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Synthesis of a Gramicidin S Analog Containing δ-Aminovaleric Acid, [4–5-δ-Aminovaleric Acid]-Gramicidin S

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In order to investigate the contribution of amide bond in peptide to its activity and conformation, a new type of analog of gramicidin S, [4–5-δ-aminovaleric acid]-gramicidin S, was synthesized, in which one of the D-phenylalanyl-L-proline residues of gramicidin S was replaced with δ-aminovaleric acid residue. The analog peptide has antimicrobial activity and shows similar ORD and CD spectra to those of gramicidin S. The linear peptide (Val–Orn–Leu–δAva–Val–Orn–Leu–D-Phe–Pro) has no activity and its ORD and CD spectra are nearly the same as those of random coil of polypeptide.

Since the primary structure of antibiotic cyclodecapeptide gramicidin S (GS) from *Bacillus brevis* was confirmed by means of successful total synthesis by Schwyzer and Sieber,¹⁾ a number of investigations have been undertaken to determine the higher structure of the antibiotic. Hodgkin and Oughton²⁾ proposed some possible models for GS from X-ray diffraction analysis. One of them, an antiparallel β-pleated sheet model, was favored by Schwyzer,³⁾ and supported by the results of nuclear magnetic resonance by Stern *et al.*⁴⁾ and others.⁵,⁶⁾ Several studies on optical rotatory dispersion (ORD)⁷⁻⁹⁾ and circular dichroism (CD)⁷,¹⁰,¹¹⁾ of GS have also been undertaken to reveal the conformation in solution. A considerable number of analogs of GS have been prepared by Izumiya *et al.* in an effort to elucidate the relationship between structure and antimicrobial activity. In almost all cases, each amino acid residue of the natural peptide was replaced with a residue having a different side chain. Their results¹²⁾ suggested some fundamental significance of the presence of side chains of constituent amino acids, especially basic amino acid.¹³⁾ It was found that the analogs formed by replacing the L-proline residues of GS with glycine¹⁴⁾ or sarcosine¹⁵⁾ residues had an activity similar to that of GS. For determining the relation between ring-size of cyclic peptide and activity, cyclic pentapeptide (half-size compounds of GS¹⁶⁾ and some analogs¹²,¹⁷⁾), cyclo-(Val–Orn–Leu–D-Phe–Pro–Gly),¹⁸,¹⁹⁾ cyclo (Val–Orn–Leu–D-Phe–Pro–Gly–Gly),¹⁹,²⁰⁾ and [5,5′-βAla]-GS²¹⁾ were synthesized by Izumiya and his co-workers,but found to show no activity. It is consequently presumed that the ring-size of such an analog is an important factor in determining whether or not the analog possesses activity.

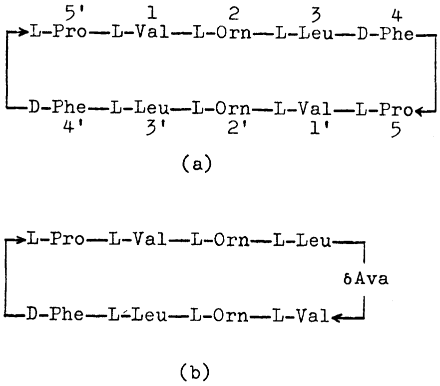

Fig. 1. Structure of GS (a) and [δAva$^{4-5}$]–GS (b).

Participation of amide bonds through hydrogen bonds between L-valine and L-leucine residues in holding the rigid ring structure of the peptide was confirmed by nmr studies.⁴,⁵⁾ However, it has not been ascertained whether all the amide bonds are indispensable for the function and conformation of the antibiotic. In order to determine this, we synthesized a new type of GS analog containing δ-aminovaleric acid residue (δAva). In this peptide, one of the D-phenylalanyl-L-proline residues of GS is replaced with δAva and, consequently, one amide group is displaced with an ethylene group ($-CH_2-CH_2-$) which has almost the same length as –CO–NH– but does not participate in the hydrogen bond. Since this analog and the

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1) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).

2) D. C. Hodgkin and B. M. Oughton, *Biochem. J.*, **65**, 752 (1957).

3) R. Schwyzer, Ciba Foundation Symp. Amino Acids and Peptides with Antimetabolic Activity, 171 (1958); R. Schwyzer, *Chimia* **12**, 53 (1958).

4) A. Stern, W. A. Gibbons, and L. C. Craig, *Proc. Nat. Acad. Sci. U. S.*, **61**, 734 (1968).

5) M. Ohnishi and D. W. Urry, *Biochem. Biophys. Res. Commun.*, **36**, 194 (1969).

6) R. Schwyzer and U. Ludescher, *Biochemistry*, **7**, 2519 (1968); R. Schwyzer and U. Ludescher, *Helv. Chim. Acta*, **52**, 2033 (1969).

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8) L. C. Craig, *Proc. Nat. Acad. Sci. U. S.*, **61**, 152 (1968).

9) T. Kato, M. Waki, S. Matsuura, and N. Izumiya *J. Biochem.* (Tokyo), **68**, 751 (1970).

