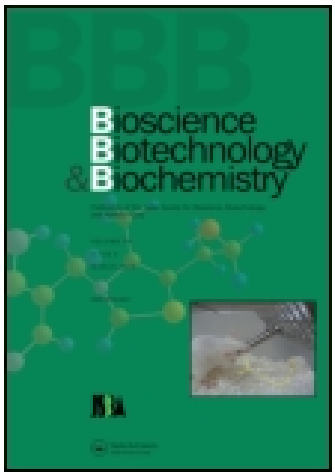

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Syntheses and Antibacterial Activities of Gramicidin S Analogs Containing L-Ornithine in Place of L-Valine

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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 Schwyzer and Sieber,[1] a large number of analogs have been synthesized to study the relationships between the structure and activity.[2] Nishino *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 *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_3COOH (**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 decapetpide (**11**) being obtained in a 24% yield (Fig. 3).

Hydrolysis of **11** with an HCl 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

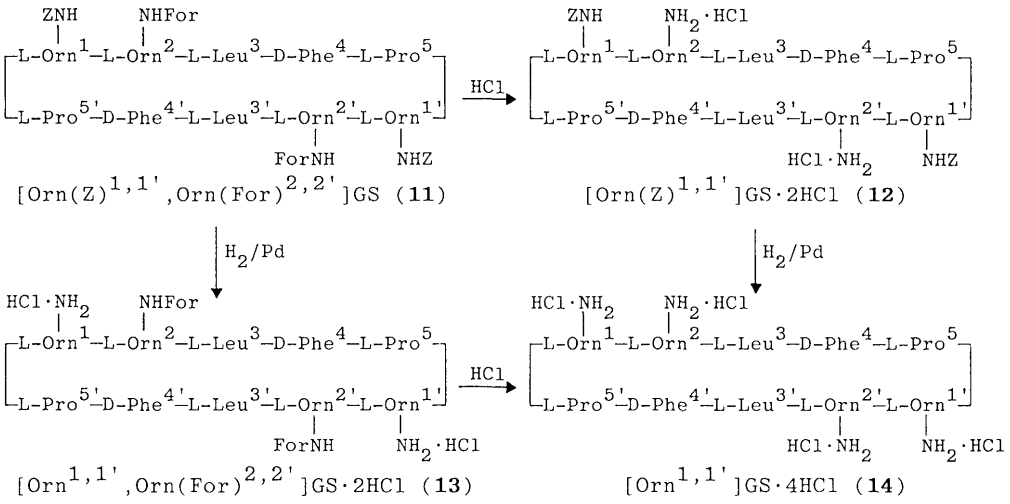

Fig. 1. Structures and Synthetic Scheme for the GS Analogs.

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Abbreviations according to IUPAC-IUB Commission, *Eur. J. Biochem.*, **138**, 9–37 (1984), are used throughout.

Additional abbreviations: Amy, α-aminomyristic acid; Boc, *tert*-butoxycarbonyl; CD, circular dichroism; DCHA, dicyclohexylamine; Dpr, 2,3-diaminopropanoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; For, formyl; GS, gramicidin S; HONSu, *N*-hydroxysuccinimide; Orn, L-ornithine; Z, benzyloxycarbonyl.

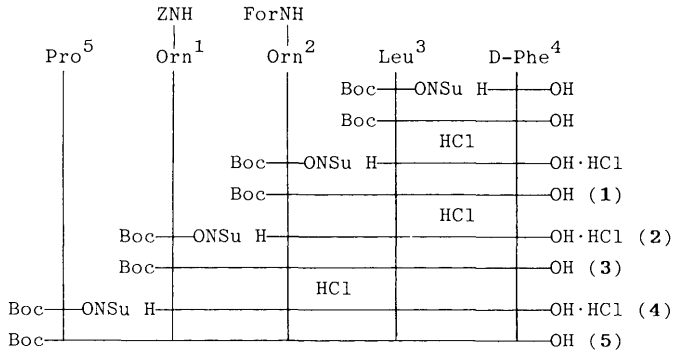

Fig. 2. Synthesis of Boc-pentapeptide-OH **5**.

