The Journal of Antibiotics (2009) 62, 597–599

www.nature.com/ja

NOTE

Gramicidin S analogs having six basic amino acid residues

Makoto Tamaki[1], Ichiro Sasaki[1], Yuki Nakao[1], Mitsuno Shindo[2], Masahiro Kimura[2] and Yoshiki Uchida[2]

The Journal of Antibiotics (2009) **62,** 597–599; doi:10.1038/ja.2009.81; published online 28 August 2009

Keywords: gramicidin S; polycation analog; antiparallel β-sheet conformation; structure–activity relationship

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. 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 amphiphilicity, which disrupts cell membrane.[6] Thus, the hydrophobic Val and Leu residues have been considered to be essential for exhibiting the strong activity of GS. Therefore, syntheses of antimicrobially active analogs of GS containing amino acid residues with hydrophilic side chain in place of Val and Leu residues have not been reported yet.[2,3,7–9]

In this account, we designed and synthesized novel GS analogs, cyclo(-Orn$^{1,1'}$-Orn$^{2,2'}$-Orn$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-)$_2$ (**1**) and cyclo (-Lys$^{1,1'}$-Orn$^{2,2'}$-Lys$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-)$_2$ (**2**), which have four basic amino acid residues (Orn and Lys residues) in place of four hydrophobic amino acid residues (Val and Leu residues), to investigate the role of amphiphilic β-sheet structure of GS for the antibiotic activity.

Syntheses of **1** and **2** were performed by a solid-phase method using oxime resin.[10] The formation of the cyclic peptides by the dimerization–cyclization of H-D-Phe-Pro-X-Orn(Z)-X-oxime on resin (X=Orn(Z), and Lys(Z)) (Z-=benzyloxycarbonyl-) gave cyclo(-X$^{1,1'}$-Orn(Z)$^{2,2'}$-X$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-)$_2$ in 83 and 92% yields, respectively. The removal of all the masking groups by 25% HBr/AcOH produced the corresponding antibiotics **1** and **2** in 53 and 67% yields, respectively. The homogeneities of **1** and **2** were confirmed by thin-layer chromatography, high performance liquid chromatography, FAB-MS and ^{1}H NMR spectrometry.

To investigate the secondary structures of **1** and **2**, CD and ^{1}H NMR spectra of **1** and **2** were measured. Compounds **1**, **2** and GS showed almost identical CD spectra in methanol (Figure 1), suggesting that **1** and **2** have a β-sheet structure similar to that of GS.

The ^{1}H NMR (400 MHz) spectra of **1** and **2** were measured at 30 °C in DMSO-*d*6 (peptide concentration: ~17 mg ml^{-1}). All protons were assigned by means of H–H COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy) and ROESY (rotating frame nuclear Overhauser enhancement spectroscopy). Only one $^{\alpha}$NH resonance appeared for X$^{1,1'}$, Orn$^{2,2'}$, X$^{3,3'}$ and D-Phe$^{4,4'}$ residues (**1**: X=Orn, **2**: X=Lys), indicating that **1** and **2** have conformations with C_2 symmetry in the NMR time average. In the ^{1}H NMR spectrum of **1**, temperature coefficient values of $^{\alpha}$NH groups for Orn$^{1,1'}$, Orn$^{2,2'}$, Orn$^{3,3'}$ and D-Phe$^{4,4'}$ were 2.8, 6.3, 2.8 and 5.7 ppb K^{-1}, respectively. These results indicated that Orn$^{1,1'}$ $^{\alpha}$NH and Orn$^{3,3'}$ $^{\alpha}$NH are shielded from the solvent and involved in two stable intramolecular hydrogen bonds, whereas Orn$^{2,2'}$ $^{\alpha}$NH and D-Phe$^{4,4'}$ $^{\alpha}$NH are exposed to the solvent. The $J_{NH\text{-}^{\alpha}CH}$ values of Orn$^{1,1'}$, Orn$^{2,2'}$, Orn$^{3,3'}$ and D-Phe$^{4,4'}$ residues were 8.1, 8.8, 7.6 and 2.0 Hz, respectively. The $J_{NH\text{-}^{\alpha}CH}$ values observed for Orn$^{1,1'}$, Orn$^{2,2'}$ and Orn$^{3,3'}$ residues were strongly indicative of an extended β-sheet conformation.[11] On the other hand, $J_{NH\text{-}^{\alpha}CH}$ value of D-Phe residue was indicative of a β-turn conformation.[11] The chemical shift perturbation[12] ($\Delta\delta H_{\alpha}$=observed δH_{α}−random coil δH_{α}) of the $^{\alpha}$H of Orn$^{1,1'}$, Orn$^{2,2'}$ and Orn$^{3,3'}$ showed positive values (>0.1 p.p.m.). On the other hand, the D-Phe$^{4,4'}$ and Pro$^{5,5'}$ residues showed negative values. The chemical shift perturbation of the $^{\alpha}$H of **1** agreed well with those of GS.[13] The results suggested that Orn$^{1,1'}$-Orn$^{2,2'}$-Orn$^{3,3'}$ sequences in **1** have a similar β-sheet conformation to that of GS sequences. Next, for detailed analysis, the spatial ROE correlations were measured. (Figure 2) ROE spatial correlations between Pro$^{5,5'}$ $^{\alpha}$CH and Orn$^{1,1'}$ $^{\alpha}$NH, Orn$^{1,1'}$ $^{\alpha}$CH and Orn$^{2,2'}$ $^{\alpha}$NH, Orn$^{2,2'}$ $^{\alpha}$CH and Orn$^{3,3'}$ $^{\alpha}$NH, Orn$^{3,3'}$ $^{\alpha}$CH and D-Phe$^{4,4'}$ $^{\alpha}$NH, and D-Phe$^{4,4'}$ $^{\alpha}$CH and Pro$^{5,5'}$ $^{\delta}$CH$_2$ were observed. The results indicated that amide bonds in **1** are

