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# A Novel Active Analogue of Gramicidin S with Smaller Ring Size

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**Abstract** A novel active gramicidin S analogue with smaller ring size, cyclo[-$\delta$-Orn(-Val-Pro-D-Phe-H)-Leu-]$_2$, was synthesized and examined the structure-activity relationship. Its analogue showed antibiotic activity against all Gram-positive microorganisms tested, and its activity was 1/2～1/8 of that of gramicidin S. The present results indicated that both structures of cyclo(-$\delta$-Orn-Leu-)$_2$ and H-D-Phe-Pro-Val sequence play the important role for showing the antibiotic activity.

**Keywords** gramicidin S analogue, smaller ring size, synthesis, antibiotic activity

Gramicidin S (GS), cyclo(-Val-Orn-Leu-D-Phe-Pro-)$_2$ [1～5], tyrocidine A (TA), cyclo(-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-) [2, 6, 7], and gratisin (GR), cyclo(-D-Phe-Pro-D-Tyr-Val-Orn-Leu-)$_2$ [5, 8～14], are potent cyclopeptide antibiotics[†] (Fig. 1). It has been proposed that their principal modes of antibiotic actions result from an interaction of these antibiotics with the cell membrane of the target microorganisms. They then adopt an antiparallel $\beta$-sheet conformation which results in disruption of its cell membrane [1～15]. In addition, no resistance has been found for the antibiotics, because it requires significant alteration of the lipid composition of the cell membrane [16]. In view of the fact that widespread antibiotic resistance has become a serious threat to public health [17], these amphiphilic antibiotics are attractive targets for a new drug discovery. In order to find drug candidates with high antimicrobial and low hemolytic activities, many analogues with various ring sizes have been synthesized [1～14]. However, their antibiotics' analogues with smaller ring sizes and antibiotic activity have not been found yet [1～14].

In studies of the biomimetic synthesis of GS, we cyclized Z-D-Phe-Pro-Val-Orn-Leu-ONSu[††], in order to examine the reactivity between the $\delta$-amino group of the Orn residue and the carboxyl group of the Leu residue [18]. In this cyclization, we isolated the two cyclic products from the reaction mixture by gel filtration using Sephadex LH-20, followed by recrystalization. These products were identified as the cyclic monomer and cyclic dimer on the basis of molecular weight, which was determined by fast-atom bombardment mass spectrometry. Recently, we found that cyclic dimer (peptide 1) isolated from the reaction mixture has antibiotic activity against the Gram-positive microorganisms tested (Fig. 1).

In the present studies, we wish to report the synthesis and the structure-activity relationship of the novel active GS analogue (**1**) with smaller ring size.

The syntheses of peptides **1** and **2** (Fig. 1) were performed by a conventional liquid phase method. Peptide **2** was synthesized in order to investigate the role of the cyclic part in peptide **1** for the antibiotic activity. Boc-$\delta$-

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[†] Amino acid residues with no prefix are of L-configuration. The abbreviations of amino acids and peptides are in accordance with the rules of IUPAC-IBU commission of Biological Nomenclature.

[††] Abbreviations used are as follows: Boc, *t*-butoxycarbonyl; Z, benzyloxy carbonyl; -OBzl, -benzoxy; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; -ONSu, *N*-hydroxysuccinimide ester; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid

