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Synthesis and Properties of Gramicidin S Analogs Containing D-Lys Residue in Place of Orn Residue

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[L-Lys$^{2,2'}$]-gramicidin S has been known to possess the same type activity and conformation as that of gramicidin S. The D-isomer of the Lys residue, instead of the Orn residue, was introduced to gramicidin S in order to investigate the contribution of the configurations of the Orn residue in gramicidin S to its activity and conformation. [D-Lys$^{2,2'}$]- and [D-Lys^{2}, L-Lys$^{2'}$]-gramicidin S were synthesized by a liquid-phase method. The CD spectra of these analogs showed a pattern similar to that of gramicidin S, suggesting that the conformation of these analogs at the β-turn parts around the D-Phe-Pro sequence resemble that of gramicidin S. These analogs have either a weak or no activity against the Gram-positive microorganisms tested, indicating the importance of the configuration of the Orn residue in gramicidin S for exhibiting strong activity.

Gramicidin S (GS)[1-2] is an antibiotic cyclodecapeptide isolated from *Bacillus brevis*. In studies of the relationship between the structure and antibiotic activity of GS, various analogs have been synthesized. From these studies it has been recognized that the basic amino groups of the Orn residues in GS are necessary for the activity. For example, the acylation of these amino groups caused a drastic decrease in the activity.[3] On the other hand, changes in the length of the side chain in the Orn residues[4-5] and a conversion of the amino groups into strongly basic guanidino groups[6] did not alter the activity. However, the effects of an alternation of the configuration of the Orn residues on the secondary structure and antibiotic actvity of GS have not been studied.

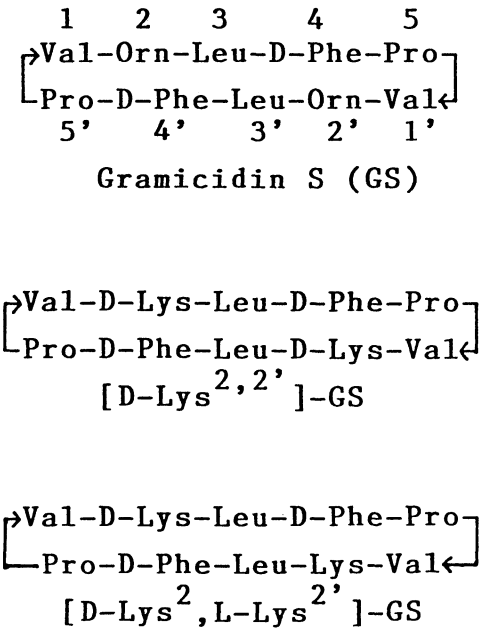

Fig. 1. Primary structure of gramicidin S and its analogs.

Waki et al.[5] synthesized [L-Lys$^{2,2'}$]-GS, in which the Orn residues at the 2- and 2'-positions are replaced with Lys residues, and reported that this analog shows the same ORD curve and antibiotic activity as those of GS.

In the present paper we describe the synthesis, antibiotic activity and CD spectra of [D-Lys$^{2,2'}$]- and [D-Lys^{2}, L-Lys$^{2'}$]-GS (Fig. 1), and discuss the structure-activity relationship of these GS analogs.