[1]Department of Chemistry, Toho University, Funabashi, Chiba, Japan and [2]Department of Food Science and Nutrition, Osaka Shoin Women's University, Higashi-Osaka, Osaka, Japan
Correspondence: Dr M Tamaki, Department of Chemistry, Toho University, Miyama 2-2-1, Funabashi, Chiba 274-8510, Japan.
E-mail: tamaki@chem.sci.toho-u.ac.jp
Received 7 April 2009; revised 10 July 2009; accepted 13 July 2009; published online 28 August 2009

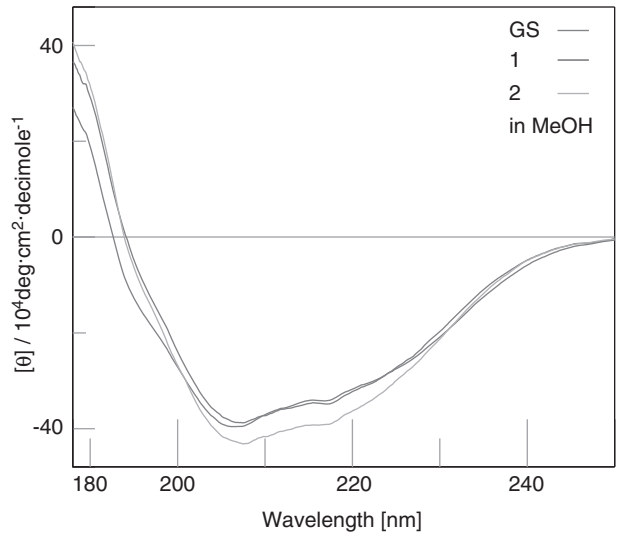

Figure 1 CD spectra of **1**, **2** and GS in methanol.

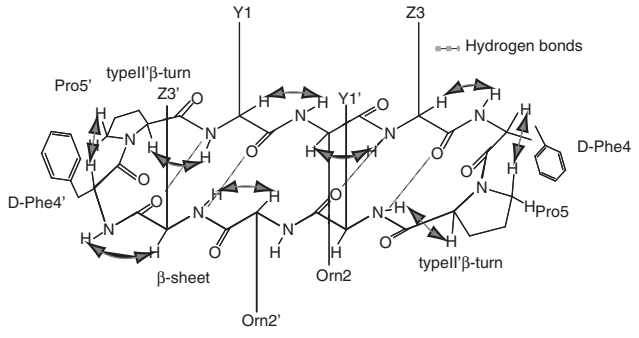

Figure 2 Proposed secondary structures of **1** ($Y^{1,1'}$ and $Z^{3,3'}$=-$(CH_2)_3NH_2$), **2** ($Y^{1,1'}$ and $Z^{3,3'}$=-$(CH_2)_4NH_2$) and GS ($Y^{1,1'}$=-$CH(CH_3)_2$, $Z^{3,3'}$=-$CH_2CH(NH_3)_2$) with ROE spatial correlations.

