Syntheses and Antibacterial Activities of Gramicidin S Analogs Containing L-Ornithine in Place of L-Valine

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A gramicidin S analog ([Orn\(^{1,1}\)]GS·4HCl) containing L-ornithine in place of L-valine at the 1,1′ positions was synthesized by the conventional solution method in order to examine whether this analog had antibacterial activity toward Gram-negative bacteria. In the synthesis of [Orn\(^{1,1}\)]GS·4HCl, two intermediate analogs ([Orn\(^{1,1}\), Orn(For)\(^{2,2}\)]GS·2HCl and [Orn(Z)\(^{1,1}\)]GS·2HCl) were obtained. [Orn\(^{1,1}\)]GS·4HCl and [Orn\(^{1,1}\), Orn(For)\(^{2,2}\)]GS·2HCl showed no activity toward either Gram-negative or Gram-positive bacteria, whereas [Orn(Z)\(^{1,1}\)]GS·2HCl showed appreciable activity toward only Gram-positive bacteria.

Gramicidin S (GS) is a cyclic decapeptide, cyclo(-Val-Orn-Leu-d-Phe-Pro-Val-Orn-Leu-d-Phe-Pro-), which is antibiotic toward Gram-positive bacteria, but inactive to Gram-negative ones. After the early synthesis of a GS analog [L-Lys\(^{2,2}\)]GS·2HCl by Schwzyer and Sieber,\(^1\) a large number of analogs have been synthesized to study the relationships between the structure and activity.\(^2\) Nishino \textit{et al.} recently found that [L-Amy\(^{3,3}\)]GS·2HCl was inactive due to the bulky side chains of the L-Amy residues.\(^3\) Izumiya \textit{et al.} has reported that [d-Dpr\(^{4,4}\)]GS·4HCl showed appreciable antibacterial activity toward several Gram-negative bacteria, suggesting that the degree of activity would be influenced by the positive charges of the molecule.\(^4\)

In this paper, we report the synthesis and antibacterial activity of [Orn\(^{1,1}\)]GS·4HCl (14) and its intermediates, [Orn(Z)\(^{1,1}\)]GS·2HCl (12) and [Orn\(^{1,1}\), Orn(For)\(^{2,2}\)]GS·2HCl (13) as shown in Fig. 1.

Firstly, a linear pentapeptide (5) was synthesized, in which the δ-amino groups of two L-Orn residues were protected by Z and For, respectively (Fig. 2).

Boc-5-OH (5) was transformed to Boc-5-ONSu (6) and H-5-OH (7), and these pentapeptides were then coupled to produce Boc-10-OH (8). Compound 8 was converted to H-10-ONSu·CF\(_3\)COOH (10) and then treated with pyridine at a high dilution. The reaction product was purified by column chromatography, using ion-exchange resins and Sephadex LH-20, the desired cyclic decapetide (11) being obtained in a 24% yield (Fig. 3).

Hydrolysis of 11 with an HC1 solution in MeOH gave crystalline [Orn(Z)\(^{1,1}\)]GS·2HCl (12) in an 84% yield. Hydrogenolysis of 11 produced crystalline [Orn\(^{1,1}\), Orn(For)\(^{2,2}\)]GS·2HCl (13) in an 81% yield. The important analog [Orn\(^{1,1}\)]GS·4HCl (14) was obtained as colorless crystals in a 75% yield. The purity of each of these analogs was ascertained by TLC, amino acid analysis, and paper

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Fig. 1. Structures and Synthetic Scheme for the GS Analogs.

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Additional abbreviations: Amy, 2-amino-5-methylimidazole; Boc, tert-butoxycarbonyl; CD, circular dichroism; DCHA, dicyclohexylamine; Dpr, 2,3-diaminopropionic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; For, formyl; GS, gramicidin S; HONSu, N-hydroxysuccinimide; Orn, l-ornithine; Z, benzoyloxycarbonyl.
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Fig. 2. Synthesis of Boc-pentapeptide-OH 5.

Fig. 3. Synthesis of cyclo(-protected decapeptide-) 11.

Fig. 4. Paper Electrophoresis of GS and Its Analogs. The conditions are described in the Experimental section.

