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Natural Homologs of Gramicidin S. II.[1] Synthesis of Gramicidin S-2 and S-3

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Two cyclic dodecapeptides corresponding to newly found members of gramicidin S family, gramicidin S-2: *cyclo*(-Val-Orn-Leu-D-Phe-Pro-Abu-Orn-Leu-D-Phe-Pro-), and gramicidin S-3: *cyclo*(-Abu-Orn-Leu-D-Phe-Pro-Abu-Orn-Leu-D-Phe-Pro-), were synthesized. In the synthesis, the linear decapeptide precursors of the antibiotics prepared by a combination of the "hold-in-solution" method and the usual liquid phase method were converted to cyclic ones in excellent yields by one-pot reaction using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 1-hydroxybenzotriazole. The synthesized antibiotics were identical with the natural products in HPLC analysis and assays of the antibiotic activities. The proposed structures for gramicidin S-2 and S-3 were confirmed. The CD spectra showed that the ring conformations of gramicidin S-2 and S-3 closely resemble each other and also that of gramicidin S-1, which has the already known structure.

In studies of gramicidin S, a peptide antibiotic produced by *Bacillus brevis* strain ATCC 9999 or the Nagano strain, it is known that replacement of the constituent amino acids in the antibiotic by other ones can occur because of the rather broad specificities of gramicidin S synthetases.[2] However, as the natural product only a single compound has been known, in which two identical pentapeptides join head to tail (Fig. 1. Gramicidin S-1).[3]

Recently we found that natural gramicidin S consists of at least 3 compounds; gramicidin S-1 as the major component with the known structure, and two minor ones, gramicidin S-2: *cyclo*(-Val-Orn-Leu-D-Phe-Pro-Abu-Orn-Leu-D-Phe-Pro-) and gramicidin S-3: *cyclo*(-Abu-Orn-Leu-D-Phe-Pro-Abu-Orn-Leu-D-Phe-Pro-) (Fig. 1).[1]

For investigation of the properties of the newly found minor components, chemical synthesis of them is required since isolation of the less abundant compounds from the mixture of the natural products exclusively depends on HPLC technique; providing of them in a sufficient amount is difficult in practice from the natural source.

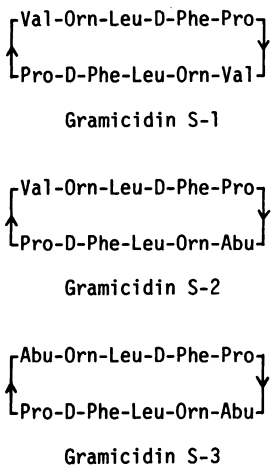

Fig. 1. Structures of gramicidin S-1, S-2, and S-3.

A convenient method was adopted for the preparation of gramicidin S-2 and S-3. The method (Fig. 2) includes rapid preparation of the tetrapeptide (**1**), effectual fragment condensation to give the C-terminal free decapeptides (**5a** and **5b**), and one-pot cyclization to afford the cyclic decapeptides (**7a** and **7b**).

The tetrapeptide (**1**) was synthesized by the "hold-in-solution" method[4] in an overall yield of 48% based on the C-terminal amino acid ester. The pentapeptides (**2a** and **2b**) derived from **1** were saponified to give the C-terminal free peptides (**3a** and **3b**), which were further converted to the N- and C-terminal free peptides (**4a** and **4b**). The coupling reaction of **3a**, which was preactivated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSCD·HCl)[5] and 1-hydroxybenzotriazole (HOBt)[6], and the amine components (**4a** and **4b**) afforded the C-terminal free decapeptides (**5a** and **5b**) in 96 and 93% yields, respectively. By acidolysis, the decapeptides (**5a** and **5b**) were converted to the N- and C-terminal free decapeptides (**6a** and **6b**), each of which was allowed to cyclize in anhydrous pyridine-DMF (1:1, 3×10^{-3} M peptide in the solvent (1 M=1 mol dm^{-3})) for 3 d at room temperature with the aid of 10 fold excess moles of WSCD·HCl and HOBt per mole of the peptide. After purification by gel filtration, the cyclic peptides, **7a** and **7b**, were obtained in 75 and 82% yields, respectively. Hydrogenolysis of the cyclic peptides (**7a** and **7b**) gave the desired peptides (**8a** and **8b**), whose homogeneities were confirmed by HPLC analysis, elemental analysis, and amino acid analysis of their acid-hydrolysates.