10) F. Quadrifoglio and D. W. Urry, *Biochem. Biophys. Res. Commun.*, **29**, 785 (1967).

11) S. Laiken, M. Printz, and L. C. Craig, *J. Biol. Chem.*, **244**, 4454 (1969).

12) M. Kondo and N. Izumiya, This Bulletin, **40**, 1975 (1967).

13) M. Waki, O. Abe, R. Okawa, T. Kato, S. Makisumi, and N. Izumiya, *ibid.*, **40**, 2904 (1967).

14) H. Aoyagi, T. Kato, M. Ohno, M. Kondo, M. Waki, S. Makisumi, and N. Izumiya, *ibid.*, **38**, 2139 (1965).

15) H. Aoyagi and N. Izumiya, *ibid.*, **39**, 1747 (1966).

16) M. Waki and N. Izumiya, *ibid.*, **40**, 1687 (1967).

17) H. Aoyagi, M. Kondo, T. Kato, S. Makisumi, and N. Izumiya, *ibid.*, **40**, 1685 (1967).

18) T. Kato, M. Kondo, M. Ohno, and N. Izumiya, *ibid.*, **38**, 1202 (1965).

19) Abbreviations with no prefix show L-amino acid residues.

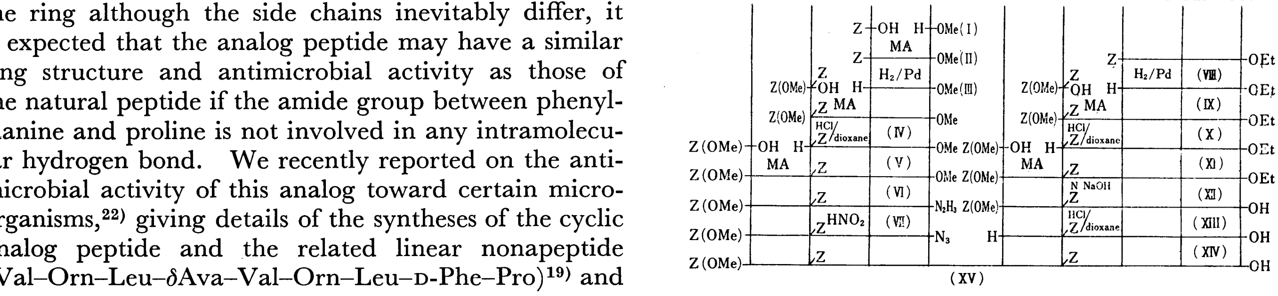
20) O. Abe, K. Kuromizu, M. Kondo, and N. Izumiya, This Bulletin, **43**, 914 (1970).

21) S. Matsuura, M. Waki, S. Makisumi, and N. Izumiya, *ibid.*, **43**, 1197 (1970).

natural peptide have the same number of members in the ring although the side chains inevitably differ, it is expected that the analog peptide may have a similar ring structure and antimicrobial activity as those of the natural peptide if the amide group between phenylalanine and proline is not involved in any intramolecular hydrogen bond. We recently reported on the antimicrobial activity of this analog toward certain microorganisms,[22] giving details of the syntheses of the cyclic analog peptide and the related linear nonapeptide (Val–Orn–Leu–δAva–Val–Orn–Leu–D-Phe–Pro)[19] and their properties in comparison with GS.

Results and Discussion

Synthesis. The synthesis of the GS analog is shown in Schemes 1 and 2. The conventional coupling methods could be applied to the synthesis of peptide containing δAva as well as α-amino acid. Two fragments VI and XII were synthesized without racemization by using the procedure of stepwise elongation with the mixed anhydride method improved by Anderson *et al.*[24] The latter was saponified to XIII. After the removal of the *N*-protecting group of XIII, the resulting peptide XIV was coupled with VII to the linear nonapeptide XV by the Rudinger modification[25] of the azide method. Preparation of XV by hydrolyzing the ethyl ester (XX) of the peptide was unsuccessful under various of conditions. Cyclization of XV was carried out following the method of Schwyzer *et al.*[1,16] for the synthesis of GS. The cyclic peptide XVI was obtained in a good yield (70%) regardless of the presence of δAva in the middle of the linear peptide. This result might indicate that the conformation of the active ester of XV is so arranged as to be favorable for the cyclization as well as the synthesis of GS.[3] The desired [4-5-δAva]-GS XVII was obtained as a crystalline dihydrochloride by means of hydrogenolysis of benzyloxycarbonyl groups on ornithine residues of the cyclic peptide XVI in the presence of hydrogen chloride, followed by lyophilization in water.



Scheme 1.[19,23]

The linear nonapeptide trihydrochloride XVIII was prepared from peptide XV. *N*-Protecting groups of XV were removed with hydrogen bromide in acetic acid, and the hydrobromide of the product was transformed into trihydrochloride XVIII.

Characterization. Thin layer chromatography of each of the synthetic peptides XVII and XVIII gave one ninhydrin-positive spot. The chromatogram of paper electrophoresis is shown in Fig. 2. The linear peptide XVIII has three amino groups and hence moves faster than the others.