Table  Antibacterial Activity of GS and Its Analogs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Minimum Inhibitory concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. subtilis*</td>
</tr>
<tr>
<td>GS·2HCl</td>
<td>6.25</td>
</tr>
<tr>
<td>[Orn(Z)1,1']GS·2HCl (12)</td>
<td>12.5</td>
</tr>
<tr>
<td>[Orn1,1', Orn(For)2,2']-GS·2HCl (13)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>[Orn(Z)1,1']GS·4HCl (14)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>[Gly1,1']GS·2HCl'</td>
<td>100</td>
</tr>
<tr>
<td>[d-Dpr4,4']GS·4HCl'</td>
<td>50</td>
</tr>
</tbody>
</table>

* Gram-positive bacteria.
* Gram-negative bacteria.
* From ref. 5.
* From ref. 4.

electrophoresis. Analogs 12 and 13 showed almost the same mobility as GS·2HCl, whereas the mobility of 14 was greater than that of GS (Fig. 4).

The CD spectrum of 12 was similar to that of GS and showed double-minima at ca. 205 and 215 nm, although the ellipticity of 12 was lower than that of GS (Fig. 5). The CD spectra of 13 and 14 showed no distinct ellipticity at 215 nm. The molecular shape of 14 may be different from that of GS due to the presence of two cationic charges at the 1- and 1'-positions.

Compound 12 showed appreciable antibacterial activity toward Gram-positive bacteria as GS did (Table). It should be noted that [Gly1,1']GS was inactive.5 These
results indicate that the bulkiness of the side chain of 1,1'-t-Orn(β-Z) residues may be important for displaying antibacterial activity. Compound 14 showed no activity against Gram-negative bacteria nor against positive ones. Assuming that 14 and GS had a similar conformation as shown in Fig. 6, the four alkyl side chains of t-Val and t-Leu may form the hydrophobic face of GS, and this face would be changed to a hydrophilic face by the two 1,1'-t-Orn residues in 14.

**Experimental**

TLC was carried out on silica gel G (Merck) with the following solvent systems (vol. %): R: 1 CHCl₃, MeOH (5:1); R: 2 CHCl₃, MeOH: AcOH (50:10:2); R: 3 n-BuOH: AcOH: pyridine: H₂O (4:1:1:2). Optical rotation values were measured with a Horiha SEPA-200 polarimeter, while amino acid analyses were performed with a Hitachi KLA-5 analyzer. Paper electrophoreses were carried out on Toyo Roshi No. 51 paper with HCOOH: AcOH: MeOH: H₂O (1:3:6:10, pH 1.9) for 3h at 600V. CD measurements were taken with a Jasco J-40A spectropolarimeter, using a cuvette with a 0.01 cm light-path width.

**H-Leu-t-Phe-OH·HCl.** A solution of H-Leu-t-Phe-OH·DCHA (22.4 g, 40 mmol) in EtOAc was treated with 10% aqueous citric acid, and the organic layer was washed with water and dried with Na₂SO₄. The filtrate was evaporated in vacuo, and the remaining oil was dissolved in 3.5 M HCl in dioxane (120 ml). The solution was left to stand at room temperature for 2h and then evaporated. The residue that had solidified during standing was collected with the help of ether: yield, 9.3 g (74%); mp 185-188°C; R: 2 0.71. H-Leu-t-Phe-OH·2H₂O has been synthesized by hydrogenating Z-Leu-t-Phe-OH.⁶

**Boc-Orn(For)Leu-t-Phe-OH (1).** H-Leu-t-Phe-OH·HCl (7.9 g, 25 mmol) was dissolved in a mixture of H₂O (50 ml) and NEt₄ (7 ml, 50 mmol). A solution of Boc-Orn(For)ONsu (9.8 g, 27.5 mmol) in dioxane (50 ml) was added to this mixture at 5-10°C, and the resulting mixture was stirred overnight at room temperature. After the reaction mixture was evaporated, 10% citric acid was added to the residue. This mixture was extracted with EtOAc, and the organic layer was washed with sat. NaCl and then dried (Na₂SO₄). The resulting filtrate was evaporated, and the solid was collected with ether: yield, 9.1 g (70%); mp 141-143°C; [α]D²⁰ = -40.6° (c 1.0, dioxane); R² 0.57, R³ 0.77. Anal. Found: C, 59.66; H, 7.70; N, 10.64%. Calcd. for C₇₂H₁₃₁O₇₆N₅: C, 59.98; H, 7.74; N, 10.76%.

**H-Orn(For)-Leu-t-Phe-OH·HCl (2).** Compound 1 (7.8 g, 15 mmol) in 3.5 M HCl in dioxane (45 ml) was treated as described for the preparation of H-Leu-t-Phe-OH·HCl: yield of solid, 6.5 g (95%); mp 122-124°C; R³ 0.19. Anal. Found: C, 53.21; H, 7.37; N, 11.41%. Calcd. for C₁₁₂H₁₇₀O₁₈N₇·HCl·2H₂O: C, 53.11; H, 7.43; N, 11.79%.