The synthetic routes of [D-Lys$^{2,2'}$]- and [D-Lys^{2}, L-Lys$^{2'}$]-GS are shown in Fig.2. In the synthesis of [D-Lys$^{2,2'}$]-GS (**5a**), the Boc-pentapeptide benzyl ester (**1a**) was synthesized from the Pro benzyl ester by a step-by-step elongation using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCD·HCl) and 1-hydroxybenzotriazole (HOBt), and then saponified to give Boc-pentapeptide (**2a**). A part of **2a** was converted into the corresponding succinimido ester **2a'** with WSCD·HCl and *N*-hydroxysuccinimide (HONSu). Coupling of **2a'** and the pentapeptide derived from **2a** afforded the decapeptide (**3a**), which was converted into the decapeptide succinimido ester (**3a'**). The Boc-group of **3a'** was removed by the action of trifluoroacetic acid (TFA); the succinimido ester was then cyclized under high dilution in pyridine at 45 °C for 5 h. The resulting product was purified by means of high-performance liquid chromatography (HPLC), followed by recrystallization. The cyclodecapeptide (**4a**) was obtained in 26% yield. The removal of all the masking groups of **4a** by hydrogenolysis yielded [D-Lys$^{2,2'}$]-GS (**5a**). [D-Lys^{2}, L-Lys$^{2'}$]-GS (**5b**) was synthesized in a similar manner. The yield of the cyclization of **3b** was 60%. Cyclization of **3a** using a succinimido active ester method gave a very low yield of the desired cyclic peptide, compared with that of **3b**. These phenomena indicate that the substitution of two Orn(*Z*) residues by two D-Lys(*Z*) residues induces a decrease in the chance for collisions between the N- and C-terminals in the linear decapeptide **3a**. The homogeneity of