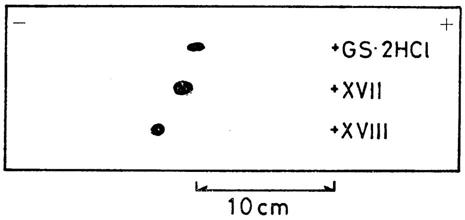


Fig. 2. Paper electrophoresis of cyclic analog (XVII), linear peptide (XVIII), and gramicidin S dihydrochloride (GS) (HCOOH-AcOH-H_2O; 4 : 15 : 180 v/v, pH 1.9, Toyo No. 50 15×40 cm, 600 v, 2 hours).

The free amino groups of the analog peptide XVII were detected by the DNS method in the usual manner.[26] After coupling the peptide with 1-dimethylamino-naphthalene-5-sulphonyl chloride (DNS-chloride) and hydrolyzing the DNS derivative, only the

XV ⟶ Linear nonapeptide (XVIII)

$\downarrow$ $SO(OC_6H_4NO_2)_2$

Z(OMe)-Val-Orn(δ-Z)-Leu-δAva-Val-Orn(δ-Z)-Leu-D-Phe-Pro-ONp

$\downarrow$ CF_3COOH

H-Val-Orn(δ-Z)-Leu-δAva-Val-Orn(δ-Z)-Leu-D-Phe-Pro-ONp·CF_3COOH

$\downarrow$ in pyridine

Cyclo-(Val-Orn(δ-Z)-Leu-δAva-Val-Orn(δ-Z)-Leu-D-Phe-Pro) (XVI)

$\downarrow$ H_2/Pd, HCl

Cyclo-(Val-Orn-Leu-δAva-Val-Orn-Leu-D-Phe-Pro)·2HCl (XVII)

Scheme 2. Diagram of the synthesis of XVII[19,23]

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[22] I. Muramatsu, S. Sōfuku, and A. Hagitani, *J. Antibiot.*, **25**, 189 (1972).

[23] The following abbreviations are from *Biochemistry*, **5**, 2485 (1966): Z-, benzyloxycarbonyl; Z(OMe)-, *p*-methoxybenzyloxycarbonyl; -ONp, *p*-nitrophenoxy; -OMe, methoxy; -OEt, ethoxy.

[24] G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **89**, 5012 (1967).

[25] K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolman, N. Yanaihara, and G. Zanetti, *ibid.*, **87**, 620 (1965); J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

[26] G. Pataki, "Dünnschichtchromatographie in der Aminosäure- und Peptid-Chemie," Walter de Gruyter & Co., Berlin (1966), p. 167.

N^δ-DNS-ornithine was detected on thin layer chromatograms,[27] which proves that the analog has a cyclic form. The IR spectrum of XVII was measured as a KBr disk and the absorption bands of amide I (1640 cm^{-1}) and II (1530 cm^{-1}) were in good agreement with those of GS. The IR spectrum of XVIII had a band at 1727 cm^{-1} (-COOH) besides bands I and II.

On the Structure and Biological Activity. Analog XVII has an antimicrobial activity in the range from the same to half that of GS toward certain microorganisms, and about one third of the acute toxicity of GS in mice when given intravenously.[22] Linear nonapeptide XVIII has no activity toward the same microorganisms. To obtain information on the conformation of this biological active analog, ORD and CD were measured. ORD studies on GS and several other GS analogs had been carried out by Craig,[8] Balasuburamanian,[7] and Kato *et al.*[9] Kato *et al.* pointed out that GS and biological active analogs showed negative Cotton effect at 232 mμ and inactive analogs gave curves differing from that of GS. The ORD of the new analog has a trough similar to GS at 232 mμ in both ethanol and water (Fig. 3). In the case of XVIII, a trough is found at 210 mμ with hypsochromic shift. The trough corresponded to that of random structure of polypeptide.[28] The CD spectra of XVII and XVIII are shown in Fig. 4. The minimum value of XVII appears at 203 mμ and a shoulder at 216 mμ. This band is observed at a wavelength slightly shorter than that of the band of GS (at 206 mμ). Laiken *et al.*[11] found that the CD of modified GS, in which D-phenylalanine residues were hydrogenated, showed two minima in the same region as that of GS. They supposed that the two minima are attributable to the common backbone conformation of both peptides. The CD of XVIII is regarded as that of random structure[29,30] in the same manner as ORD. Thus, the CD band of XVII in the region of 203—216 mμ seems to be attributable to the backbone conformation. The CD spectrum resembles one typical of the α-helix conformation of polypeptide but the wavelength of the minimum differs from that of α-helix and also β-structure.[29,30] It seems reasonable to assume that the CD of GS or its analog is characteristic for such cyclic oligopeptides. The CD spectra in the region near 260 mμ were measured and the bands[31] caused by phenylalanine residue were found. We might conclude that the analog XVII has a conformation similar to that of GS both from the results of ORD and CD measurements and from a valuation of the biological activity. The results also support the antiparallel β-pleated sheet structure of GS proposed by Stern *et al.* and Schwyzer, in which amide groups of phenylalanylproline residues do not take part in any intramolecular hydrogen bond.

