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A Novel, Antimicrobially Active Analog of Gramicidin S without Amphiphilic Conformation

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Abstract A novel gramicidin S analog, cyclo(-Val-Leu-Leu-Orn-Leu-D-Phe-Pro-)$_2$, was synthesized, its antibiotic activity compared with gramicidin S and shown to be as potent as gramicidin S when compared with the susceptibility toward five Gram-positive microorganisms. It exceeded the activity of gramicidin S against *Bacillus megaterium* ATCC 19213 by a factor of two. Circular dichroism and NMR data suggested this analog to adopt an antiparallel β-sheet conformation without amphiphilic character.

Keywords gramicidin S analog, a potent cyclotetradecapeptide antibiotic, antiparallel β-sheet conformation, no amphiphilic character, circular dichroism, NMR

Gramicidin S (GS), cyclo(-Val-Orn-Leu-D-Phe-Pro-)$_2$ [1~5], is a potent cyclopeptide antibiotic (Fig. 1). 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, which disrupts cell membrane [1~5]. So far, no resistance has been found for the antibiotic, because it requires significant alteration of the lipid composition of the cell membrane [6]. In view of widespread antibiotic resistance that has become a serious threat to public health [7], amphiphilic antibiotics are attractive targets for drug discovery. To find candidates with high antimicrobial and low hemolytic activities, many GS analogs of various ring sizes have been designed and synthesized [1~5].

In recent studies of the biomimetic synthesis of GS, we performed the cyclization of H-D-Phe-Pro-Val-Leu-Leu-Orn-Leu-oxime on resin, in order to examine the role of the length of linear precursor peptide [8]. Using Boc-solid phase peptide synthesis, a fully deprotected linear peptide oxime, H-D-Phe-Pro-Val-Leu-Leu-Orn-Leu-oxime was synthesized on resin (Loading of oxime group: 0.48 mmol/g resins). The formation of the cyclic peptide by the cyclization-cleavage of oxime on resin was performed in 1,4-dioxane with 2 equiv. of *N*,*N*-diisopropylethylamine and AcOH for 1 day at 25°C (concentration of peptide in solution; 3×10^{-3} M). The purification of cyclo(-Val-Leu-Leu-Orn-Leu-D-Phe-Pro-)$_2$ (**1**) (Fig. 1) from the cyclization mixtures were performed using preparative reverse phase HPLC, followed by recrystllization from methanol - ether. The analytical data for **1** were shown as follows: mp 291~294°C; $[\alpha]_D^{20}$ −213.06° (*c* 0.1, MeOH); LRMS(FAB), Calcd for $C_{84}H_{136}O_{14}N_{16}$: 1594 Found: 1594. In the HPLC studies of **1**, we found that **1** eluted slower from the reverse phase (ODS) column than GS, indicating that **1** has a higher effective hydrophobicity than GS. HPLC analyses of **1** and GS were performed using analytical reverse phase HPLC system, 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 mm particle size, Tosoh Co., Japan). Chromatographies were carried out by a linear gradient of 55~95% methanol/0.1%TFAaq over 60 minutes with a flow rate of 1 ml/minute at 30°C. From the studies of the HPLC

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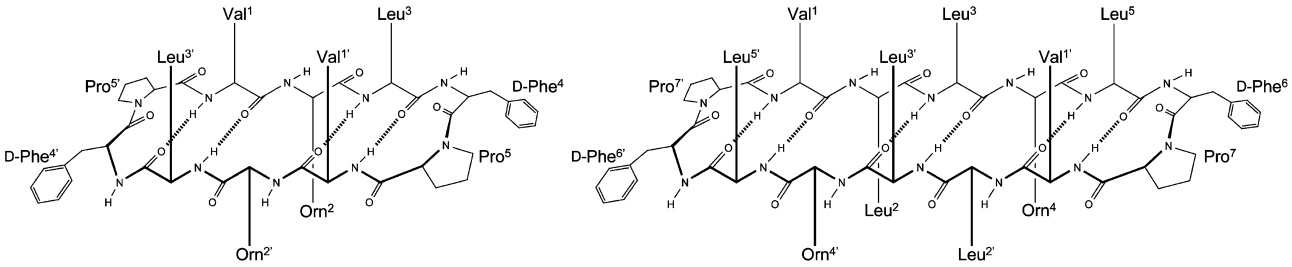

Fig. 1 Secondary structures of GS and its analog **1**.*

* Amino acid residues without prefix are of the L-configuration.