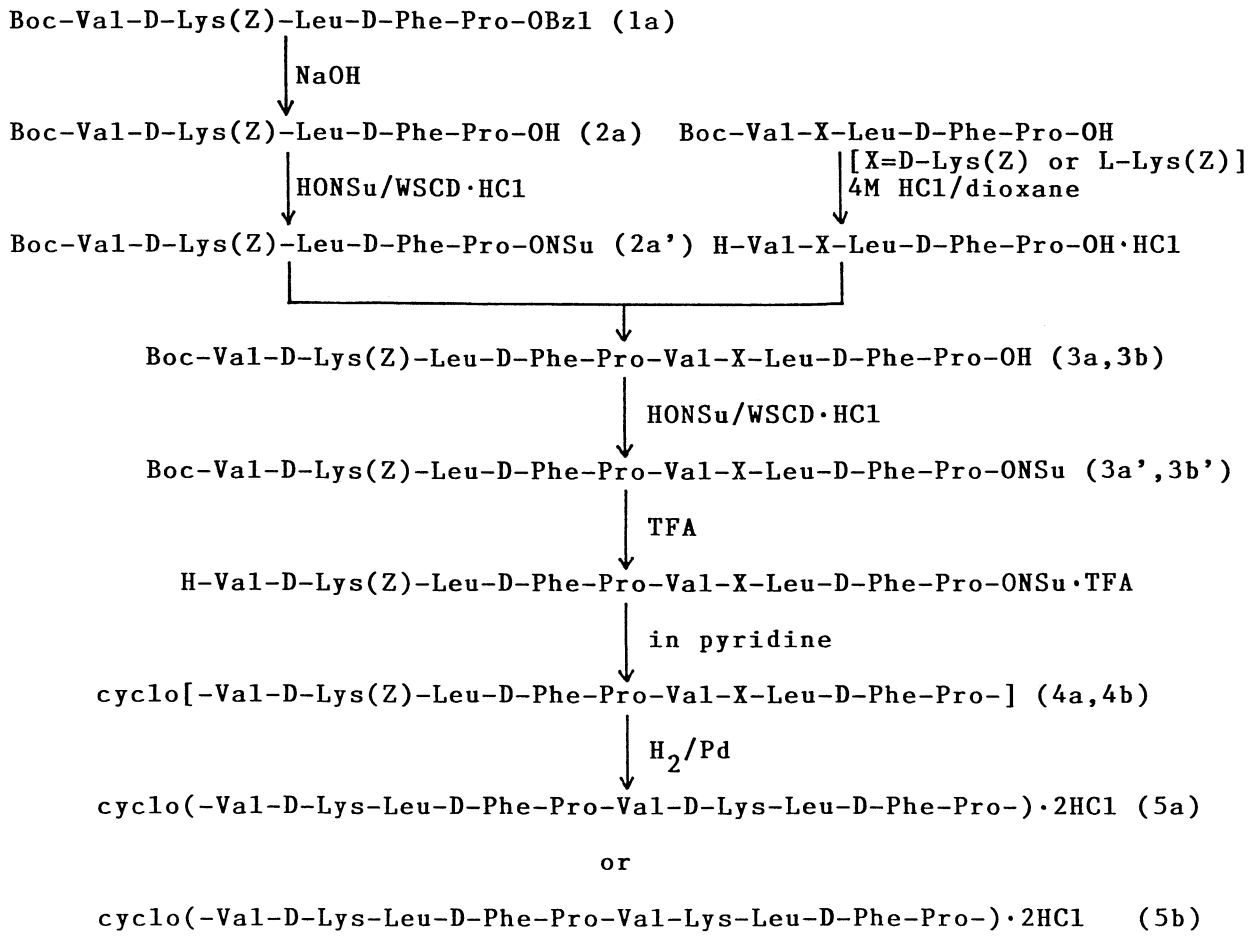

Fig. 2. Synthesis of [D-Lys$^{2,2'}$]-GS and [D-Lys^{2}, L-Lys$^{2'}$]-GS.

[D-Lys$^{2,2'}$]- and [D-Lys^{2}, L-Lys$^{2'}$]-GS was confirmed by means of thin-layer chromatography (TLC), amino acid analysis, HPLC, elemental analysis, and fast atom bombardment (FAB) mass spectrometry.

The CD spectra of GS and the analogs (**5a** and **5b**) were measured in aqueous solutions (Fig. 3). Though the shape of the CD spectrum of **5b** was similar to that of GS, each depth of the two troughs was about 1/2 that of GS. Though two troughs in the CD parttern of **5a** were also observed, their depths were much shallower. In studies regarding an alternation of the configurations of the constituent amino acid residue in GS, it was reported that [D-Val$^{1,1'}$]-,[7] [L-Phe$^{4,4'}$]-,[8] and [D-Pro$^{5,5'}$]-GS,[9] in which L-Val, D-Phe, or L-Pro residues in GS was replaced with D-Val, L-Phe, or D-Pro residues, respectively, possessed a CD spectra different from those of GS in aqueous solutions. These results indicated that the configuration of the amino acid residues at the β-turn parts of GS influences its conformation more than does that of the Orn residue. In CD studies of GS and gratisin, we also reported that their CD spectra mainly reflect the ring features near the Pro residue, but not the entire structure of the molecule.[7-10] The present results suggest that though there exists a similarity among the CD pattern of these synthetic analogs and the GS results from the presence of a D-Phe-Pro sequence of these analogs, the stability of their conformation decreases as the D-Lys residue is introduced.

The antibiotic activity of these synthetic peptides is summarized in Table 1. [D-Lys$^{2,2'}$]-GS possessed about 1/8 of the activity of the natural GS against *C. diphtheriae* P.W.8, and no activity toward the other Gram-positive microorganisms tested. On the other hand, [D-Lys^{2}, L-Lys$^{2'}$]-GS showed activity against all of the Gram-positive microorganisms tested, although it was very weak. Further, it was reported that [D-Val$^{1,1'}$]-,[7] [L-Phe$^{4,4'}$]-,[8] and [D-Pro$^{5,5'}$]-GS[9] shows either a weak or no activity. These results point out the importance of the configuration of the constituent amino acid residue of GS for exhibiting activity. In ESR studies of GS, Ovchinikov et al. reported that the average distance between the NH_3^+ groups of the Orn residues in GS and its analogs with activity is about 8—10 Å, and that the spatial situation is held by a rigid conformation containing two D-Phe-Pro type-II' β-turns.[11] From the present studies, it is deduced that the introduction of one or two D-Lys residues at

Table 1. Antibiotic Activity of GS and Its Analogs[a)]