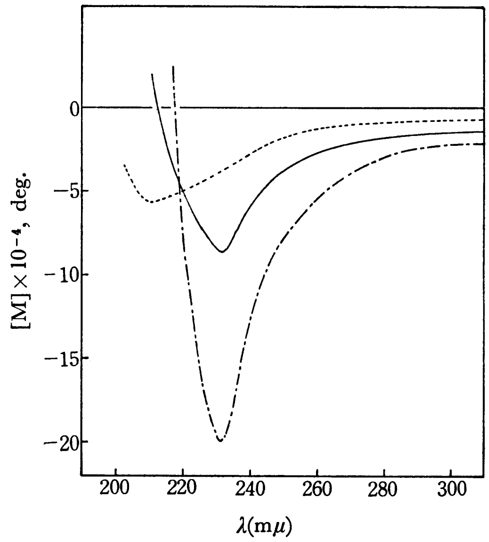

Fig. 3. Optical rotatory dispersions of cyclic analog (XVII), linear peptide (XVIII), and gramicidin S dihydrochloride (GS) in water, XVII (—), XVIII (……), and GS (—·—). Measurments were made using a 1 mm quartz cell at room temperature.

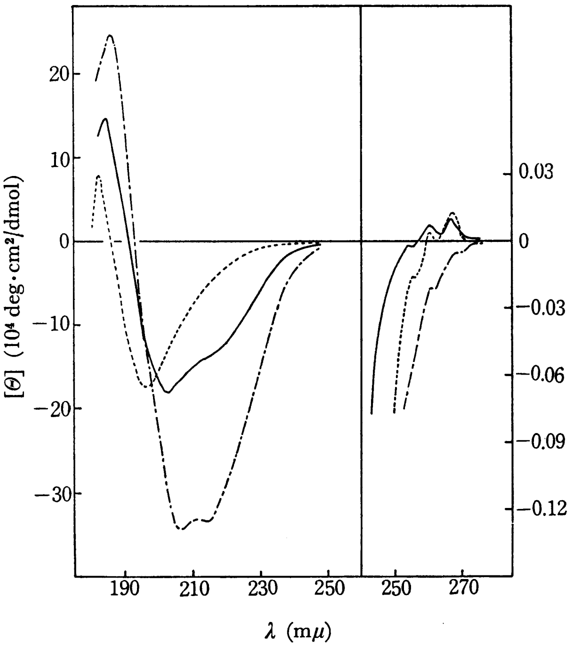

Fig. 4. Circular dichroism spectra of cyclic analog (XVII), linear peptide (XVIII), and gramicidin S dihydrochloride (GS) in water, XVII (——), XVIII (……), and GS(——). Measurments were made using 0.5 mm (<250 mμ) and 10 cm (>240 mμ) quartz cells at room temperature.

Experimental

All melting points are uncorrected. ORD measurements were performed with a JASCO model UV/ORD-5 and the results were expressed in terms of molar optical rotations.

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[27] DNS-amino acid was detected as a green spot under UV (254 mμ). R_f values of δ-DNS-ornithine were 0.5 on silica gel plate with n-butanol-acetic acid-water (10 : 1 : 1, v/v) and 0.3 on polyamide plate with benzene-acetic acid-n-butanol (9 : 2 : 1, v/v).

[28] E. R. Blout, I. Schmier, and N. S. Simmons, *J. Amer. Chem. Soc.*, **84**, 3193 (1962).

[29] G. Holzwarth and P. Doty, *ibid.*, **87**, 218 (1965).

[30] R. Townend. T. F. Kumosinski, S. N. Timasheff, G. D. Fasman, and B. Davidson, *Biochem. Biophys. Res. Commun.*. **23**, 163 (1966).

[31] M. Shiraki, *Sci. Papers College of Gen. Education, Univ. of Tokyo*, **19**, 151 (1969).

CD spectra were measured with a JASCO model J-20 and represented by molar ellipticity values. IR spectra were recorded on a Hitachi grating infrared spectrophotometer model 215. The molecular weight of XVI was determined with a Hitachi-Perkin-Elmer vapor pressure osmometer model 115, using methanol. Amino acid analyses were carried out with an automatic amino acid analyzer,[32] after hydrolysis of XVII and XVIII in 6 N hydrochloric acid for 19 hours at 110°C. The purity of the peptide derivatives at each step was confirmed by thin layer chromatography on a silica gel plate with the following solvent systems (v/v): Solv. 1, chloroform–methanol–acetic acid (95 : 5 : 3); Solv. 2, chloroform–methanol (9 : 1); Solv. 3, chloroform–methanol–acetic acid–pyridine (95 : 5 : 3 : 4); Solv. 4, chloroform–methanol–acetic acid (12 : 1 : 1); Solv. 5, *n*-butanol–acetic acid–water (4 : 1 : 1); Solv. 6, *n*-butanol–acetic acid–pyridine–water (4 : 1 : 1 : 2). Compounds possessing free amino groups were detected by spraying ninhydrin reagent. *N*-Protected compounds were also detected with ninhydrin after deblocking with concd. hydrobromic acid.