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 [Gly$^{1,1'}$]GS was inactive.[5] These

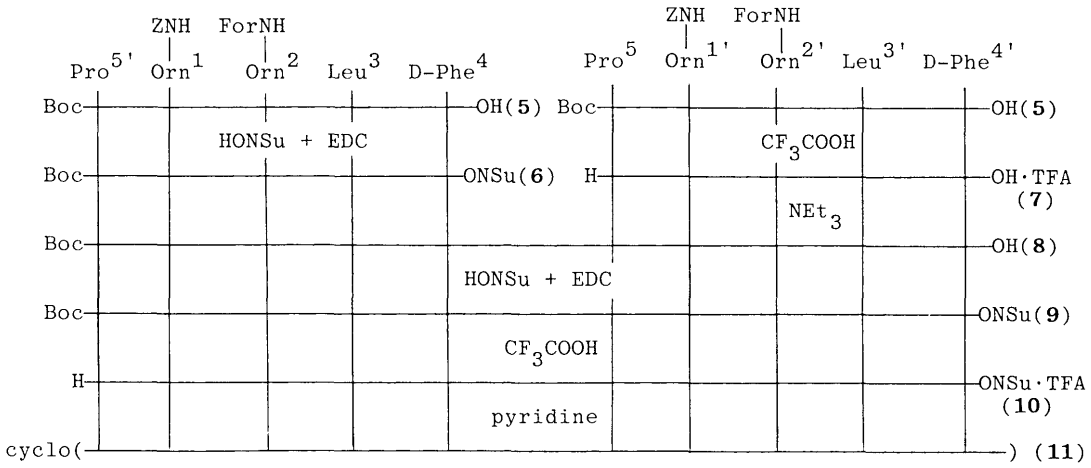

Fig. 3. Synthesis of cyclo(-protected decapeptide-) **11**.

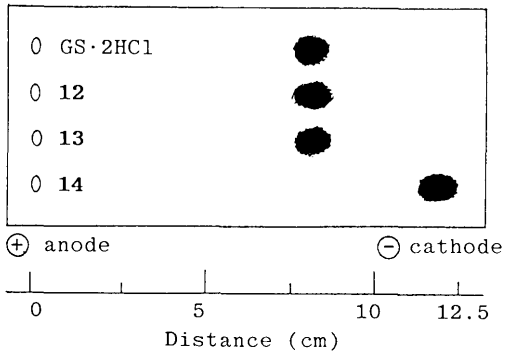

Fig. 4. Paper Electrophoresis of GS and Its Analogs.
The conditions are described in the Experimental section.

Table Antibacterial Activity of GS and Its Analogs

Peptide	Minimum Inhibitory concentration (μg/ml)		
	B. subtilis[a]	*S. aureus*[a]	*E. coli*[b]
GS·2HCl	6.25	12.5	>100
[Orn(Z)$^{1,1'}$]GS·2HCl (**12**)	12.5	25	>100
[Orn$^{1,1'}$, Orn(For)$^{2,2'}$]-GS·2HCl (**13**)	>100	>100	>100
[Orn$^{1,1'}$]GS·4HCl (**14**)	>100	>100	>100
[Gly$^{1,1'}$]GS·2HCl[c]	100	>100	>100
[D-Dpr$^{4,4'}$]GS·4HCl[d]	50	100	25

[a] Gram-positive bacteria.
[b] Gram-negative bacteria.
[c] From ref. 5.
[d] From ref. 4.