Test organisms	GS	[D-Lys$^{2,2'}$]-	[D-Lys^{2}, L-Lys$^{2'}$]-
S. aureus MS353	3.13	>100	50
S. aureus Smith	3.13	>100	50
S. epidermidis ATCC 27626	3.13	>100	50
S. pyogenes N. Y. 5	3.13	>100	50
S. agalatiae 1020	3.13	>100	50
M. luteus ATCC 9341	3.13	>100	100
C. diphtheriae P. W. 8	3.13	25	12.5
B. subtilis ATCC 6633	3.13	100	12.5
E. coli NIHJ JC-2	>100	>100	>100
P. aeruginosa PA01	>100	>100	>100

a) The minimum inhibitory concentration ($\mu g\,ml^{-1}$) was determined by means of an agar-dilution method with 10^6 organisms per milliliter.

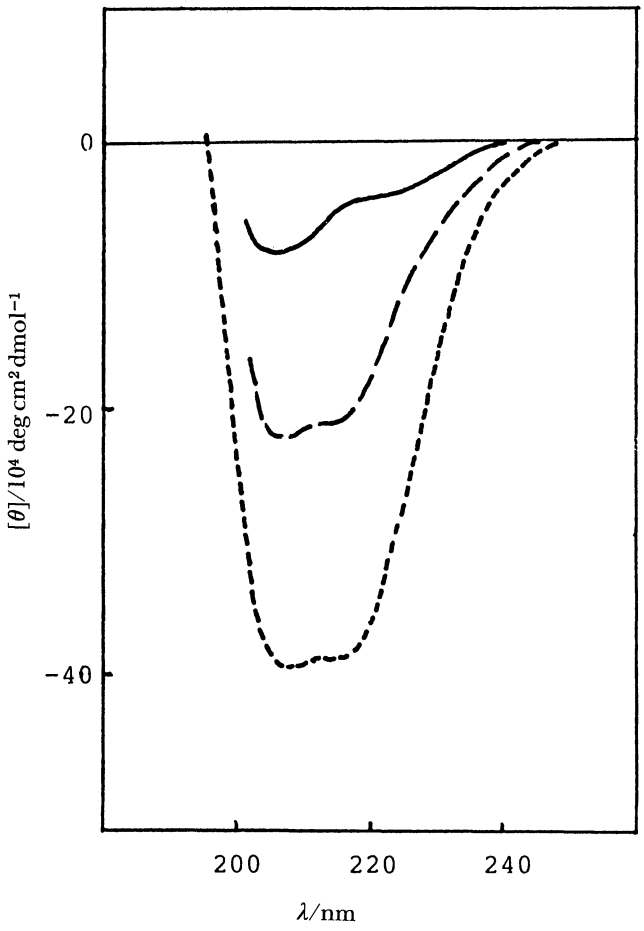

Fig. 3. CD spectra of GS and its analogs in aqueous solution.
——, [D-Lys$^{2,2'}$]-GS: ——, [D-Lys^{2}, L-Lys$^{2'}$]: ----, GS.

positions 2 and 2′ in GS decreases the stability of the type-II′ β-turn around D-Phe-Pro sequences, resulting in a distortion of the spatial arrangement of the NH_3^+ groups required for antibiotic activity.

Experimental

All melting points are uncorrected. The CD spectra were measured by a JASCO spectropolarimeter (model J-500) using a 0.5 mm quarts cell at room temperature. The CD spectroscopy of GS and its analogs was carried out as aqueous solutions of their dihydochlorides at a concentration of 1.5—2.0×10^{-4} M (1 M=1 mol dm^{-3}). The molecular weights of these synthetic peptides were determined by FAB mass spectrometry using a JEOL JMS-D-300 mass spectrometer. Amino acid analyses were carried out using a Hitachi 835 amino acid analyzer, after hydrolysis in 6 M HCl at 110 °C for 24 h. HPLC was performed by an octadecylsilica (ODS) column (φ 4.6×250 mm) using MeOH-5% $NaClO_4$aq (5:1) as an elution solvent. TLC was performed on Merck silica-gel F_{254} plates with the following solvent systems (v/v): R_f^1, $CHCl_3$-MeOH (9:1); R_f^2, $CHCl_3$-MeOH-AcOH (95:5:3); R_f^3, *n*-BuOH-AcOH-H_2O (4:1:1); R_f^4, *n*-BuOH-pyridine-AcOH-H_2O (4:1:1:2). The yields of **1a** and **1b** were calculated on the basis of the amount of Pro-OBzl as a starting material.