all-*trans* conformation. The signal of D-Phe $^{\beta}CH_2$ was two multiplets, indicating that they are nonequivalent and fixed in certain arrangement. The chemical shifts of H resonances for the diastereotopic β-, γ- and δ-CH_2 of Pro residues were separated by 0.29, 0.00 and 0.83 p.p.m., respectively, suggesting that the aromatic ring of D-Phe residue orients in close proximity to Pro $^{\delta}CH$. Similar results were obtained from the NMR studies of **2**. The NMR data indicated that **1** and **2** have GS-like antiparallel β-sheet structures with a type II′ β-turn around D-Phe-Pro as shown in Figure 2, and that the hydrophobic side chains of Val and Leu residues are not necessary for holding the rigid β-sheet conformation of GS.

The antibiotic activities and hemolytic activities of **1**, **2** and GS were summarized in Table 1. The difference of antibiotic activities among **1**, **2** and GS reflects the characters of side chains of the amino acid residues at positions 1, 1′, 3 and 3′ because the antibiotics have similar β-sheet structure to each other. The antibiotic activities of **1** and **2** were 1/4 and 1/8 of GS against *Bacillus subtilis* NBRC 3513 and *Bacillus megaterium* ATCC 19213, respectively, and less against *Staphylococcus epidermidis* NBRC 12933 and *Staphylococcus aureus* NBRC 12732. On the other hand, **1** and **2** showed no activity against Gram-negative microorganisms tested. The results indicated that the presence of hydrophobic side chains of Val and Leu residues are important for exhibiting the strong activity of GS. In addition, it is interesting to note that **1** and **2** showed some selectivity against

Table 1 Antibiotic activities[a] and hemolytic activity[b] of 1, 2 and GS

	MIC μg ml^{-1}						*(%)*
Peptides	*A*	*B*	*C*	*D*	*E*	*F*	*G*
GS	3.13	3.13	3.13	3.13	25	25	100
1	12.5	12.5	100	50	>100	>100	0.84
2	25	25	100	100	>100	>100	1.49

[a]MIC value in μg ml^{-1}. 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.
[b]G is hemolytic percentage of the peptides (50 μM) in buffer solution against sheep erythrocytes.

different microorganisms. Then, **1** and **2** showed almost no hemolytic activity toward sheep red blood cells (Table 1),[14] indicating that the replacement of the Val and Leu hydrophobic side chains into the Orn and Lys basic side chains could result in substantial reduction of hemolytic activities.

In these studies, we reported the structure–activity relationship of GS analogs **1** and **2** containing Orn and Lys residues with basic side chains in place of four hydrophobic Val and Leu residues. Currently, we are investigating the design and syntheses of other antimicrobially active analogs of GS without the hydrophobic Val and Leu residues on one side of the molecule on the basis of these studies in order to find new types of drug candidates with high antimicrobial and low hemolytic activities.

EXPERIMENTAL SECTION

Melting points were measured on Mel-Temp II melting point apparatus (Laboratory Devices, Cambridge, MA, USA) and are uncorrected. Low-resolution mass spectra (LR-MS) were obtained by using FAB mass spectrometry on a JEOL600H mass spectrometer (Jeol, Tokyo, Japan). CD spectra were recorded on a Jasco J-820 spectropolarimeter (Jasco, Tokyo, Japan) using a quarts cell of 0.5-mm pathlength. The CD spectra in methanol were measured at a peptide concentration of 1.10×10^{-4} M at room temperature. ^{1}H NMR spectra were measured in DMSO-d_6 at 30 °C (peptide concentration ca. 17 mg ml^{-1}) on a JEOL JNM-ECP400 spectrometer (Jeol) using standard pulse sequences and software. The chemical shifts were determined with respect to internal TMS (tetramethylsilane).

cyclo(-Orn-Orn-Orn-D-Phe-Pro-)$_2$ 6HBr (1)

A protected linear precursor oxime, H-D-Phe-Pro-Orn(Z)-Orn(Z)-Orn(Z)-oxime, was prepared by using Boc-solid phase peptide synthesis on resin (Loading of oxime group: 0. 35 mmol g^{-1} resins). The formation of the cyclic peptide by the dimerization–cyclization of H-D-Phe-Pro-Orn(Z)-Orn(Z)-Orn(Z)-oxime on resin was performed in 1,4-dioxane with two equiv. of triethylamine and acetic acid for 1 day at room temperature.[10] The cyclizations gave cyclo(-Orn(Z)-Orn(Z)-Orn(Z)-D-Phe-Pro-)$_2$ in yield of 83%. The removal of all the masking groups was performed by 25% HBr/acetic acid. The product was purified by gel filtration on a Sephadex LH-20 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and by reprecipitation from methanol–ether to give a white powder of **1** in 53% yield.