The present synthesis of gramicidins S demonstrates utility of the WSCD·HCl/HOBt procedure in preparation of the antibiotics. The procedure is quite effective not only for fragment condensation but also for cyclization. Recently successful one-pot cyclization using 5-nitro-2-pyridyl diphenylphosphinate was reported in a preparation of gramicidin S-1.[7] The

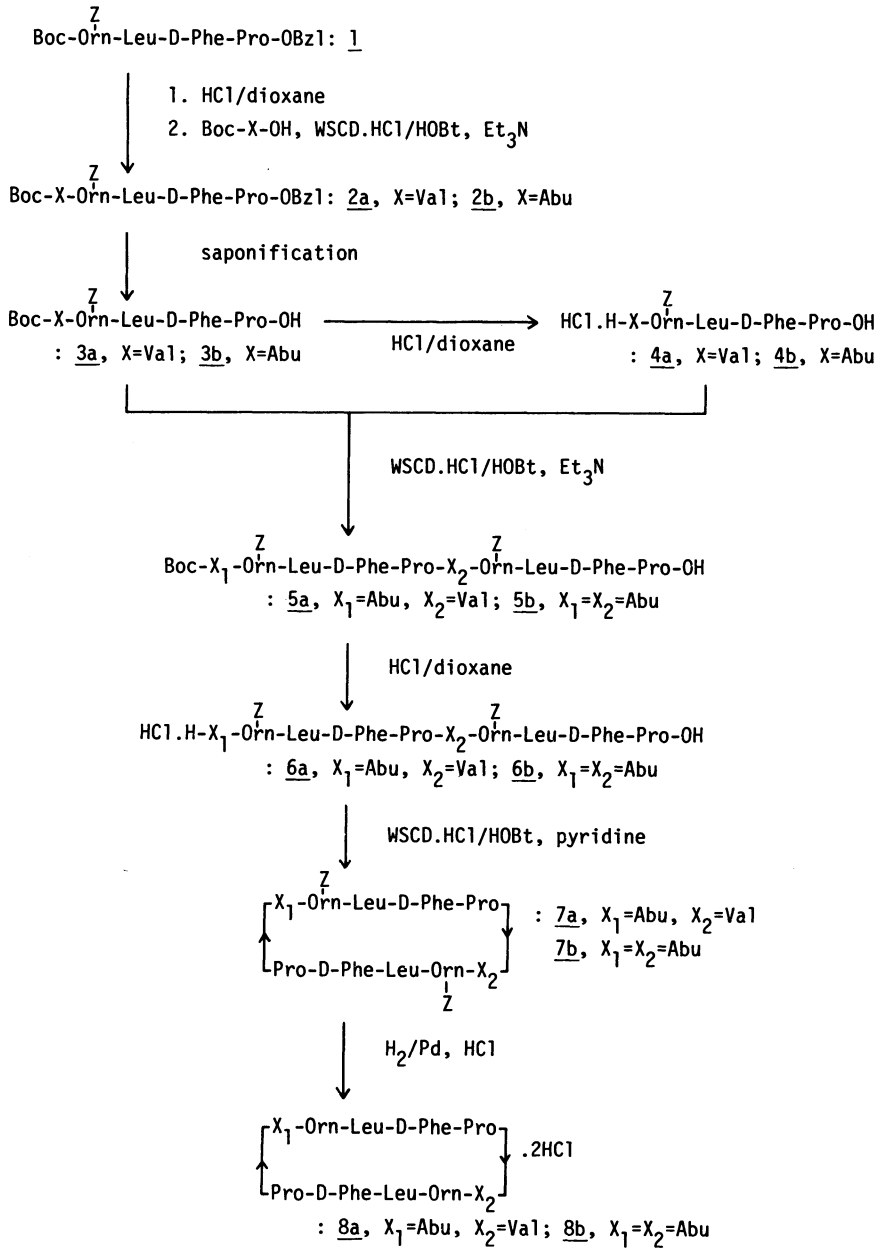

Fig. 2. Synthesis of gramicidin S-2 and S-3.

present convenient method employing the WSCD·HCl/HOBt procedure for one-pot cyclization may also be useful for synthesis of many cyclic peptides.