Boc-Val-D-Lys(Z)-Leu-D-Phe-Pro-OBzl (1a). Pro-OBzl·HCl (1.20 g, 5 mmol) was dissolved in $CHCl_3$ (20 ml). The solution was then washed with aqueous 5% Na_2CO_3 and water under cooling in an ice bath. To the organic layer were added Boc-D-Phe (1.32 g, 5 mmol), HOBt (0.75 g, 5.5 mmol) and WSCD·HCl (0.96 g, 5 mmol) at 0 °C. This solution was stirred for 1 h at 0 °C and overnight at room temperature. The reaction mixture was washed successively with aqueous 5% citric acid, water, and aqueous 5% Na_2CO_3, and water; the solvent was then evaporated in vacuo. The residue was dissolved in 4 M HCl/dioxane (20 ml) at 0 °C. After stirring for 30 min at room temperature, the solution was concentrated in vacuo. The residue was dissolved in $CHCl_3$ (30 ml); the solution was then washed with aqueous 5% Na_2CO_3 and water under cooling in an ice bath. To this solution was added Boc-Leu (1.25 g, 5 mmol), HOBt (0.75 g, 5.5 mmol) and WSCD·HCl (0.96 g, 5 mmol) at 0 °C. The same procedure as described above was repeated for this reaction mixture. Further, Boc-D-Lys(*Z*) and Boc-Val were successively coupled by the same method. All reactions were followed by TLC on a silica-gel plate. The crude protected pentapeptide obtained from the final reaction mixture was purified by chromatography on a silica-gel column (φ 1.5×60 cm) using a solvent system of $CHCl_3$-MeOH (50:1) and reprecipitation from AcOEt-ether.; overall yield, 3.38 g (73% from Pro-OBzl·HCl); mp, 70—74 °C; $[\alpha]_D^{27}$ −30.8° (*c* 1.1, DMF); R_f^1 0.90, R_f^2 0.54.

Found: C, 65.33; H, 7.65; N, 8.71%. Calcd for $C_{51}H_{70}$-

$N_6O_{10}\cdot 0.5H_2O$: C, 65.43; H, 7.64; N, 8.98%.

Boc-Val-D-Lys(Z)-Leu-D-Phe-Pro-OH (2a). To a solution of **1a** (3.00 g, 3.24 mmol) in MeOH (40 ml), 1 M NaOH (8 ml) was added; the solution was then stirred for 7 h at room temperature. After the addition of water (10 ml), the solution was concentrated in vacuo at low temperature, and aqueous 5% citric acid (100 ml) added to the reaction mixture. The resulting solid was collected by filtration, washed with water and dried. The product was recrystallized from MeOH-ether-hexane; yield, 2.64 g (97%); mp, 82—87 °C; $[\alpha]_D^{27}$ −29.6° (*c* 1.1, DMF); R_f^1 0.38, R_f^2 0.52.

Found: C, 62.47; H, 7.88; N, 9.61%. Calcd for $C_{44}H_{64}N_6O_{10}\cdot 0.5H_2O$: C, 62.47; H, 7.74; N, 9.93%.