Mp 234–235 °C. LR-FAB-MS (matrix: *m*-NBA (*m*-nitro benzyl alcohol)) Calcd for $C_{58}H_{92}N_{16}O_{10}$ [M]$^+$=1173, Found *m/z* 1174 ([M+H]$^+$, 0.90%), 1196 ([M+Na]$^+$, 0.32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.30 (d, 2H, NH_{α} D-Phe$^{4,4'}$, $^3J_{NH\text{-}H\alpha}$=2.0 Hz), 8.75 (d, 2H, NH_{α} Orn$^{2,2'}$, $^3J_{NH\text{-}H\alpha}$=8.8 Hz), 8.16 (d, 2H, NH_{α} Orn$^{3,3'}$, $^3J_{NH\text{-}H\alpha}$=7.6 Hz), 7.78 (m, 4H, NH_{δ} Orn$^{1,1'}$, m, 4H, NH_{δ} Orn$^{3,3'}$), 7.69 (m, 4H, NH_{δ} Orn$^{2,2'}$), 7.33–7.27 (m, 10H, H_{ar} D-Phe$^{4,4'}$), 7.31 (d, 2H, NH_{α} Orn$^{1,1'}$, $^3J_{NH\text{-}H\alpha}$=8.1 Hz), 4.82 (m, 2H, H_{α} Orn$^{2,2'}$), 4.55 (m, 2H, H_{α} Orn$^{1,1'}$), 4.49 (m, 2H, H_{α} Orn$^{3,3'}$), 4.43 (m, 2H, H_{α} D-Phe$^{4,4'}$), 4.35 (m, 2H, H_{α} Pro$^{5,5'}$), 3.52 (m, 2H, H_{δ} Pro$^{5,5'}$), 3.00 (m, 2H H_{β} D-Phe$^{4,4'}$), 2.87 (m, 2H H_{β} D-Phe$^{4,4'}$), 2.80 (m, 4H, H_{δ} Orn$^{1,1'}$, m, 4H, H_{δ} Orn$^{2,2'}$, m, 4H, H_{δ} Orn$^{3,3'}$), 2.69 (m, 2H, H_{δ} Pro$^{5,5'}$), 1.97 (m, 2H, H_{β} Pro$^{5,5'}$), 1.74 (m, 2H, H_{β} Orn$^{1,1'}$), 1.70 (m, 2H, H_{β} Orn$^{2,2'}$), 1.69 (m, 2H, H_{β} Orn$^{1,1'}$), 1.61 (m, 2H, H_{β} Orn$^{2,2'}$, m, 2H, H_{β} Orn$^{3,3'}$, m, 2H, H_{β} Pro$^{5,5'}$, m, 4H, H_{γ} Pro$^{5,5'}$), 1.47 (m, 2H, H_{β} Orn$^{3,3'}$, m, 4H, H_{γ} Orn$^{3,3'}$), 1.45 (m, 4H, H_{γ} Orn$^{1,1'}$, m, 4H, H_{γ} Orn$^{2,2'}$).

cyclo(-Lys-Orn-Lys-D-Phe-Pro-)$_2$ 6HBr (2)

cyclo(-Lys(Z)-Orn(Z)-Lys(Z)-D-Phe-Pro-)$_2$ was synthesized in 92% yield as has been described for the preparation of cyclo(-Orn(Z)-Orn(Z)-Orn(Z)-D-Phe-Pro-)$_2$. The removal of all the masking groups by 25% HBr/acetic acid produced **2** in 67% yield.