Methyl δ-Aminovalerate Hydrochloride (I). A solution of δ-valerolactam (45.0 g) in 500 ml of methanol was saturated with dry hydrogen chloride and refluxed for 3 hours. The reaction mixture was concentrated *in vacuo* to an oily residue which was dissolved in methanol. The colorless product was crystallized by the addition of ethyl ether, and collected by filtration; yield, 58.0 g (76.2%). This ester was also obtained by Fischer's esterification from δ-aminovaleric acid in a 95% yield. Mp 145—146°C (lit,[33] mp 145—146°C); Tlc: 0.75 (Solv. 6). (Found: C, 42.94; H, 8.32; N, 8.65%).

Methyl Benzyloxycarbonyl-L-leucyl-δ-aminovalerate (II). Benzyloxycarbonyl-L-leucine obtained from the corresponding dicyclohexylammonium salt[34] (9.0 g, 20.0 mmol) was dissolved in 100 ml of tetrahydrofuran, and 1.74 ml of *N*-methylmorpholine and 2.68 ml of isobutyl chloroformate were added at −15°C. After 5 min, a chilled mixture of 3.34 g (21.5 mmol) of I and 2.8 ml of triethylamine in 15 ml of chloroform was added at −15° to a solution of the mixed anhydride prepared in the preceding step. The reaction mixture was stirred at 5°C for one hour and then at room temperature for one hour. After removal of insoluble materials by filtration, the filtrate was concentrated *in vacuo.* The residue was dissolved in ethyl acetate, and the solution was washed successively with 5% sodium bicarbonate, water, 0.5 N hydrochloric acid and water, and then dried over sodium sulfate. After the solvent was evaporated *in vacuo*, the product was recrystallized from ethyl acetate and petroleum ether. Yield, 5.40 g (71.4%); mp 89—90°C; $[\alpha]^{22}_{D}$ −15.3° (*c* 1.5, ethanol); Tlc: R_f 0.7 (Slolv. 1).

Found: C, 63.37; H, 8.27; N, 7.64%. Calcd for $C_{20}H_{30}N_2O_5$: C, 63.47; H, 7.99; N, 7.40%.

Methyl L-Leucyl-δ-aminovalerate Hydrochloride (III). II (4.5 g, 11.9 mmol) was hydrogenolyzed in the usual manner over 0.2 g of palladium black in a mixture of 40 ml of ethanol and 6 ml of 2 N hydrochloric acid. After 4 hours, the filtrate from the catalyst was concentrated to dryness *in vacuo.* The oily product was obtained in a theoretical yield. Tlc: R_f 0.1 (Solv. 1).

Methyl N$^{\alpha}$-p-*Methoxybenzyloxycarbonyl*-N$^{\delta}$-*benzyloxycarbonyl-L-ornithyl-L-leucyl-δ-aminovalerate (IV).* This was prepared from *N*$^{\alpha}$-*p*-methoxybenzyloxycarbonyl-*N*$^{\delta}$-benzyloxycarbonyl-L-ornithine[35] (30.0 mmol) and III (30.0 mmol) by a method similar to that for the synthesis of II. The product was recrystallized from ethanol. Yield, 14.56 g (73.9%); mp 154—157°C; $[\alpha]^{20}_{D}$ −7.7°C (*c* 1, dimethylformamide); Tlc: R_f 0.6 (Solv. 1).

Found: C, 62.42; H, 7.44; N, 8.40%. Calcd for $C_{34}H_{48}N_4O_9$: C, 62.17; H, 7.37; N, 8.53%.

Methyl N$^{\alpha}$-*Benzyloxycarbonyl-L-ornithyl-L-leucyl-δ-aminovalerate Hydrochloride (V).* To a solution of IV (5.5 g, 8.4 mmol) in 33 ml of chloroform were added 3.73 N hydrogen chloride in dioxane (11.3 ml) and 2.5 ml of anisole. The solution was stirred at room temperature for 10 min and then concentrated *in vacuo.* The residue was washed several times with ethyl ether and dried *in vacuo.* The product was obtained in a theoretical yield. Tlc: R_f 0.65 (Solv. 5).

Methyl p-*Methoxybenzyloxycarbonyl-L-valyl*-N$^{\delta}$-*benzyloxycarbonyl-L-ornithyl-L-leucyl-δ-aminovalerate (VI).* *p*-Methoxybenzyloxycarbonyl-L-valine[35] (12.0 mmol) was coupled with V (8.4 mmol) by the mixed anhydride method. After removal of the precipitate in the reaction mixture by filtration, the filtrate was concentrated to dryness *in vacuo.* The residue washed with petroleum ether by decantation was combined with the above precipitate. The solids were washed on a filter funnel successively with water, 5% sodium bicarbonate, water, 10% citric acid and water, and dried under reduced pressure. The product was recrystallized from methanol and ethyl ether. Yield, 4.93 g (77.2%); mp 204—205°C; $[\alpha]^{20}_{D}$ −8.5° (*c* 2, dimethylformamide); Tlc: R_f 0.9 (Solv. 6).