By HPLC analysis, **8a** and **8b** showed identical retention times with those of natural gramicidin S-2 and S-3 (hydrochlorides), respectively. The acetyl derivatives of **8a** and **8b** also gave identical HPLC profiles with those of the derivatives obtained from the natural antibiotics. The synthetic antibiotics showed the same degree of antibiotic activities as the natural ones (perchlorates) (Table 1). The CD spectra of **8a** and **8b** in ethanol were closely similar to those of the natural peptides (perchlorates). The data indicate that **8a** and **8b** are identical with natural gramicidin S-2 and S-3, respectively. The structures proposed for gramicidin S-2 and S-3 were confirmed.

The CD spectra of **8a** and **8b**, and of natural gramicidin S-1 (hydrochloride) in ethanol closely resembled each other with troughs near 207 nm and shoulders near 219 nm (Fig. 3-A). Addition of water to the peptides in ethanol produced slight decreases of the negative ellipticities near 207 nm, while the similarity found in the spectra of the three peptides was held unchanged (Fig. 3-B). The facts show that the ring conformations of the three gramicidins S are closely similar to each other.

The antibiotic activities of gramicidin S-1, S-2, and S-3 toward some test microorganisms resembled each other (Table 1).

Table 1. Antibiotic activities of synthetic and natural gramicidins S

Test organisms	Minimum inhibitory concentration (μg/ml)					
	Gramicidin S-1[a)]	**8a**	**8b**	Gramicidin S-1[b)]	Gramicidin S-2[b)]	Gramicidin S-3[b)]
Staphylococcus aureus JC-1	0.78	3.13	3.13	3.13	3.13	3.13
Staphylococcus epidermidis ATCC 14990	0.78	1.56	1.56			
Bacillus anthracis No. 119	3.13	3.13	3.13			
Bacillus subtilis ATCC 6633	1.56	3.13	3.13	3.13	3.13	1.56
Streptococcus pneumoniae IP-692	12.5	6.25	6.25			
Streptococcus pyogenes Cook Group A	3.13	3.13	3.13	6.25	6.25	6.25
Streptococcus faecalis O-0114	6.25	6.25	6.25			
Corynebacterium dyphtheriae Type Gravis	1.56	1.56	1.56	3.13	3.13	3.13
Listeria monocytogenes 4b F4	3.13	3.13	3.13			
Escherichia coli JC-2	>100	>100	100	>50	>50	>50
Salmonella typhi O-901-W	50	50	50			
Salmonella enteritidis No. 11	100	50	50			
Klebsiella pneumoniae PCI 602	>100	100	50			
Proteus vulgaris OX-19	>100	100	50			

a) The natural product. Hydrochloride. b) The natural product. Perchlorate. The data of the activity are cited from Lit. 1.

