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Studies of Peptide Antibiotics. XX. Synthesis of Tyrocidine B[1]

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A cyclic decapeptide, *cyclo*-(L-Trp-D-Phe-L-Asn-L-Gln-L-Tyr-L-Val-L-Orn-L-Leu-D-Phe-L-Pro-) (XVIII), having the amino acid sequence of natural tyrocidine B has been synthesized. *p*-Methoxybenzyloxycarbonyl-Trp-D-Phe-OH was transformed into an acyldipeptide hydroxysuccinimide ester by reagents of hydroxysuccinimide-dicyclohexylcarbodiimide, and the acyldipeptide ester obtained was coupled with a neutral octapeptide. An acyldecapeptide acid thus obtained was subjected to the cyclization reaction. The antibacterial activity of the synthetic product (XVIII) toward Gram positive microorganisms is found to be nearly the same as that of synthetic tyrocidine A, indicating that the L-phenylalanine residue in tyrocidine A can be replaced by L-tryptophan without causing decrease in activity.

In 1940 Hotchkiss and Dubos discovered a basic polypeptide antibiotics named tyrocidine from autolyzed cultures of *Bacillus brevis*.[2] By means of countercurrent distribution,[3] Battersby and Craig showed that the crystalline tyrocidine hydrochloride contained at least three major components, tyrocidine A (TA), B (TB) and C. In 1955 King and Craig isolated a crystalline hydrochloride of TB and determined its amino acid composition.[4] The same authors determined its structure shown as TB in Fig. 1[5] by analyses on partial hydrolyzate with hydrochloric acid.[6]

The synthesis of a linear heptapeptide, Z-Val-Orn(δ-Ts)-Leu-Phe-Pro-Trp-Phe-OMe, related to TB has been described by Zahn and Brandenburg,[7] but the total synthesis of TB has never been accomplished. There are no reports on the quantitative feature of antibacterial activity nor data of measurements such as specific rotation and melting point on the natural TB.

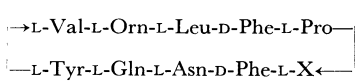

Fig. 1. Structures of tyrocidine A (TA) and B (TB).
X represents an amino acid residue such as Phe (TA) and Trp (TB or XVIII).

We reported the syntheses of TA,[8] tyrocidine C,[9] and E,[10] and have been attempting to synthesize other components of the tyrocidine family. This paper will describe the synthesis of the cyclic decapeptide hydrochloride (XVIII·HCl) having the amino acid sequence of TB, and the chemical and biological properties of the synthetic product.

Figure 2 indicates the scheme for the synthesis of the cyclic decapeptide (XVIII). The synthesis of a tripeptide derivative, Z(OMe)-Asn-Gln-Tyr-$NHNH_2$ (VII), was achieved by stepwise elongation from the carboxyl toward the amino end as shown in Fig. 2. The condensation of the azide derived from the tripeptide derivative (VII) with a neutral pentapeptide (XI) gave an acyloctapeptide acid (XII) in 87% yield. Action of trifluoroacetic acid on XII afforded amorphous trifluoroacetate of a neutral octapeptide (XIII) in an almost quantitative yield.

The synthesis of an acyldecapeptide acid (XIV) was achieved as follows. *p*-Methoxybenzyloxycar-

1) This work was presented at the 7th Symposium on Peptide Chemistry at the University of Tokyo, Tokyo, 21 November 1969, and communicated briefly; K. Kuromizu and N. Izumiya, *Experientia*, in press.

2) R. D. Hotchkiss and R. J. Dubos, *J. Biol. Chem.*, **132**, 791 (1940).

3) A. R. Battersby and L. C. Craig, *J. Amer. Chem. Soc.*, **74**, 4019 (1952).

4) T. P. King and L. C. Craig, *ibid.*, **77**, 6624 (1955).

5) Abbreviations; Z. benzyloxycarbonyl; Z(OMe), *p*-methoxybenzyloxycarbonyl; ONp, *p*-nitrophenyl ester; HOSu, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; TsOH, *p*-toluenesulfonic acid; CMC, carboxymethylcellulose; DMF, dimethylformamide; DMSO, dimethyl sulfoxide. Amino acid symbols except D-Phe denote the L configuration.

6) T. P. King and L. C. Craig, *J. Amer. Chem. Soc.*, **77**, 6627 (1955).

7) H. Zahn and D. Brandenburg, *Ann.*, **692**, 220 (1966).

8) M. Ohno and N. Izumiya, *J. Amer. Chem. Soc.*, **88**, 376 (1966); M. Ohno, T. Kato, S. Makisumi and N. Izumiya, This Bulletin, **39**, 1738 (1666).

9) K. Kuromizu and N. Izumiya, presented at the 7th Symposium on Peptide Chemistry at the University of Tokyo, 21 November 1969.