**Boc-Pro-Zn-Orn(For)-Leu-t-Phe-OH (3).** Compound 2 (4.57 g, 10 mmol) was dissolved in a mixture of H₂O (100 ml) and NEt₄ (2.8 ml, 20 mmol). A solution of Boc-Pro-Zn-Orn(For)ONsu (5.1 g, 11 mmol) in dioxane (100 ml) was added to this mixture, which was then reseeded as described for 1. After evaporating, the solid was collected with ether, and recrystallized from MeOH and ether: yield, 5.46 g (71%); mp 152-156°C; [α]D²⁰ = -34.4° (c 1.0, EtOH); R² 0.71, R³ 0.75. Anal. Found: C, 61.06; H, 7.62; N, 10.81%. Calcd. for C₉₂H₁₅₀O₁₈N₅·C₆H₁₂O₇: C, 60.92; H, 7.43; N, 10.93%.

**H-Orn(For)-Zn-Orn(For)-Leu-t-Phe-OH·HCl (4).** Compound 3 (3.85 g, 5 mmol) was dissolved in 3.5 M HCl in dioxane (14 ml), and the solid was collected with ether as described for H-Leu-t-Phe-OH·HCl: yield, 3.32 g (88%); mp 227-229°C (dec); R² 0.25, R³ 0.63. Anal. Found: C, 55.43; H, 6.93; N, 11.42%. Calcd. for C₁₁₂H₁₇₀O₁₈N₇·2H₂O: C, 55.09; H, 7.22; N, 11.34%.

**Boc-Pro-Pro-Zn-Orn(For)-Leu-t-Phe-OH (5).** Compound 4 (2.93 g, 4 mmol) was dissolved in 50% aqueous dioxane (200 ml) with NEt₄ (1.12 ml, 8 mmol). A solution of Boc-Pro-ONsu (1.37 g, 4.4 mmol) in dioxane (20 ml) was then added, and the resulting mixture was stirred overnight at room temperature. The mixture was evaporated, the residue was triturated with 10% citric acid, and the solid was collected: yield, 2.85 g (81%); mp 182-185°C; [α]D²⁰ = -84.5° (c 0.5, EtOH); R² 0.85. Amino acid hydrolyzate of 5: Orn 1.92, Leu 1.03, Phe 1.00, Pro 0.88. Anal. Found: C, 59.69; H, 7.37; N, 11.04%. Calcd. for C₉₂H₁₅₀O₁₈N₅·H₂O·C₆H₁₂O₇: C, 59.79; H, 7.43; N, 11.10%.

**Boc-Pro-Pro-Zn-Orn(For)-Leu-t-Phe-OH·ONsu (6).** A solution of 5 (1.77 g, 2 mmol), EDC·HCl (0.767 g, 4 mmol) and HONsu (0.464 g, 4 mmol) in DMF (15 ml) was stirred at 0°C for 2h. After evaporating, the residue was triturated with cold water, collected, and dried in a desiccator: yield, 1.69 g (86%); R² 0.25.

**Boc-Pro-Pro-Zn-Orn(For)-Leu-t-Phe-OH·TFA (7).** A solution of 5 (1.77 g, 2 mmol) in CF₃COOH (30 ml) was left to stand at 0°C and then at room temperature for 30 min each. The mixture was evaporated, and the solid was dried in a desiccator: yield, 1.76 g (100%); R² 0.31.

**Boc-Pro-Zn-Orn(For)-Leu-t-Phe-OH·2HCl (8).** To a solution of 7 (1.94 g, 2.2 mmol) and NEt₄ (0.66 ml, 4.4 mmol) in DMF (20 ml) was added a solution of 6 (1.76 g, 2 mmol) in DMF (20 ml) at 0°C. The mixture was left to stand at 0°C for 1h and then at room temperature, and evaporated. The residue was triturated with 10% citric acid, and the solid was collected (3.13 g). This solid material was purified in a Sephadex LH-20 column (1.8 x 180 cm), MeOH being used as the eluent. The fractions containing 8 were evaporated, and the residue was recrystallized from MeOH·ether: yield, 1.79 g (52%); mp 147-151°C; [α]D²⁰ = -48.5° (c 1.0, DMF); R² 0.73. Anal. Found: C, 60.16; H, 7.25; N, 11.97%. Calcd. for C₇₂H₁₃₂O₁₇·2H₂O: C, 60.42; H, 7.35; N, 11.89%.