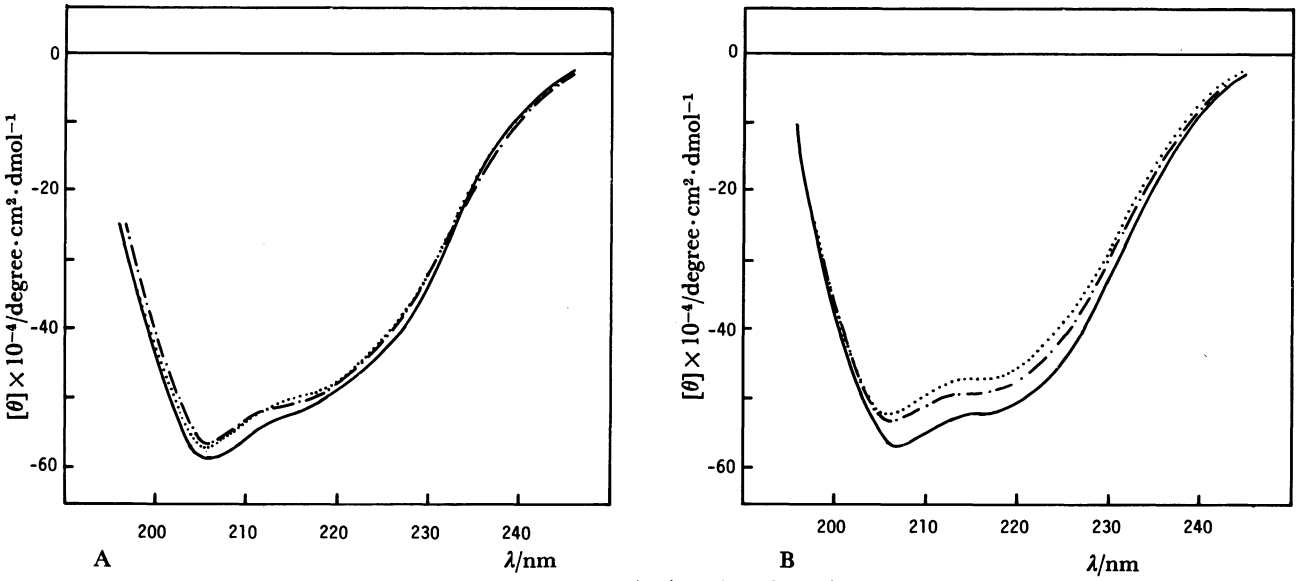

Fig. 3. CD spectra of gramicidins S. A: in ethanol, B: in ethanol-water, 1 : 1. Gramicidin S-1 hydrochloride: ——, **8a**: —·—·—, **8b**: ·····.

Experimental

Melting points were determined on a Mel-Temp apparutus and were uncorrected. CD spectra were obtained on a J-20A automatic recording spectropolarimeter (JASCO) using a calibrated 0.5 mm quartz cell. Amino acid analysis was carried out on a JLC-6AS automatic amino acid analyzer (JEOL). HPLC analysis was performed on a Partisil-10 ODS-3 column (4×250 mm, Whatman Ltd.) at a flow rate of 1.83 ml/min, which was maintained by a Milton Roy instrument minipump equipped with a JASCO PG-350D pulse dampener. The column effluent was monitored at 220 nm by a JASCO Uvidec 100-II spectrophotometer. The following solvent systems were used for the analysis; a: MeOH-water, 5/1; b: MeOH-water, 7/1; c: MeOH-5% $NaClO_4$, 4/1; d: MeOH-5% $NaClO_4$, 5/1; e: MeOH-4% ammonium acetate, 4/1; f: 2-propanol-5% $NaClO_4$, 2/3. For TLC, precoated silica-gel plates (Merck & Co., #5715) were used with the following solvent systems; a: 1-butanol-AcOH-water, 4/1/1; b: $CHCl_3$-MeOH-AcOH, 95/5/1. For elemental analysis, the samples were dried over P_2O_5 in high vaccum at 40 °C for 1 h unless otherwise noted. The natural peptides were isolated as the perchlorates by a similar method described in lit. 1. The hydrochlorides were obtained as follows: Gramicidin S-1 hydrochloride; The perchlorate was converted to the free peptide by reprecipitation from MeOH-0.5M $NaHCO_3$. The peptide was further converted to the hydrochloride by reprecipitation from MeOH-1 M HCl. Gramicidin S-2 and S-3 hydrochlorides;

Each perchlorate was dissolved in $CHCl_3$ and washed successively with 0.5 M $NaHCO_3$ and water. After removal of the solvent, the residue was dissolved in MeOH–1 M HCl and the solvent was evaporated to give a solid, which was reprecipitated from MeOH–ether.