Found: C, 61.82; H, 7.56; N, 9.21%. Calcd for $C_{39}H_{57}N_5O_{10}$: C, 61.97; H, 7.60; N, 9.27%.

p-*Methoxybenzyloxycarbonyl-L-valyl*-N$^{\delta}$-*benzyloxycarbonyl-L-ornithyl-L-leucyl-δ-aminovaleryl Hydrazine (VII).* To a solution of 2.27 g (3.0 mmol) of VI in 18 ml of dimethylformamide was added 5.63 g (90 mmol) of 80% hydrazine hydrate and the solution was stirred for 2 days at room temperature. The hydrazide, precipitated by the addition of 340 ml of water, was filtered and washed with water. A white product dried under reduced pressure was obtained in a yield of 2.26 g (99.6%). Mp 215—217.5°C; $[\alpha]^{18}_{D}$ −9.0° (*c* 1, hexamethylphosphoramide); Tlc: R_f 0.7 (Solv. 1).

Found: C, 60.17; H, 7.84; N, 12.92%. Calcd for $C_{38}H_{57}N_7O_9$: C, 60.38; H, 7.60; N, 12.97%.

Ethyl N$^{\alpha}$-p-*Methoxybenzyloxycarbonyl*-N$^{\delta}$-*benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolinate (X).* Ethyl L-leucyl-D-phenylalanyl-L-prolinate hydrochloride (IX)[36] obtained from VIII (31.0 mmol) by hydrogenolysis was coupled with *N*$^{\alpha}$-*p*-methoxybenzyloxycarbonyl-*N*$^{\delta}$-benzyloxycarbonyl-L-ornithine[35] (31.5 mmol) by the mixed anhydride method. The product was purified in a manner similar to that for II and weighed 19.47 g (77.0%). Mp 135—138°C; $[\alpha]^{30}_{D}$ −52.4° (*c* 1, methanol); Tlc: R_f 0.55 (Solv. 1).

Found: C, 64.59; H, 7.17; N, 8.58%. Calcd for $C_{44}H_{57}N_5O_{10}$: C, 64.77; H, 7.04; N, 8.58%.

Ethyl N$^{\delta}$-*Benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolinate Hydrochloride (XI).* To a solution of X (12.24 g, 15 mmol) in 120 ml of dioxane was added 5.5 M hydrogen chloride in dioxane (68.2 ml) in an ice bath, and the solution was stirred at room temperature. After 45 min a white precipitate appeared and the reaction was completed in

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[32] D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.* **30**, 1190 (1958).

[33] W. Oelofsen and C. H. Li, *J. Org. Chem.*, **33**, 1581 (1968).

[34] E. Klieger, E. Schröder, and H. Gibian, *Ann. Chem.*, **640**, 157 (1961).

[35] S. Sōfuku, M. Mizumura, and A. Hagitani, This Bulletin, **43**, 177 (1970).

[36] M. Ohno, T. Kato, S. Makisumi, and N. Izumiya, *ibid.*, **39**, 1738 (1966).

4 hours. The product, a white hygroscopic precipitate, was obtained in a reasonable yield after evaporation of the solvent *in vacuo* and washing with ethyl ether by decantation. Mp 126—130°C; Tlc: R_f 0.05 (Solv. 1).

Ethyl p-Methoxybenzyloxycarbonyl-L-valyl-N$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolinate (XII). This was obtained from *p*-methoxybenzyloxycarbonyl-L-valine[35] (15.0 mmol) and XI (15.0 mmol) by the method for VI. The crude product was recrystallized from ethyl acetate. Yield 12.27 g (89.4%); mp 153—156°C; $[\alpha]_D^{23}$ −29.5° (*c* 1, dimethylformamide), (lit,[16] mp 149—150°C; $[\alpha]_D^{24}$ −26.8° (*c* 2, dimethylformamide)), Tlc: R_f 0.65 (Solv. 3). (Found: C, 64.33; H, 7.38; N, 9.02%).

p-Methoxybenzyloxycarbonyl-L-valyl-N$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline (XIII).[16] XII (2.29 g, 2.5 mmol) was saponified according to Ref. 16. The reaction mixture was acidified to pH 4.2 with 25 ml of 2.2% citric acid on cooling, and concentrated *in vacuo*. The residue was washed with 5% citric acid and water, and dried under reduced pressure. Recrystallization from methanol and ethyl ether gave 1.75 g (78.8%) of the product. Mp 149—150.5°C; $[\alpha]_D^{21}$ −31.9° (*c* 1, dimethylformamide); Tlc: R_f 0.5 (Solv. 3).

Found: C, 62.68; H, 7.19; N, 9.54%. Calcd for $C_{47}H_{62}N_6O_{11}\cdot H_2O$: C, 62.36; H, 7.13; N, 9.29%.