Boc-[Val-D-Lys(Z)-Leu-D-Phe-Pro-]$_2$-OH (3a). To a solution of **2a** (800 mg, 0.96 mmol) in DMF (5 ml) were added HONSu (231 mg, 2 mmol) and WSCD·HCl (383 mg, 2 mmol) at 0 °C; the mixture was then stirred for 5 h at room temperature. After the solution was evaporated in vacuo, water was added to the residue. The resulting solid, Boc-Val-D-Lys(Z)-Leu-D-Phe-Pro-ONSu (**2a'**), was collected by filtration, washed with water and then dried. Another crop of **2a** (800 mg, 0.96 mmol) was dissolved in 4 M HCl/dioxane (15 ml) at 0 °C. After stirring at room temperature for 30 min, the solution was concentrated in vacuo. Ether (50 ml) was added to the residue, and the resulting solid was collected by filtration. To a solution of HCl·H-Val-D-Lys(Z)-Leu-D-Phe-Pro-OH (prepared as mentioned above) and triethylamine (0.26 ml, 1.92 mmol) in DMF (20 ml), **2a'** derived from **2a** at 0 °C was added. The mixture was left standing for 2 h at 0 °C and then overnight at room temperature. The solution was evaporated; aqueous 5% citric acid was then added to the residue. The resulting solid was collected by filtration, washed with water and dried. The product was recrystallized from AcOEt-ether; yield, 1.40 g (93%); mp, 120—125 °C; $[\alpha]_D^{27}$ −37.9° (*c* 1.1, DMF); R_f^1 0.40, R_f^2 0.53.

Found: C, 62.28; H, 7.70; N, 10.51%. Calcd for $C_{83}H_{118}N_{12}O_{17}\cdot 2.5H_2O$: C, 62.27; H, 7.74; N, 10.50%.

Cyclo[-Val-D-Lys(Z)-Leu-D-Phe-Pro-]$_2$ (4a). Compound **3a** (750 mg, 0.48 mmol) was converted into the *N*-hydroxysuccinimido ester (**3a'**) by methods described for **2a**. It was then dissolved in TFA (15 ml) at 0 °C. The mixture was stirred for 40 min at room temperatue and then concentrated in vacuo. The residue, the trifluoroacetate of the decapeptide active ester, was triturated with ether and collected by filtration and dissolved in DMF (10 ml). The solution was poured, dropwise, in pyridine (500 ml) at 45 °C. After stirring for 3 h at 45 °C, the solution was concentrated. The addition of water to the reisdue afforded precipitates, which were filtered and washed with water. The purification of this compound was performed by HPLC on ODS column (ϕ 7.6×250 mm) using a solvent system of MeOH-H_2O (6:1), followed by reprecipitation from MeOH-ether; yield, 180 mg (26%); mp, 220—222 °C; $[\alpha]_D^{27}$ −137.7° (*c* 0.65, DMF); R_f^1 0.83, R_f^2 0.56.

Found: C, 63.32; H, 7.64; N, 11.35%. Calcd for $C_{78}H_{108}N_{12}O_{14}\cdot 2H_2O$: C, 63.57; H, 7.66; N, 11.40%.

Cyclo(-Val-D-Lys-Leu-D-Phe-Pro-)$_2$·2HCl (5a). Compound **4a** (120 mg, 0.083 mmol) was dissolved in 90% aq MeOH (40 ml) and then 1 M HCl (0.17 ml) was added to the solution. The compound was hydrogenolyzed in the presence of palladium black for 15 h. After removing the catalyst, the filtrate was concentrated in vacuo. The product was purified by gel filtration on a Sephadex LH-20 column (ϕ 1.2×120 cm) using MeOH as a solvent, and by reprecipitation from MeOH-ether; yield, 90 mg (87%); mp, 246—248 °C; $[\alpha]_D^{27}$ −158.3° (*c* 0.70, EtOH); R_f^3 0.44, R_f^4 0.76.

Amino acid ratios: Val, 0.97; Lys, 0.98; Leu, 1.03; Phe, 1.08; Pro, 0.93. MS (FAB), *m/z* 1169 ($C_{62}H_{96}N_{12}O_{10}$, MH^+).

Found: C, 54.81; H, 8.04; N, 12.40%. Calcd for $C_{62}H_{96}N_{12}O_{10}\cdot 2HCl\cdot 6.5H_2O$: C, 54.78; H, 8.23; N, 12.36%.