Boc-Orn(Z)-Leu-D-Phe-Pro-OBzl (1). H-Pro-OBzl·HCl (1.209 g, 5 mM) in 1,2-dichloroethane (30 ml) was acylated successively by Boc-D-Phe-OH, Boc-Leu-OH, and Boc-Orn(Z)-OH (6 mM each) according to the procedures described in the literature.[4] The following reagents were used; Operation A: HOBt (6 mM), WSCD·HCl (7 mM). Operation B: 0.5 M $NaHCO_3$ (20 ml×2), water (20 ml), 0.1 M HCl (20 ml×2), water (20 ml×3). Operation C: 5.47 M HCl/dioxane (12 ml). Operation D: 2 M Na_2CO_3 (18 ml). Operation E: water (20 ml×3). After the final acylation, the reaction mixture was subjected to operation B and the organic layer was concentrated to give an oil. The desired peptide was obtained as a solid after chromatography on a silica-gel column (2.2×15 cm, $CHCl_3$–MeOH) followed by trituration in hexane: 1.960 g (48%), mp 76—85 °C (dec), $[\alpha]_D^{28}$ −59.9° (*c* 1, EtOH). Found: C, 66.22; H, 7.49; N, 8.26%. Calcd for $C_{45}H_{59}O_9N_5$: C, 66.40; H, 7.31; N, 8.60%.

Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OBzl (2a). Boc-Val-OH (0.434 g, 2.0 mM), HOBt (0.270 g, 2.0 mM), HCl·H-Orn(Z)-Leu-D-Phe-Pro-OBzl (0.841 g, 1.12 mM, derived from **1**), Et_3N (0.16 ml, 1.12 mM) and WSCD·HCl (0.383 g, 2.0 mM) were allowed to react in DMF (3 ml) at room temperature overnight to give a mass, which was dissolved in AcOEt and washed successively with 0.1 M HCl, water, 0.5 M $NaHCO_3$, and water. The organic layer was concentrated and diluted with hexane to give precipitates, which were recrystallized from MeOH–AcOEt–hexane: mp 178—182 ° (dec), 0.667 g (65%), $[\alpha]_D^{29}$ −71.0° (*c* 1, EtOH). Found: C, 65.54; H, 7.78; N, 9.13%. Calcd for $C_{50}H_{68}O_{10}N_6$: C, 65.77; H, 7.51; N, 9.20%

Boc-Abu-Orn(Z)-Leu-D-Phe-Pro-OBzl (2b). Prepared in a similar way described above. Recrystallized from MeOH–AcOEt–hexane; mp 189—191 °C (dec), yield: 63%, $[\alpha]_D^{28}$ −68.8° (*c* 1, EtOH). Found: C, 65.22; H, 7.60; N, 9.24%. Calcd for $C_{49}H_{66}O_{10}N_6$: C, 65.46; H, 7.40; N, 9.35%.

Boc-Val-Orn(Z)-Leu-D-Phe-Pro-OH (3a). To a suspension of **2a** (0.612 g, 0.670 mM) in EtOH (30 ml) was added 2 M NaOH (3 ml) and the mixture was stirred for 2 h at room temperature. After addition of 1 M HCl (6 ml), the mixture was concentrated to a small volume, diluted with AcOEt and washed with 0.05 M HCl and water. The organic layer was concentrated and ether was added to give a solid: 0.468 g (85%), mp 122—125 °C (dec), $[\alpha]_D^{29}$ −41.2° (c 1, DMF). Lit:[8] mp 122—125 °C, $[\alpha]_D^{20}$ −39.0° (c 2.05, DMF).

Boc-Abu-Orn(Z)-Leu-D-Phe-Pro-OH (3b). Prepared from **2b** in a similar way described above. Reprecipitated from AcOEt–ether; mp 115—130 °C (dec), yield: 89%, $[\alpha]_D^{29}$ −41.6° (*c* 1, DMF). Found: C, 61.89; H, 7.79; N, 10.27%. Calcd for $C_{42}H_{60}O_{10}N_6$: C, 62.36; H, 7.48; N, 10.39%.