L-Valyl-N$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline Hydrochloride (XIV). When XIII (1.24 g, 1.6 mmol) was subjected to the same treatment as for V, the product was obtained in a theoretical yield. Mp 153—157°; Tlc: R_f 0.1 (Solv. 1).

p-Methoxybenzyloxycarbonyl-L-valyl-N$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-leucyl-δ-aminovaleryl-L-valyl-N$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline (XV). To a suspension of 1.21 g (1.6 mmol) of VII in 26 ml of dimethylformamide were added 5.5 N hydrogen chloride in dioxane (1.12ml) and 0.43 ml of *n*-butyl nitrite.[37] To this mixture a solution of 1.6 mmol of XIV and 0.44 ml of triethylamine in 38 ml of dimethylformamide was added dropwise at −20—−30°C, and the stirring was continued at this temperature for 15 min and at 0°C for 2 days. The crude product precipitated on pouring the reaction mixture into 500 ml of cold water was filtered and washed with cold 5% citric acid and water and dried. It was dissolved in 130 ml of dimethylformamide and the insoluble material was removed by filtration. The product was precipitated by addition of ethyl ether into the filtrate. After filtering off and drying it weighed 1.614 g (69.7%). Mp 225—227°C (decomp.). It was then recrystallized from a large quantity of methanol. Yield, 1.354 g (58.5%); mp 226°C (decomp.); $[\alpha]_D^{21}$ −34.4° (*c* 0.5, dimethylformamide); Tlc: R_f 0.55 (Solv. 4).

Found: C, 63.19; H, 7.73; N, 10.69%. Calcd for $C_{76}H_{107}N_{11}O_{17}$: C, 63.09; H, 7.46; N, 10.65%.

Cyclo-L-valyl-N$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-leucyl-δ-aminovaleryl-L-valyl-N$^{\delta}$-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl (XVI). The reaction of XV (723 mg, 0.5 mmol) and dinitrophenylsulfite[38] (1.62 g, 5 mmol) in a mixture of pyridine (6 ml) and dimethylformamide (20 ml) at room temperature for 41 hours gave the *p*-nitrophenyl ester of XV. After evaporation of the reaction mixture *in vacuo* and treatment of the residue with a mixture of *n*-hexane and ethyl ether (1 : 1), the dried colorless product was dissolved in trifluoroacetic acid (5 ml) containing anisole (0.8 ml) in an ice-salt bath. After 20 min, the reaction mixture was concentrated *in vacuo* at 0°C. The residue was triturated with ethyl ether and the supernatant liquid removed by decantation. A solution of the residue in dimethylformamide (10 ml) containing acetic acid (0.13 ml) was added dropwise into 330 ml of anhydrous pyridine with stirring at 58—60°C over 2.5 hours. The reaction mixture was further stirred for 5 hr at this temperature and then evaporated to dryness *in vacuo*. The yellow residue was dissolved in a mixture of methanol and water (7 : 1) and this solution was passed successively through columns of Dowex-1 (OH^- form) and Dowex-50 (H^+ form) which had been washed with the same solvent. The effluent (total 300 ml) was concentrated to dryness *in vacuo* and a white crystalline product was obtained in a 81% yield, mp 212—215°C. It was recrystallized from 90% methanol. Yield, 446.7 mg (70.6%); mp 221—223°C; $[\alpha]_D^{27}$ −207.0° (*c* 0.5, methanol); Tlc: R_f 0.6 (Solv. 1), 0.65 (Solv. 4), 0.95 (Solv. 6); MW, Found: 1224.5 (Calcd for $C_{67}H_{97}N_{11}O_{13}$: 1264.6).

Found: C, 63.43; H, 7.87; N, 11.93%. Calcd for $C_{67}H_{97}N_{11}O_{13}$: C, 63.64; H, 7.73; N, 12.18%.

Cyclo-L-valyl-L-ornithyl-L-leucyl-δ-aminovaleryl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl Dihydrochloride (XVII). XVI (252.9 mg, 0.2 mmol) in *ca.* 8 ml of methanol containing 0.89 N methanolic hydrogen chloride (0.68 ml) was hydrogenolyzed in the presence of palladium black (30 mg) for 8 hours. After removal of the catalyst, the filtrate was concentrated to dryness *in vacuo*. The residue was dissolved in water and the solution was filtered through active charcoal. A white crystalline product was obtained by lyophilization of the filtrate which was dried in a desiccator over calcium chloride. Yield, 192.9 mg (90.2%); mp 251—252 °C (decomp.); $[\alpha]_D^{28}$ −204.0° (*c* 0.1, ethanol); Tlc: R_f 0.75 (Solv. 6); IR: 1530, 1640 cm^{-1} (KBr). Amino acid ratios: Val, 2.09; Orn, 1.90; Leu, 2.09; Phe, 1.00; δAva, 0.83. When the sample dried in a vacuum over phosphorous pentoxide at 105°C for 6 hours was left standing in the air, it rapidly absorbed moisture. The increase in weight was 9.6%, which was in good agreement with one (10.5%) calculated from change of the anhydrous form to the heptahydrate.

Found: C, 51.16; H, 8.59; N, 12.71; Cl, 6.38%. Calcd for $C_{51}H_{87}N_{11}O_9Cl_2\cdot 7H_2O$: C, 51.24; H, 8.52; N, 12.89; Cl, 5.93%.