10) N. Mitsuyasu and N. Izumiya, *Experientia*, **26**, 476 (1970); N. Mitsuyasu, S. Matsuura, M. Waki, M. Ohno, S. Makisumi and N. Izumiya, This Bulletin, **43**, 1829 (1970).

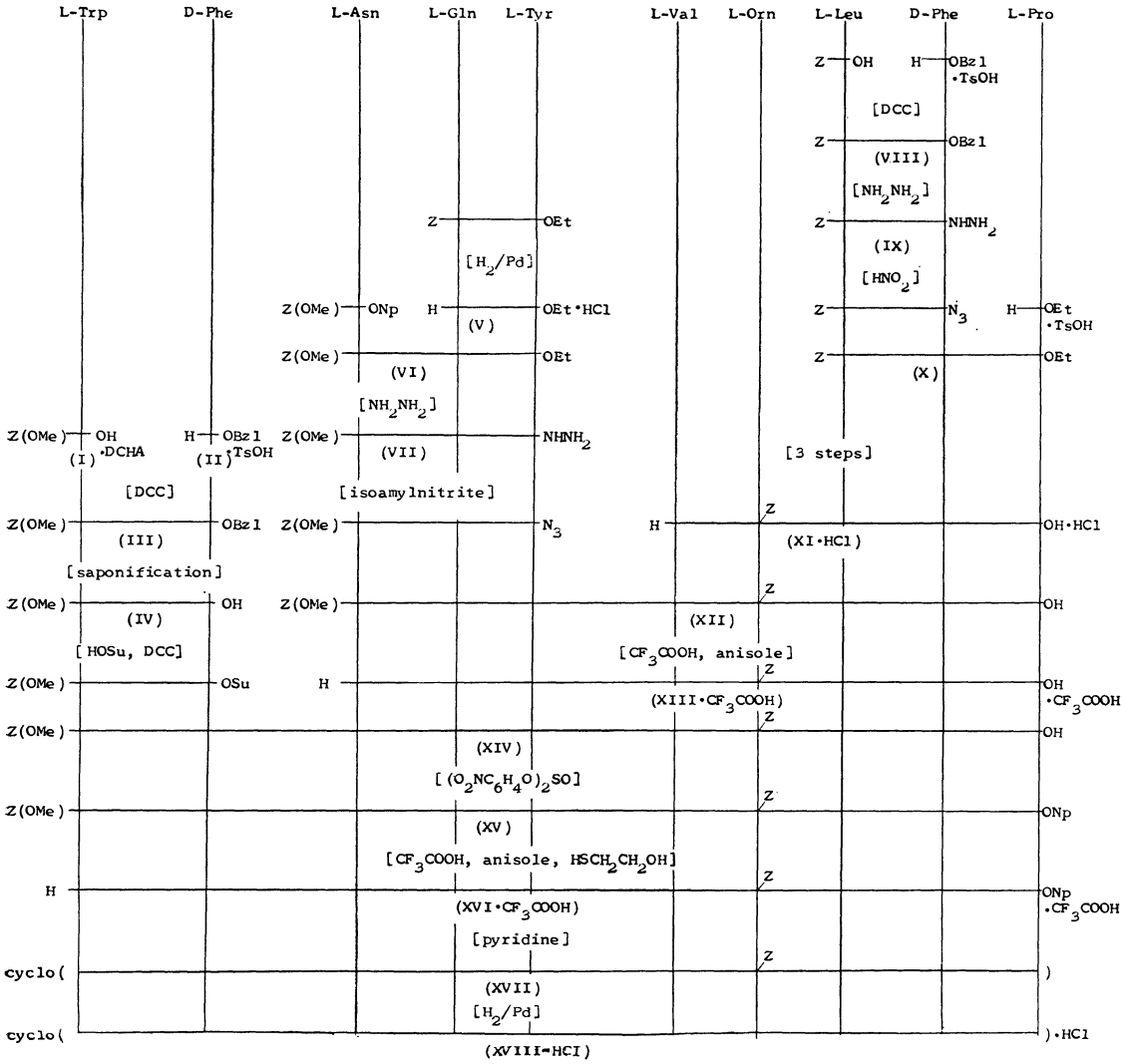

Fig. 2. Synthesis of a cyclic decapeptide hydrochloride (XVIII·HCl).