Mp. 231.5–233.0 °C. LR-FAB-MS (matrix: *m*-NBA) Calcd for $C_{62}H_{100}N_{16}O_{10}$ $[M]^+$=1229, Found *m/z* 1230 ($[M+H]^+$, 1.01%), 1252 ($[M+Na]^+$, 0.33%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.22 (d, 2H, NH_α D-Phe$^{4,4'}$, $^3J_{NH\text{-}H\alpha}$=1.6 Hz), 8.69 (d, 2H, NH_α Orn$^{2,2'}$, $^3J_{NH\text{-}H\alpha}$=7.8 Hz), 8.16 (d, 2H, NH_α Lys$^{3,3'}$, $^3J_{NH\text{-}H\alpha}$=8.1 Hz), 7.84 (m, 4H, NH_ε Lys$^{1,1'}$, m, 4H, NH_δ Orn$^{2,2'}$, m, 4H, NH_ε Lys$^{3,3'}$), 7.32–7.27 (m, 10H, H_{ar} D-Phe$^{4,4'}$), 7.28 (d, 2H, NH_α Lys$^{1,1'}$, $^3J_{NH\text{-}H\alpha}$=8.3 Hz), 4.79 (m, 2H, H_α Orn$^{2,2'}$), 4.50 (m, 2H, H_α Lys$^{1,1'}$), 4.47 (m, 2H, H_α Lys$^{3,3'}$), 4.41 (m, 2H, H_α D-Phe$^{4,4'}$), 4.32 (m, 2H, H_α Pro$^{5,5'}$), 3.59 (m, 2H, H_δ Pro$^{5,5'}$), 3.00 (m, 2H H_β D-Phe$^{4,4'}$), 2.89 (m, 2H H_β D-Phe$^{4,4'}$), 2.83 (m, 4H, H_δ Orn$^{2,2'}$), 2.74 (m, 4H, H_ε Lys$^{3,3'}$), 2.68 (m, 4H, H_ε Lys$^{1,1'}$), 2.52 (m, 2H, H_δ Pro$^{5,5'}$), 1.98 (m, 2H, H_β Pro$^{5,5'}$), 1.74 (m, 2H, H_β Orn$^{2,2'}$), 1.71 (m, 4H, H_β Lys$^{1,1'}$), 1.69 (m, 2H, H_β Orn$^{2,2'}$), 1.57 (m, 4H, H_γ Lys$^{1,1'}$, m, 4H, H_γ Orn$^{2,2'}$, m, 4H, H_β Lys$^{3,3'}$, m, 2H, H_β Pro$^{5,5'}$, m, 4H, H_γ Pro$^{5,5'}$), 1.33 (m, 4H, H_γ Lys$^{3,3'}$), 1.27 (m, 4H, H_δ Lys$^{1,1'}$), 1.19 (m, 4H, H_δ Lys$^{3,3'}$).

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. & von Dohren, H.) *Fed Rep, Ger* 205–244 (de Gruyter, Berlin, 1990).

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* **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 gramicidine S. *J. Am. Chem. Soc.* **124,** 12684–12688 (2002).

6 Katsu, T. *et al.* Structure-activity relationship of gramicidin S analogues on membrance permeability. *Biochim. Biophys. Acta.* **899,** 159–170 (1987).

7 Arai, T. *et al.* Synthesis of [Hexafluorovalyl$^{1,1'}$]gramicidin S. *Bull. Chem. Soc. Jpn.* **69,** 1383–1389 (1996).

8 Mihara, H. *et al.* A pair of pyrene groups as a conformational probe for antiparallel β-sheet structure formed in cyclic peptides. *J. Chem. Soc., Perkin Trans.* **2,** 517–522 (1997).

9 Solanas, C. *et al.* Therapeutic Index of gramicidin S is strongly modulated by D-phenylalanine analogues at the β-turn. *J. Med. Chem.* **52,** 664–674 (2009).

10 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).

11 Pardi, A., Billeter, M. & Wüthrich, K. Calibration of the angular dependence of the amide proton-C$^\alpha$proton coupling constants, ^{3}JHNα, in a globular protein. *J. Mol. Biol.* **180,** 741–751 (1984).

12 Wishart, D. S., Sykes, B. D. & Richards, F. M. The chemical shift index: a fast and simple method for assignment of protein secondary structures through NMR spectroscopy. *Biochemistry* **31,** 1647–1651 (1992).

13 Grotenbreg, G. M. *et al.* Synthesis and biological evalution of novel turn-modified gramicidine S analogues. *Bioorg. Med. Chem.* **11,** 2835–2841 (2003).

14 Tamaki, M. *et al.* Syntheses of low-hemolytic antimicrobial gratisin peptides. *Bioorg. Med. Chem. Lett.* **19,** 2856–2859 (2009).