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

Test organisms	GS	**1**
Bacillus subtilis IFO 3513	3.1	6.3
B. megaterium ATCC 19213	3.1	1.6
Staphylococcus epidermidis IFO 12933	3.1	3.1
S. aureus IFO 12732	3.1	3.1
Enterococcus faecalis IFO 3989	6.3	13
Escherichia coli IFO 12734	25	>100
Pseudomonas aeruginosa IFO 3080	25	>100

[a)] Minimum inhibitory concentration (μg/ml) was determined by a microplate culture method with 10^6 organisms per milliliter.

separation of GS and its analogs, it was reported that the hydrophobicity plays a predominant role in the antibiotic activity, and that antibiotics with a stronger activity show a higher degree of effective hydrophobicity [9, 10]. The HPLC data of **1** suggested the possibility that **1** might elicit antibiotic activity.

In the present account, we report the antibacterial activity of cyclo(-Val-Leu-Leu-Orn-Leu-D-Phe-Pro-)$_2$ (**1**) and its secondary structure supported by circular dichroism (CD) and NMR spectra.

The antibiotic activities of **1** and GS are summarized in Table 1. **1** showed activity at the same level as GS against all Gram-positive microorganisms tested; its activity against *Bacillus megaterium* ATCC 19213 is two times higher than GS. On the other hand, **1** showed no activity against all Gram-negative microorganisms tested at the concentration of 100 μg/ml.

To investigate the structure-activity relationship of **1**, CD and NMR spectra of **1** were measured. The CD spectrum of **1** in ethanol was similar to that of GS, and the two troughs at 207 nm and 217 nm were rather deep in comparison with those of GS. Next, NMR spectra of **1** were measured by 400 MHz ^{1}H-NMR in DMSO-d_6. All protons were assigned by means of COSY and HOHAHA, and ROESY. **1** has C_2 symmetry in the NMR time average, because only one amide proton resonance appears for each residue. The values of temperature coefficient of NH groups for Val$^{1,1'}$, Leu$^{2,2'}$, Leu$^{3,3'}$, Orn$^{4,4'}$, Leu$^{5,5'}$ and D-Phe$^{6,6'}$ residues are 0.2, 5.0, 2.6, 5.2, 2.8 and 6.9 ppb/K, respectively. The $J_{\text{NH-}\alpha\text{CH}}$ values of Val$^{1,1'}$, Leu$^{2,2'}$, Leu$^{3,3'}$, Orn$^{4,4'}$, Leu$^{5,5'}$ and D-Phe$^{6,6'}$ residues are 9.2, 9.2, 9.2, 8.8, 8.8 and 2.0 Hz, respectively [11～13]. The CD [1～5] and NMR [11～13] data of **1** suggested that **1** adopts a β-sheet conformation similar to that of GS with Leu and Orn side chains on one side of the molecular as shown in Fig. 1.

In the antiparallel β-sheet conformation of GS, two hydrophilic Orn side chains are on one side of the molecule and four hydrophobic Leu and Val side chains are on the opposite side. It has been proposed that this specific side chain arrangement, in the form of an amphiphlic β-structure, is necessary for antibiotic activity [1～5]. On the other hand, **1** has the antiparallel β-sheet conformation, that excludes amphiphilicity (Fig. 1). Thus, this antibiotic is the first example of GS analog without amphiphilic β-structure and shows the activity at the same level as that of GS.

Currently, we are investigating the design and synthesis of other active GS analogs that have the antiparallel β-sheet conformation without amphiphilicity.

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