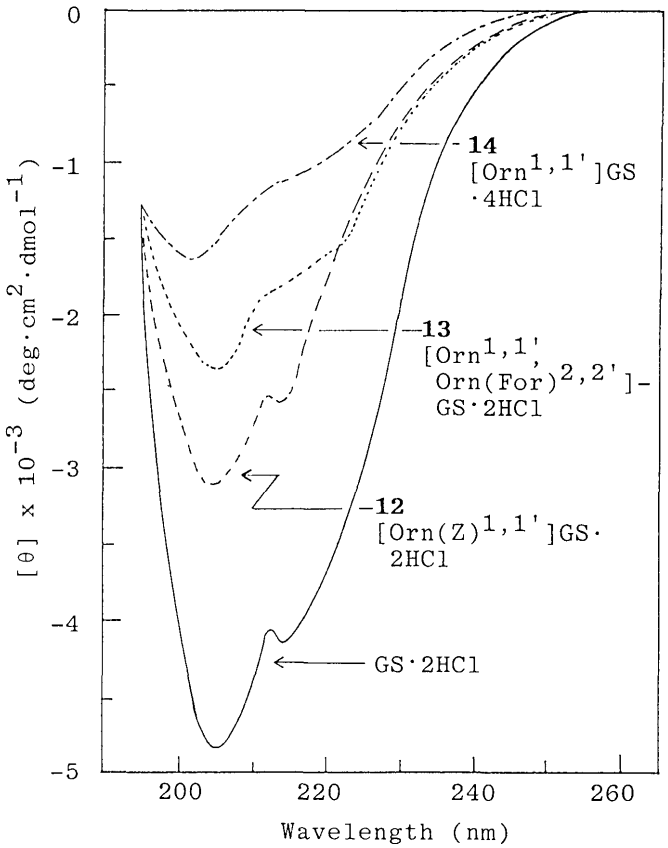

Fig. 5. CD Curves for GS and Its Analogs.
Methanol was used as the solvent.

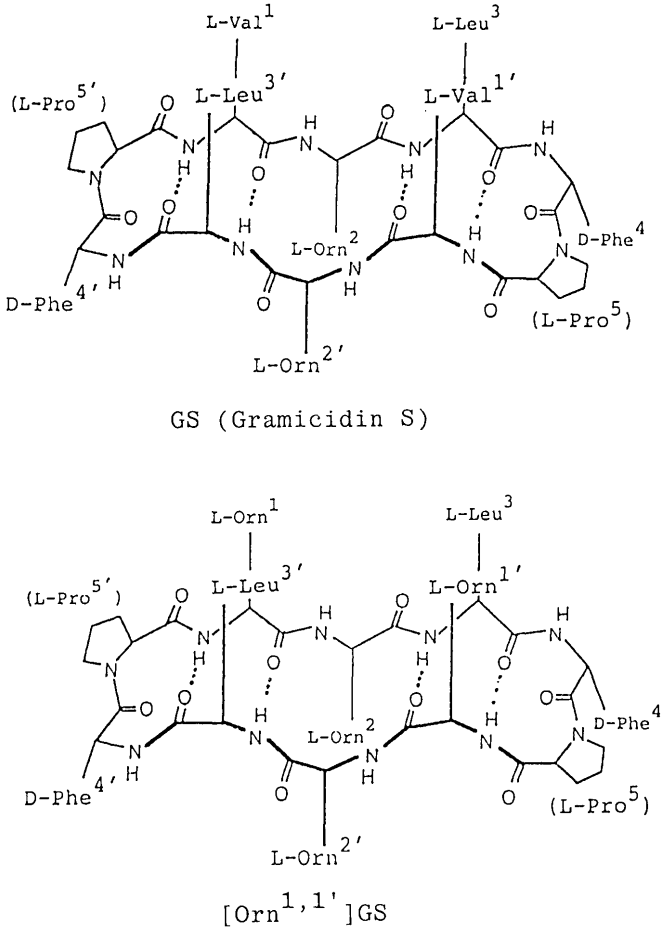

Fig. 6. Conformation of GS (top) and the Assumed Conformation of [Orn$^{1,1'}$]GS (bottom).

results indicate that the bulkiness of the side chain of 1,1'-L-Orn(δ-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 L-Val and L-Leu may form the hydrophobic face of GS, and this face would be changed to a hydrophilic face by the two 1,1'-L-Orn residues in **14**.