L-Valyl-L-ornithyl-L-leucyl-δ-aminovaleryl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline Trihydrochloride (XVIII). XV (434 mg, 0.3 mmol) was added into 25% hydrogen bromide in acetic acid (2.5 ml) in an ice bath. The solution was shaken at room temperature for one hour and concentrated *in vacuo*. Treatment of the oily residue with cold ethyl ether afforded a hygroscopic solid, which was collected by filtration and dried rapidly under reduced pressure. An aqueous solution of the product was charged to a column (1.5×13 cm) of Dowex-50 (H^+ form) and the column was washed with water. Pyridine-acetate buffer was used for elution and the effluent was collected in *ca.* 7 ml fractions, each of which was tested by thin layer chromatography with ninhydrin. Buffers[39] of pH 4.87, 4.96, and 5.13 were used for fraction numbers 1—37, 38—50, 51—65, respectively. Fraction nos. 21—65 were combined and concentrated *in vacuo*. Addition of water to the residue and evaporation were repeated several times to remove pyridine and acetic acid completely. The product was dissolved in a small amount of methanol. Hydrogen chloride in tetrahydrofuran (4.09 N, 3 ml) was added to it. The solution was

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[37] W. A. Noyes, "Organic Syntheses," Coll. Vol. II, p. 108 (1943).

[38] B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1760 (1960).

[39] J. L. Bailey, "Techniques in Protein Chemistry," Elsevier Publishing Co., Amsterdam (1967), p. 105.

concentrated to dryness *in vacuo* and the residue was recrystallized from methanol and ethyl ether. Yield, 223 mg (66.2%); mp 223—225°C (decomp.); $[\alpha]^{18}_{\mathrm{D}}$ $-46.3°$ (*c* 0.2, ethanol); Tlc: *R*$_f$ 0.1 (Solv. 5), 0.8 (Solv. 6); IR: 1531, 1640, 1727 cm^{-1} (KBr). Amino acid ratios: Val, 1.85; Orn, 1.91; Leu, 2.06; Phe, 1.00; δAva, 1.11.

Found: C, 50.43; H, 8.27; N, 12.69; Calcd for $C_{51}H_{90}N_{11}O_{10}Cl_3 \cdot 5H_2O$: C, 50.47; H, 8.30; N, 12.96%.

Antimicrobial activity of XVIII for some microorganisms (*Bacilus subtilis*, *Staphylococcus aureous*, *Micrococcus flavus*, *Sarcina lutea*, *Proteus vulgaris etc.*) was examined by the agar dilution method, but no inhibition was observed in concentrations below 100 μg/ml.

Ethyl L-*Valyl*-N$^{\delta}$-*benzyloxycarbonyl*-L-*ornithyl*-L-*leucyl*-D-*phenylanalyl*-L-*prolinate Hydrochloride (XIX).* To a solution of XII (2.29 g, 2.5 mmol) in 20 ml of dioxane was added 3.73 N hydrogen chloride in dioxane (20 ml). The solution was stirred at room temperature for 3 hours and then concentrated to dryness *in vacuo*. The product was obtained as oil in a theoretical yield. Tlc: *R*$_f$ 0.35 (Solv. 2).

Ethyl p-*Methoxybenzyloxycarbonyl*-L-*valyl*-N$^{\delta}$-*benzyloxycarbonyl*-L-*ornithyl*-L-*leucyl*-δ-*aminovaleryl*-L-*valyl*-N$^{\delta}$-*benzyloxycarbonyl*-L-*ornithyl*-L-*leucyl*-D-*phenylalanyl*-L-*prolinate (XX).* VII (1.89 g, 2.5 mmol) was dissolved in 60 ml of dimethylformamide containing 10 ml of acetic acid; 190 mg of sodium nitrite in 4 ml of water and 7.5 ml of 1 N hydrochloric acid were then added at 0—5°C under vigorous stirring. After 15 min, the reaction mixture was poured into cold water (700 ml). In a cold room the white precipitate was filtered and washed on a funnel successively with cold water, 5% sodium bicarbonate, and water. This peptide azide was dried in a desiccator over phosphorous pentoxide. The azide was added to a solution of XIX (2.5 mmol) and 0.35 ml of triethylamine in 85 ml of dimethylformamide, and the mixture was stirred for 7 days at 0°C. The insoluble substance was removed by filtration and the filtrate was poured into water. The precipitate was collected, and washed with 5% citric acid, water, 5% sodium bicarbonate, and water. After drying, the product was purified from dimethylformamide and ethyl ether. Yield, 2.18 g (59.2%); mp 218—225°C (decomp.); $[\alpha]^{21}_{\mathrm{D}}$ $-32.4°$ (*c* 0.5, dimethylformamide); Tlc: *R*$_f$ 0.6 (Solv. 2).

Found: C, 63.52; H, 7.59; N, 10.45%. Calcd for $C_{78}H_{111}N_{11}O_{17}$: C, 63.64; H, 7.85 N, 11.29%.

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