Boc-Val-D-Lys(Z)-Leu-D-Phe-Pro-Val-Lys(Z)-Leu-D-Phe-Pro-OH (3b). This compound was prepared from **2a** (600 mg, 0.72 mmol) and Boc-Val-Lys(Z)-Leu-D-Phe-Pro-OH (700 mg, 0.84 mmol) in a manner similar to that described for the preparation of **3a**. Purification of the product was performed by reprecipitation from AcOEt-ether-hexane: yield, 1.03 g (92%); mp, 104—112 °C; $[\alpha]_D^{27}$ −40.9° (*c* 1.2, DMF); R_f^1 0.51, R_f^2 0.57.

Found: C, 62.87; H, 7.77; N, 10.53%. Calcd for $C_{83}H_{118}N_{12}O_{17}\cdot 1.5H_2O$: C, 62.98; H, 7.70; N, 10.62%.

Cyclo[-Val-D-Lys(Z)-Leu-D-Phe-Pro-Val-Lys(Z)-Leu-D-Phe-Pro-] (4b). This compound was prepared from **3b** (750 mg, 0.48 mmol), as described regarding the preparation of **4a**; yield, 417 mg (60%); mp, 133—137 °C; $[\alpha]_D^{27}$ −154.4° (*c* 1.0, DMF); R_f^1 0.76, R_f^2 0.55.

Found: C, 64.32; H, 7.68; N, 11.45%. Calcd for $C_{78}H_{108}N_{12}O_{14}\cdot H_2O$: C, 64.35; H, 7.62; N, 11.54%.

Cyclo(-Val-D-Lys-Leu-D-Phe-Pro-Val-Lys-Leu-D-Phe-Pro-)·2HCl (5b). This compound was prepared from **4b** (200 mg, 0.14 mmol) as described regarding the preparation of **5a**; yield, 150 mg (87%); mp, 228—235 °C; $[\alpha]_D^{27}$ −223.4° (*c* 0.82, EtOH); R_f^3 0.69, R_f^4 0.79.

Amino acid ratios: Val, 0.98; Lys, 0.99; Leu, 1.05; Phe, 1.04; Pro, 0.94.

MS(FAB), *m/z* 1169 ($C_{62}H_{96}N_{12}O_{10}$, MH^+).

Found: C, 56.51; H, 8.08; N, 12.75%. Calcd for $C_{62}H_{96}N_{12}O_{10}\cdot 2HCl\cdot 4H_2O$; C, 56.65; H, 8.13; N, 12.79%.

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References

1) A. R. Battersby and L. C. Craig, *J. Am. Chem. Soc.*, **73**, 1887 (1951).

2) Amino acid residues with no plefix 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, benzyloxycarbonyl; OBzl, benzyloxy; DMF, *N,N*-dimethylformamide; AcOEt, ethyl acetate.

3) A. S. Kaprel' yants, V. V. Nikiforov, A. I. Miroshinov, L. G. Snezhkova, V. A. Eremin, and D. N. Ostrovski, *Biochemistry* (translated from Russian), **42**, 252 (1977).

4) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 1582 (1958).

5) M. Waki, O. Abe, R. Okawa, T. Kato, S. Makisumi, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **40**, 2904 (1967).

6) A. B. Silaev and V. N. Stepanov, *Dokl. Acad. Nauk SSSR*, **112**, 297 (1957).

7) M. Tamaki, T. Okitsu, M. Araki, H. Sakamoto, M.

Takimoto, and I. Muramatsu, *Bull. Chem. Soc. Jpn.*, **58**, 531 (1985).
8) M. Rothe and F. Eisenbeiss, *Angew. Chem.*, **80**, 907 (1968).
9) K. Sato, R. Kato, and U. Nagai, *Bull. Chem. Soc. Jpn.*, **59**, 535 (1986).
10) M. Tamaki, *Bull. Chem. Soc. Jpn.*, **57**, 3210 (1984).
11) Yu. A. Ovchinikov and V. T. Ivanov, *Tetrahedron*, **31**, 2117 (1975).