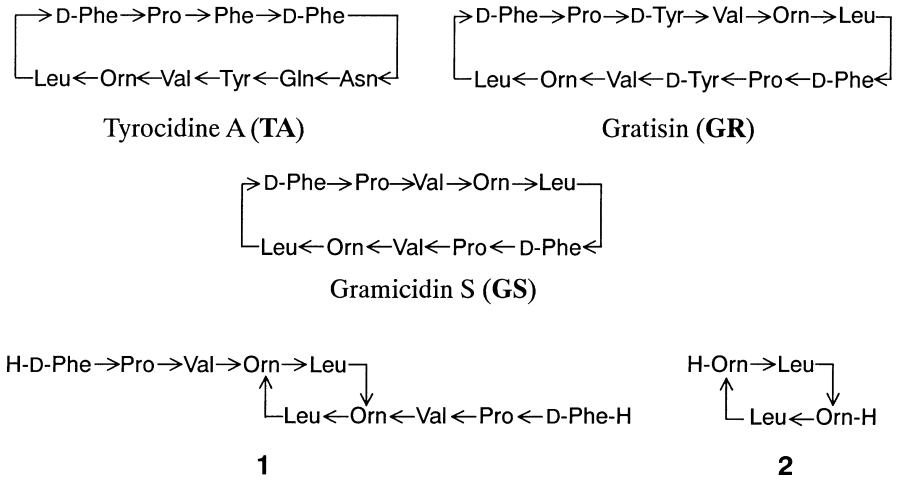


**Fig. 1** Primary structures of GS, TA, GR and its analogues **1** and **2** with smaller ring size.

**Table 1** Antibiotic activities of GS and its analogues **1** and **2**[a)]

| Test organisms | GS | **1** | **2** |
|---|---|---|---|
| *Staphylococcus aureus* FDA 209P JC-1 | 1.56 | 6.25 | >100 |
| *Staphylococcus aureus* MS353 CS6S. | 1.56 | 6.25 | >100 |
| *Staphylococcus aureus* MS15009 | 1.56 | 6.25 | >100 |
| *Staphylococcus epidermidis* ATCC 15305 | 3.13 | 25 | >100 |
| *Streptococcus pyogenes* N.Y.5 | 1.56 | 6.25 | >100 |
| *Enterococcus faecalis* ATCC29212 | 3.13 | 25 | >100 |
| *Enterococcus faecium* ATCC 19432 | 3.13 | 6.25 | >100 |
| *Bacillus subtilis* ATCC 6633 | 1.56 | 6.25 | >100 |
| *Escherichia coli* NIHJ-JC2 | >100 | >100 | >100 |
| *Klebsiella pneumoniae* NCTC9632 | >100 | >100 | >100 |
| *Pseudomonas aeruginosa* PA01 | >100 | >100 | >100 |

a) Minimum inhibitory concentration ($\mu$g/ml) was determined by an agar dilution method with $10^6$ organisms per milliliter.

Orn(Z)-Leu-$\delta$-Orn(Z)-Leu-OBzl was synthesized from Leu-OBzl by step-by-step elongation using EDCI and HOBt, and then saponified to give Boc-$\delta$-Orn(Z)-Leu-$\delta$-Orn(Z)-Leu-OH. The obtained Boc-tetrapeptide-OH was converted into the corresponding succinimide ester with EDCI and HONSu. The Boc group of Boc-$\delta$-Orn(Z)-Leu-$\delta$-Orn(Z)-Leu-ONSu was removed by the action of TFA, and then the succinimide ester was cyclized in pyridine (concentration of peptide in pyridine: $3\times10^{-3}$ M) at 25°C for 1 day, and gave cyclo(-$\delta$-Orn(Z)-Leu-)$_2$ in a yield of 71%. The protecting groups of $\alpha$-NH of the Orn residue were removed with 25% HBr in acetic acid, and cyclo(-$\delta$-Orn-Leu-)$_2\cdot$2HBr (**2**) was purified by reprecipitation from methanol-ether. Boc-D-Phe-Pro-Val-OH was synthesized from Val-OBzl in a similar manner as described regarding the synthesis of Boc-$\delta$-Orn(Z)-Leu-$\delta$-Orn(Z)-Leu-OH. Coupling of Boc-D-Phe-Pro-Val-OH with cyclo(-$\delta$-Orn-Leu-)$_2$ was performed by EDCI and HOBt to give the corresponding decapeptides. The removal of the Boc groups by the action of TFA yielded peptide **1**. The homogeneities of synthetic peptides **1** and **2** were confirmed by means of fast atom bombardment mass spectrometry, amino acid analysis, and high-performance liquid chromatography. In addition, the analytical data of peptide **1** [19] agreed with those of cyclic dimer obtained from the cyclization mixture of Z-D-Phe-Pro-Val-Orn-Leu-ONSu.

The antibiotic activities of GS, peptides **1** and **2** toward several organisms are summarized in Table 1. Peptide **1** showed antibiotic activity against all Gram-positive microorganisms tested, and its activity was 1/2～1/8 of that of GS. Peptide **1** may be the first example of an active GS analogue with a smaller ring size than that of GS [1～14]. On the other hand, peptide **2** showed no antibiotic activity.

In order to investigate the structure-activity relationship of peptide **1**, NMR spectra of peptides **1** and **2** were measured by 400 MHz $^1$H-NMR in DMSO-$d_6$ [20]. NMR data [20] indicated that peptide **2** has a rigid $C_2$ symmetric structure, and the amide protons of Leu residues are involved in a rigid intramolecular hydrogen bond. Further, the $J^{\alpha}$CH-NH value of Leu residues is 8.5 Hz. These results suggested strongly that peptide **2** adopts a $\beta$-sheet conformation stabilized by two intramolecular hydrogen bonds between the Leu residues in DMSO-$d_6$ [21～23]. In addition, NMR data [20] of peptide **1** indicated that cyclo(-$\delta$-Orn-Leu-)$_2$ (**2**) in peptide **1** holds a $\beta$-sheet conformation.

The present results indicated that both structures of cyclo(-$\delta$-Orn-Leu-)$_2$ and H-D-Phe-Pro-Val sequence play an important role for showing antibiotic activity.

Currently, we are investigating the design and synthesis of other active GS analogues having cyclo(-$\delta$-Orn-Leu-)$_2$ (**2**) as a scaffold in order to find new types of drug candidates with high antimicrobial and low hemolytic activities.

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