bonyldipeptide acid (IV) (1.2 equiv.) was treated with HOSu (1.4 equiv.) and DCC (1.1 equiv.). The acyldipeptide active ester, Z(OMe)-Trp-D-Phe-OSu, thus obtained was coupled with the neutral octapeptide (XIII)(1 equiv.) in the presence of triethylamine (1 equiv.) to afford the acyldecapeptide acid (XIV) in 70% yield. It was assumed that no racemization occurred on the D-Phe residue of H-Trp-D-Phe-Asn- sequence in XIV. Weygand *et al.* indicated that no racemization on a C-terminal amino acid residue in an acylpeptide acid was observed when the acylpeptide acid was treated with HOSu and DCC and subsequently the acylpeptide hydroxysuccinimide ester was coupled with an amine component.[11] Several investigators recognized also that the procedure with reagents of HOSu-DCC safely avoided racemization.[12,13] Furthermore, it was observed that the use of HOSu-DCC under the same conditions for preparation of acyldecapeptide acid (XIV) caused no racemization by an assay method using an amino acid analyzer.[13]

The protected cyclic decapeptide (XVII) was prepared as follows. Acyldecapeptide acid (XIV) was transformed to an acyldecapeptide *p*-nitrophenyl ester (XV), and its *p*-methoxybenzyloxycarbonyl group was removed by the action of trifluoroacetic acid. The cyclization reaction of the

11) F. Weygand, D. Hoffmann and E. Wünsch, *Z. Naturforsch.*, *B*, **21**, 426 (1966).

12) J. E. Zimmerman and G. W. Anderson, *J. Amer. Chem. Soc.*, **89**, 7151 (1967); G. S. Omenn and C. B. Anfinsen, *ibid.*, **90**, 657 (1968); R. Geiger, G. Jäger, W. König and A. Volk, *Z. Naturforsch.*, *B*, **24**, 999 (1969).

13) N. Izumiya and M. Muraoka, *J. Amer. Chem. Soc.*, **91**, 2391 (1969).

Table I. Inhibitory activity of the compounds on microorganisms
Minimum inhibitory concentration, μg/ml

	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Proteus vulgaris*	*Salmonella paratyphi*	*Pseudomonas aeruginosa*	*Shigella sonnea*	*Candida albicans*
XVIII	>100	3.13	100	>100	>100	>100	>100	>100
TA	>100	3.13	100	>100	>100	>100	>100	12.5
GS	1.56	0.78	25	>100	50	50	12.5	3.13

decapeptide ester (XVI) gave a benzyloxycarbonyl-substituted cyclic decapeptide (XVII); its yield was 43% from XIV. The molecular weight determination demonstrated that the molecular size of XVII corresponded to that of cyclic decapeptide.

The hydrogenation of XVII in the presence of an equivalent amount of hydrogen chloride in methanol afforded the crystalline cyclic decapeptide hydrochloride (XVIII·HCl) in 78% yield. The homogeneity of XVIII was confirmed by elemental analysis, chromatography using several different methods (Figs. 5 and 6) and amino acid analysis. The antibacterial activities of XVIII toward several microorganisms are listed in Table 1. It was found that the degree of the activities of XVIII toward the Gram positive microorganisms was nearly the same as that of TA, indicating that the L-Phe residue in a TA molecule can be replaced with the L-Trp without seriously influencing the activity.

Experimental

Melting points were uncorrected. The optical rotations were determined with a Yanagimoto Photometric Polarimeter, OR-20 type. Thin layer chromatography was performed on Merck silica gel G with the following solvent systems: R_f^1, *n*-butanol - acetic acid - pyridine - water 4 : 1 :1 : 2 v/v; R_f^2, chloroform - methanol 5 : 1 v/v. Paper chromatography was performed on Toyo Roshi No. 52 with the following solvent systems: R_f^I, *n*-butanol - acetic acid - pyridine - water 4 : 1 : 1 : 2 v/v; R_f^{II}, *n*-butanol - formic acid - water 15 : 3 : 2 v/v. Spots of materials possessing free amino groups on a thin layer plate were detected by spraying ninhydrin, and those of the amino group-blocked materials, by spraying 47% hydrobromic acid and then ninhydrin. Prior to analysis, the compounds were dried over phosphorus pentoxide at 60°C and 2 mmHg to a constant weight unless otherwise described.

Z(OMe)-Trp-OH·DCHA (I). To a chilled solution of L-tryptophan (10.2 g, 50 mmol) and 2N sodium hydroxide (25 ml) were added *p*-methoxybenzyloxycarbonyl chloride in ether[14] (15 ml, 60 mmol) and 2N sodium hydroxide (30 ml) portionwise at −5°C. After 2 hr, the reaction mixture was extracted with ether and acidified with 0.5M citric acid solution at 0°C. The oily material formed was extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulfate and evaporated *in vacuo*. The residual oil was dissolved in ether (200 ml) and dicyclohexylamine (9.98 ml, 50 mmol) was added. The resulting crystals were collected by filtration, and recrystallized from ethanol - ethyl acetate - ether; yield, 19.6 g (71%); mp 151—152°C; $[\alpha]_D^{20}$ +11.5° (*c* 1, methanol); R_f^2 0.54.