Boc-Abu-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-OH (5a). Compound **3b** (0.400 g, 0.494 mM), HOBt (0.101 g, 0.75 mM), and WSCD·HCl (0.096 g, 0.5 mM) were allowed to react in DMF (3 ml) at room temperature for 40 min. To the solution was added **4a** (0.395 g, 0.521 mM, derived from **3a**, R_f^a 0.62), Et_3N (0.20 ml) and DMF (0.5 ml), and the mixture was stirred for 2 d. After removal of the solvent the desired peptide was solidified by addition of 0.1 M HCl. Reprecipitated from DMF–water: 0.727 g (96%), mp 174—180 °C (dec), R_f^b 0.36, $[\alpha]_D^{29}$ −67.3° (*c* 1, DMF). Found: C, 62.62; H, 7.67, N, 11.02%. Calcd for $C_{80}H_{112}O_{17}N_{12}\cdot H_2O$: C, 62.73; H, 7.50; N, 10.97%.

Boc-Abu-Orn(Z)-Leu-D-Phe-Pro-Abu-Orn(Z)-Leu-D-Phe-Pro-OH (5b). Prepared from **3b** (0.400 g, 0.494 mM) and **4b** (0.392 g, 0.529 mM, derived from **3b**, R_f^a 0.63) in a similar way described above. Reprecipitated from DMF–water: 0.701 g (93%), mp 111—116 °C (dec), R_f^b 0.32, $[\alpha]_D^{29}$ −53.5° (*c* 1, DMF). Found: C, 62.37; H, 7.41; N, 11.26%. Calcd for $C_{79}H_{110}O_{17}N_{12}\cdot H_2O$: C, 62.51; H, 7.44; N, 11.07%.

Cyclo*(-Abu-Orn(Z)-Leu-D-Phe-Pro-Val-Orn(Z)-Leu-D-Phe-Pro-) (7a).* A mixture of **6a** (0.207 g, 0.136 mM, derived from **5a**. Found in air-dried sample: C, 59.25; H, 7.48; N, 11.19%. Calcd for $C_{75}H_{104}O_{15}N_{12}\cdot HCl\cdot 4H_2O$: C, 59.14; H, 7.48; N, 11.03%), HOBt (0.193 g, 1.43 mM), and WSCD·HCl (0.274 g, 1.43 mM) were stirred in anhydrous DMF–pyridine (21 ml each, dried over Molecular Sieve 4 Å, Merck & Co., #5708) for 3 d at room temperature. After removal of the solvent, the residue was dissolved in $CHCl_3$ and was washed successively with 1 M HCl, water, 0.5 M $NaHCO_3$, and water. Removal of the solvent gave a crude product, which was purified by gel filtration on a Sephadex LH-20 column (1.2×96 cm, MeOH). Reprecipitated from MeOH–ether–hexane; mp 265—269 °C (dec), Retention time on the HPLC $(RT)^b$ 15.68 min, 0.146 g (75%), $[\alpha]_D^{28}$ −283° (*c* 0.3, EtOH). Found: C, 62.49; H, 7.40; N, 11.63%. Calcd for $C_{75}H_{102}O_{14}N_{12}\cdot 2H_2O$: C, 62.92; H, 7.46; N, 11.74%.

Cyclo*(-Abu-Orn(Z)-Leu-D-Phe-Pro-Abu-Orn(Z)-Leu-D-Phe-Pro-) (7b).* Prepared from **6b** (0.208 g, 0.138 mM, derived from **5b**. Found in air-dried sample: C, 59.23; H, 7.36; N, 11.15%. Calcd for $C_{74}H_{102}O_{15}N_{12}\cdot HCl\cdot 4H_2O$: C, 58.89; H, 7.41; N, 11.14%) in a similar way described above. Reprecipitated from MeOH–ether–hexane; mp 256—261 °C (dec), RT^b 12.30 min, 0.160 g (82%), $[\alpha]_D^{28}$ −289° (*c* 0.3, EtOH). Found: C, 62.84; H, 7.35; N, 11.91%. Calcd for $C_{74}H_{100}O_{14}N_{12}\cdot 2H_2O$: C, 62.69; H, 7.39; N, 11.86%.