Experimental

TLC was carried out on silica gel G (Merck) with the following solvent systems (vol. %): R_f^1, $CHCl_3$–MeOH (5:1); R_f^2, $CHCl_3$–MeOH–AcOH (50:10:2); R_f^3, *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2). Optical rotation values were measured with a Horiba 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–H_2O (1:3:6:10, pH 1.9) for 3 h at 600 V. CD measurements were taken with a Jasco J-40A spectropolarimeter, using a cuvette with a 0.01 cm light-path width.

H-Leu-D-Phe-OH·HCl. A solution of Boc-Leu-D-Phe-OH·DCHA (22.4 g, 40 mmol)[6] in EtOAc was treated with 10% aqueous citric acid, and the organic layer was washed with water and dried with Na_2SO_4. The filtrate was evapoated *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 2 h 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_f^2 0.71. H-Leu-D-Phe-OH·$2H_2O$ has been synthesized by hydrogenating Z-Leu-D-Phe-OH.[7]

Boc-Orn(For)-Leu-D-Phe-OH (**1**). H-Leu-D-Phe-OH·HCl (7.9 g, 25 mmol) was dissolved in a mixture of H_2O (50 ml) and NEt_3 (7 ml, 50 mmol). A solution of Boc-Orn(For)-ONSu (9.8 g, 27.5 mmol)[8] in dioxane (50 ml) was added to this mixture at 5–10°C, and the resulting mixture was stirred overnight at room temperature. Afrer 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 (Na_2SO_4). The resulting filtrate was evaporated, and the solid was collected with ether: yield, 9.1 g (70%); mp 141–143°C; $[\alpha]_D^{20}$ −40.6° (*c* 1.0, dioxane); R_f^2 0.57, R_f^3 0.77. *Anal.* Found: C, 59.66; H, 7.70; N, 10.64%. Calcd. for $C_{26}H_{40}O_7N_4$: C, 59.98; H, 7.74; N, 10.76%.

H-Orn(For)-Leu-D-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-D-Phe-OH·HCl: yield of solid 2, 6.5 g (95%); mp 122–124°C; R_f^3 0.19. *Anal.* Found: C, 53.21; H, 7.37; N, 11.41%. Calcd. for $C_{21}H_{32}O_5N_4 \cdot HCl \cdot H_2O$: C, 53.11; H, 7.43; N, 11.79%.

Boc-Orn(Z)-Orn(For)-Leu-D-Phe-OH (**3**). Compound **2** (4.57 g, 10 mmol) was dissolved in a mixture of H_2O (100 ml) and NEt_3 (2.8 ml, 20 mmol). A solution of Boc-Orn(Z)-ONSu (5.1 g, 11 mmol)[9] in dioxane (100 ml) was added to this mixture, which was then reacted 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; $[\alpha]_D^{20}$ −34.4° (*c* 1.0, EtOH); R_f^2 0.71, R_f^3 0.75. *Anal.* Found: C, 61.06; H, 7.62; N, 10.81%. Calcd. for $C_{39}H_{56}O_{11}N_6$: C, 60.92; H, 7.43; N, 10.93%.

H-Orn(Z)-Orn(For)-Leu-D-Phe-OH·HCl (**4**). Compound **3** (3.85 g, 5 mmol) was dissolved in 3.5 M HCl/dioxane (14 ml), and the solid was collected with ether as described for H-Leu-D-Phe-OH·HCl: yield, 3.22 g (88%); mp 227–229°C (dec.); R_f^2 0.25, R_f^3 0.63. *Anal.* Found: C, 55.43; H, 6.93; N, 11.42%. Calcd. for $C_{34}H_{48}O_8N_6 \cdot HCl \cdot 2H_2O$: C, 55.09; H, 7.22; N, 11.34%.

Boc-Pro-Orn(Z)-Orn(For)-Leu-D-Phe-OH (**5**). Compound **4** (2.93 g, 4 mmol) was dissolved in 50% aqueous dioxane (200 ml) with NEt_3 (1.12 ml, 8 mmol). A solution of Boc-Pro-ONSu (1.37 g, 4.4 mmol)[10] 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; $[\alpha]_D^{20}$ −84.5° (*c* 0.5, EtOH); R_f^1 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 $C_{44}H_{63}O_{11}N_7 \cdot H_2O$: C, 59.79; H, 7.43; N, 11.10%.

