3-Bom)-Leu-His(3-Bom)-]$_2$ by 25% HBr in AcOH for 5 day yield **1**.

White powder, yield 97 mg (34%), m.p. 227–230 °C.

LR-FAB-MS (matrix: thioglycerin) calcd for $C_{64}H_{82}N_{18}O_{10}$: $[M]^+$=1263, found m/z. 1263 ($[M]^+$, 57.2%), 1264, ($[M+H]^+$, 43.5%). $R_f^1$ 0.54, $R_f^2$ 0.38.

**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 (Z-) of the protected precursor of **2**–**5** were removed by 25% HBr in AcOH for 1 day.

### *CYCLO*(-LYS-LEU-LYS-D-PHE-PRO-)$_2$4HBR (2)

White powder, yield 156 mg (56%), m.p. 254–257 °C.

LR-FAB-MS (matrix: thioglycerin) calcd for $C_{64}H_{102}N_{14}O_{10}$: $[M]^+$=1227, found m/z 1227 ($[M]^+$, 52.2%), 1228 ($[M+H]^+$, 39.2%). $R_f^1$=0.54, $R_f^2$=0.19.

### *CYCLO*(-ORN-LEU-ORN-D-PHE-PRO-)$_2$4HBR (3)

White powder, yield 134 mg (50%), m.p. 240–243 °C.

LR-FAB-MS (matrix: thioglycerin) calcd for $C_{60}H_{94}N_{14}O_{10}$: $[M]^+$=1171, found m/z. 1171 ($[M]^+$, 100%), 1172, ($[M+H]^+$, 98.1%). $R_f^1$=0.41, $R_f^2$=0.30.

### *CYCLO*(-DAB-LEU-DAB-D-PHE-PRO-)$_2$4HBR (4)

White powder, yield 111 mg (43%), m.p. 259–260 °C.

LR-FAB-MS (matrix: thioglycerin) calcd for $C_{56}H_{86}N_{14}O_{10}$: $[M]^+$=1115, found m/z 1115 ($[M]^+$, 20.8%), 1116, ($[M+H]^+$, 16.0%). $R_f^1$=0.97,$R_f^2$=0.54.

### *CYCLO*(-DAP-LEU-DAP-D-PHE-PRO-)$_2$4HBR (5)

White solid, yield 94 mg (38%), m.p. 251–252°C.

LR-FAB-MS (matrix: thioglycerin) calcd for $C_{52}H_{78}N_{14}O_{10}$: $[M]^+$=1059, found m/z 1059 ($[M]^+$, 100%), 1060, ($[M+H]^+$, 100 %). $R_f^1$=0.75,$R_f^2$=0.44.

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1 Gause, G. F. & Brazhnikova, M. G. Gramicidin S and its use in the treatment of infected wounds. *Nature* **154,** 703 (1944).
2 Izumiya, N., Kato, T., Aoyagi, H., Waki, M. & Kondo, M. *Synthetic Aspects of Biologically Active Cyclic Peptide-Gramicidin S and Tyrocidines*, Kodansha Tokyo and Halsted press, New York, 1979.
3 Waki, M. & Izumiya, N. *Biochemistry of Peptide Antibiotics* eds Kleinkauf, H. and von Dohren, H., de Gruyter, Berlin, Fed Rep, Ger. (1990) pp. 205–244.
4 Hull, S. E., Karlson, R., Main, P., Woolfson, M. M. & Dodson, E. J. The crystal structure of a hydrated gramicidin S-urea complex. *Nature (London)* **275,** 206–207 (1978).
5 Yamada, K. *et al.* Stereochemistry of protected ornithine side chains of gramicidin S derivatives: X-ray crystal structure of the bis-Boc-tetra-N-methyl derivative of gramicidin S. *J. Am. Chem. Soc* **124,** 12684–12688 (2002).
6 Tamaki, M. *et al.* Gramicidin S analogues having six basic amino acid residues. *J. Antibiotics* **62,** 597–599 (2009).
7 Ando, S., Nishikawa, H., Takaguchi, H. & Izumiya, N. Syntheses of cyclic decapeptides with four ornithyl residues related to Gramicidin S. *Bull. Chem. Soc. Jpn* **59,** 1201–1206 (1986).
8 Tamaki, M., Honda, K., Kikuchi, S. & Ishii, R. Biomimetic formation of gramicidin S by dimerization-cyclization pentapeptide precursor on solid support. *Tetrahedron Lett.* **47,** 8475–8478 (2006).
9 Lee, S. *et al.* Conformations and CD curves of cyclo(L- or D-Phe-L-Pro-Aca): cyclized models for specific types of β-bends. *FEBS Lett.* **174,** 310–313 (1984).
10 Greenfield, N. & Fasman, G. D. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8,** 4108–4116 (1969).
11 Urakawa, H. *et al.* Structure-activity relationships of bacterial outer-membrane permeabilizers based on polymyxin B heptapeptides. *Bioorg. Med. Chem. Lett.* **20,** 1771–1775 (2010).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)