Cyclo*(-Abu-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-)·2HCl (8a).* Compound **7a** (0.109 g) was hydrogenated over Pd black (0.047 g) in EtOH (20 ml)–AcOH (10 ml). After removal of the catalyst by filtration, 1 M HCl (4 ml) was added to the filtrate, and the solvent was evaporated to give the desired peptide. Reprecipitation from EtOH–ether, 0.093 g (93%). Mp 270—273 °C (dec), $[\alpha]_D^{27}$ −267° (*c* 0.3 EtOH). RT^c 10.10 min, RT^e 17.97 min, RT^f 25.63 min. (Natural gramicidin S-2 hydrochloride: RT^c 10.12 min, RT^e 18.05 min, RT^f 25.62 min). Amino acid composition in acid-hydrolysate (6 M HCl, 110 °C, 20 h): Pro 2.09, Abu 1.07, Val 0.98, Leu 2.04, Phe 1.94, Orn 1.89. Found: C, 54.40; H, 7.92; N, 12.80%. Calcd for $C_{59}H_{90}O_{10}N_{12}\cdot 2HCl\cdot 6H_2O$: C, 54.16; H, 8.01; N, 12.85%. Acetyl derivative; mp 265—275 °C (dec). Found: C, 59.32; H, 7.88; N, 13.13%. Calcd for $C_{63}H_{94}O_{12}N_{12}\cdot 4H_2O$: C, 58.95; H, 8.01; N, 13.09%. RT^a 13.20 min, RT^d 12.60 min. SIMS *m/z*: 1211 $(M+H)^+$. (Acetyl derivative from the natural product: RT^a 13.20 min, RT^d 12.65 min. SIMS *m/z*: 1211 $(M+H)^+$).

Cyclo*(-Abu-Orn-Leu-D-Phe-Pro-Abu-Orn-Leu-D-Phe-Pro-)·2HCl (8b).* Prepared from **7b** (0.111 g) in a similar way as described above. Reprecipitated from

EtOH–ether, 0.090 g (89%), mp 272—274 °C (dec), $[\alpha]_D^{27}$ −263° (*c* 0.3, EtOH). RT^c 7.60 min, RT^e 13.12 min, RT^f 18.30 min. (Natural gramicidin S-3 hydrochloride: RT^c 7.62 min, RT^e 13.10 min, RT^f 18.37 min). Amino acid composition: Pro 2.05, Abu 2.07, Leu 2.04, Phe 1.93, Orn, 1.91. Found: C, 53.55; H, 7.75; N, 12.89%. Calcd for $C_{58}H_{88}O_{10}N_{12}\cdot 2HCl\cdot 6H_2O$: C, 53.82; H, 7.94; N, 12.99%. Acetyl derivative; mp 274—279 °C (dec). Found: C, 58.18; H, 7.69; N, 13.05%. Calcd for $C_{62}H_{92}O_{12}N_{12}\cdot 5H_2O$: C, 57.84; H, 7.99; N, 13.05%. RT^a 10.05 min, RT^d 9.57 min. SIMS *m/z*: 1197 $(M+H)^+$. (Acetyl derivative from the natural product: RT^a 10.08 min, RT^d 9.58 min. SIMS *m/z*: 1197 $(M+H)^+$).

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References

1) Part I: S. Nozaki and I. Muramatsu, *J. Antibiot.*, **37**, 689 (1984).

2) M. Altmann, H. Koischwitz, J. Salnikow, and H. Kleinkauf, *FEBS Lett.*, **93**, 247 (1978); Reviewed in E. Katz and A. L. Demain, *Bacteriol. Rev.*, **41**, 449 (1977).

3) Amino acids with no prefix are of L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission of Biochemical Nomenclature. Other abbreviations are as follows: Boc=*t*-butoxycarbonyl, Z=benzyloxycarbonyl, OBzl=benzyl ester.

4) S. Nozaki and I. Muramatsu, *Bull. Chem. Soc. Jpn.*, **55**, 2165 (1982).

5) J. C. Sheehan, P. A. Cruickshank, and G. L. Boshart, *J. Org. Chem.*, **26**, 2525 (1961).

6) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

7) T. Mukaiyama, K. Kamekawa, and Y. Watanabe, *Chem. Lett.*, **1981**, 1367.

8) M. Ohno, T. Kato, S. Makisùmi, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **39**, 1738 (1966).