Boc-Pro-Orn(Z)-Orn(For)-Leu-D-Phe-ONSu (**6**). A solution of **5** (1.77 g, 2 mmol), EDC·HCl (0.767 g, 4 mmol) and HONSu (0.46 g, 4 mmol) in DMF (15 ml) was stirred at 0°C for 2 d. After evaporating, the residue was triturated with cold water, collected, and dried in a desiccator: yield, 1.69 g (86%); R_f^2 0.25.

H-Pro-Orn(Z)-Orn(For)-Leu-D-Phe-OH·TFA (**7**). A solution of **5** (1.77 g, 2 mmol) in CF_3COOH (20 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%); R_f^2 0.31.

Boc-(Pro-Orn(Z)-Orn(For)-Leu-D-Phe)$_2$-OH (**8**). To a solution of **7** (1.94 g, 2.2 mmol) and NEt_3 (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 1 h and then overnight 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 solumn (1.8 × 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; $[\alpha]_D^{20}$ −48.5° (*c* 1.0, DMF); R_f^2 0.73. *Anal.* Found: C, 60.16; H, 7.25; N, 11.97%. Calcd. for $C_{83}H_{116}O_{19}N_{14} \cdot 2H_2O$: C, 60.42; H, 7.35; N, 11.89%.

Boc-(Pro-Orn(Z)-Orn(For)-Leu-D-Phe)$_2$-ONSu (**9**). A solution of **8** (3.43 g, 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%); R_f^2 0.73.

H-(Pro-Orn(Z)-Orn(For)-Leu-D-Phe)$_2$-ONSu·TFA (**10**). A solution of **9** (1.71 g, 1 mmol) in TFA (10 ml) was left to stand for 2 h at 0°C, and then evaporated. The solid was collected by ether: yield, 1.66 g (96%); R_f^2 0.42.

cyclo(-*Orn*(*Z*)-*Orn*(*For*)-*Leu*-D-*Phe*-*Pro*-)$_2$ (**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 d and then evaporated. The residue was dissolved in a mixture of MeOH and H_2O (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; $[\alpha]_D^{20}$ −215 (*c* 0.2, MeOH); R_f^1 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 $C_{78}H_{106}O_{16}N_{14}\cdot 6H_2O$: C, 58.41; H, 7.43; N, 12.23%.

[*Orn*(*Z*)$^{1,1'}$]*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.); R_f^1 0.22, R_f^3 0.78. *Anal.* Found: C, 57.56; H, 7.34; N, 12.31%. Calcd. for $C_{76}H_{106}O_{14}N_{14}\cdot 2HCl\cdot 4H_2O$: C, 57.60; H, 7.27; N, 12.38%.

[*Orn*$^{1,1'}$, *Orn*(*For*)$^{2,2'}$]*GS*·*2HCl* (**13**). Compound **11** (80 mg, 0.05 mmol) was dissolved in a mixture of MeOH (10 ml), AcOH (1 ml) and H_2O (1 ml), and 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.); R_f^1 0.07, R_f^3 0.71. *Anal.* Found: C, 54.09; H, 7.50; N, 14.09%. Calcd. for $C_{62}H_{94}O_{12}N_{14}\cdot 2HCl\cdot 4H_2O$: C, 54.26; H, 7.51; N, 14.29%.

[*Orn*$^{1,1'}$]*GS*·*4HCl* (**14**). Compound **11** (160 mg, 0.1 mmol) was dissolved in a mixture of MeOH–AcOH–H_2O (10 : 1 : 1), and then hydrogenated. The resulting filtrate was evapoated, 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.); R_f^1 0.04, R_f^3 0.53. *Anal.* Found: C, 49.22; H, 7.53; N, 13.28%. Calcd. for $C_{60}H_{94}O_{10}N_{14}\cdot 4HCl\cdot 8H_2O$: 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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