**Boc-Pro-Zn-Orn(For)-Leu-t-Phe-OH·ONsu (9).** A solution of 8 (3.43 g, 2.2 mmol), HONsu (0.46 g, 4 mmol) and EDC·HCl (0.77 g, 4 mmol) in DMF (20 ml) was treated in the same way as that described for 6: yield, 3.24 g (95%); R² 0.73.

**H-Orn(For)-Zn-Orn(For)-Leu-t-Phe-OH·TFA (10).** A solution of 9 (1.71 g, 1 mmol) in TFA (10 ml) was left to stand for 2h at 0°C, and then evaporated. The solid was collected by ether: yield, 1.66 g (96%); R² 0.42.
cyclot-Orn(Z)-Orn(For)-Leu-β-Phe-Pro-β1 (11). A solution of 10 (1.66 g, ca. 0.95 mmol) dissolved in DMF (20 ml) was added to pyridine (300 ml) at room temperature. The mixture was stirred for 2 h and then evaporated. The residue was dissolved in a mixture of MeOH and H₂O (5:1), and this solution was put into a column of Dowex 50 × 8 (1.8 × 10 cm), eluting with the same solvent. The eluate was treated in a column of Dowex 1 × 8 (1.8 × 10 cm) and evaporated, the residue then being dissolved in MeOH and purified by LH-20 as described for the purification of 8. Recrystallization from MeOH–ether afforded pure 11 in a 0.366 g (24%) yield; mp 132-134°C; [α]²⁰ DM = -215 (c 0.2, MeOH); R₃ 0.55. Amino acid hydrolyzate of 11: Orn 2.04, Leu 0.97, Phe 1.00, Pro 0.94. Anal. Found: C, 58.73; H, 7.35; N, 12.41%. Calcd. for C₁₈H₁₀₆O₁₅N₁₂ · 6H₂O: C, 58.41; H, 7.43; N, 12.23%.

Orn(Z)⁻¹⁻ GS: 2HCl (12). A solution of 11 (80 mg, 0.05 mmol) in 0.5 M HCl in MeOH (10 ml) was left to stand for 3 d at room temperature. The solution was then evaporated, and the residual crystals were collected with the help of a mixture of MeOH and ether (1:4): yield, 68 mg (84%); mp 162-165°C (dec.); R₅ 0.22, R₃ 0.78. Anal. Found: C, 57.56; H, 7.34; N, 12.31%. Calcd. for C₁₉H₁₀₈O₁₄N₁₄·2HCl·4H₂O: C, 57.60; H, 7.27; N, 12.38%.

Orn⁻¹⁻, Orn(For)⁻¹⁻ GS: 2HCl (13). Compound 11 (80 mg, 0.05 mmol) was dissolved in a mixture of MeOH (10 ml), AcOH (1 ml) and H₂O (1 ml), then hydrogenated on Pd black. The resulting filtrate was evaporated, and the crystalline residue was dried in a desiccator. The crystals were dissolved in MeOH (5 ml) containing HCl (0.1 mmol), and the solution was evaporated. Crystals of 13 were collected with a mixture of MeOH and ether (1:4): yield, 58 mg (81%); mp 167-170°C (dec.); R₅ 0.07, R₃ 0.31. Anal. Found: C, 54.09; H, 7.50; N, 14.09%. Calcd. for C₁₉H₁₀₈O₁₄·2HCl·4H₂O: C, 54.26; H, 7.51; N, 14.29%.

Orn⁻¹⁻ GS: 4HCl (14). Compound 11 (160 mg, 0.1 mmol) was dissolved in a mixture of MeOH·AcOH·H₂O (10:1:1), and then hydrogenated. The resulting filtrate was evaporated, and the residue was treated with 0.5 M HCl in MeOH (20 ml) as described for 12: yield, 112 mg (75%); mp 240-242°C (dec.); R₅ 0.04, R₃ 0.53. Anal. Found: C, 49.22; H, 7.53; N, 13.28%. Calcd. for C₁₉H₁₀₆O₁₅N₁₂·4HCl·8H₂O: C, 49.31; H, 7.60; N, 13.42%.

A small amount of 14 was also obtained by the route via 11 and 12.

Antibacterial activity. The minimum amounts of peptides necessary for completely inhibiting growth were determined by a dilution method, using a bouillon agar medium. The results are shown in the Table.

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References