Found: C, 69.55; H, 7.83; N, 7.59%. Calcd for $C_{20}H_{20}O_5N_2 \cdot C_{12}H_{23}N$: C, 69.91; H, 7.88; N, 7.64%.

H-D-Phe-OBzl·TsOH (II). A mixture of D-phenylalanine (16.5 g), *p*-toluenesulfonic acid monohydrate (20.9 g), benzyl alcohol (50 ml) and benzene (100 ml) was heated following the general procedure by Izumiya and Makisumi.[15] Yield, 36.8 g (86%); mp 166—167°C; $[\alpha]_D^{20}$ +7.5° (*c* 2, DMF) (Found: C, 64.53; H, 5.80; N, 3.26%). Reported values for the L-isomer; mp 165°C[16] and 170.5—171.5°C;[17] $[\alpha]_D$ +7.2° (DMF)[16,18] and −7.2° (methanol).[17]

Z(OMe)-Trp-D-Phe-OBzl (III). To a solution of I (2.75 g, 5 mmol) and II (2.14 g, 5 mmol) in chloroform (20 ml) was added DCC (1.04 g, 5 mmol) at 0°C. After being stirred for 3 hr at 0°C, the reaction mixture was left to stand overnight at room temperature. The mixture was then evaporated *in vacuo*, and ethyl acetate was added to the residue. The filtrate from dicyclohexylurea was washed successively with 10% citric acid, 4% sodium bicarbonate solution and water, and then dried over sodium sulfate. The filtrate was evaporated *in vacuo* and the residue was crystallized by the addition of ether. It was recrystallized from ethyl acetate - ether - petroleum ether; yield, 2.49 g (82%); mp 137—139°C; $[\alpha]_D^{20}$ +3.6° (*c* 0.5, methanol); R_f^1 0.92, R_f^2 0.80.

Found: C, 71.47; H, 5.96; N, 6.85%. Calcd for $C_{36}H_{35}O_6N_3$: C, 71.38; H, 5.83; N, 6.93%.

Z(OMe)-Trp-D-Phe-OH (IV). To a solution of III (1.66 g, 2.75 mmol) in methanol (5 ml) and dioxane (8 ml) was added N sodium hydroxide (5.5 ml). The solution was allowed to stand for 2 hr at room temperature. After the addition of water (8 ml), the solution was acidified with 0.5M citric acid under cooling, and concentrated *in vacuo* to remove organic solvent at 10—15°C. After the addition of water (80 ml), the mixture was stored in a refrigerator for several hours and the crystals were collected by filtration. The product was recrystallized from methanol - ether - petroleum ether; yield, 1.14 g (80%); mp 84—86°C; $[\alpha]_D^{20}$ −25.0° (*c* 0.24, methanol); R_f^2 0.46.

14) S. Sakakibara, I. Honda, M. Naruse and M. Kanaoka, *Experientia*, **25**, 576 (1969).

15) N. Izumiya and S. Makisumi, *Nippon Kagaku Zasshi*, **78**, 1768 (1957).

16) N. Izumiya and S. Makisumi, *ibid.*, **78**, 662 (1957).

17) L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).

18) We wish to correct the value of the L-isomer as $[\alpha]_D^{13}$ −7.2° (*c* 2, DMF).

Found: C, 65.10; H, 5.75; N, 7.63%. Calcd for $C_{29}H_{29}O_6N_3 \cdot H_2O$: C, 65.27; H, 5.85; N, 7.87%.

H-Gln-Tyr-OEt·HCl (V). A solution of Z-Gln-Tyr-OEt[8] (6.89 g, 14.5 mmol) dissolved in DMF (60 ml) containing N ethanolic hydrogen chloride (16 ml) was hydrogenated in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo*, and the residual crystals were collected by filtration with the aid of ether. It was recrystallized from *n*-butanol - ether - petroleum ether; yield, 5.15 g (94%); mp 157—158°C; $[\alpha]_D^{20}$ +22.4° (*c* 1, methanol); R_f^1 0.67.

Found: C, 51.42; H, 6.47; N, 11.01%. Calcd for $C_{16}H_{23}O_5N_3 \cdot HCl$: C, 51.40; H, 6.47; N, 11.24%.

Z(OMe)-Asn-Gln-Tyr-OEt (VI). To a solution of V (4.51 g, 11.3 mmol) dissolved in DMF (20 ml) and triethylamine (1.58 ml) was added Z(OMe)-Asn-ONp[19] (4.17 g, 10 mmol) in DMF (10 ml). After the solution had been stood overnight at room temperature, it was concentrated *in vacuo* and diluted with water (300 ml). The crystalline product deposited was collected by filtration and washed successively with 10% citric acid, 3% sodium bicarbonate solution and water. It was recrystallized from DMF-ether; yield, 3.90 g (63%); mp 213—214°C (decomp.); $[\alpha]_D^{20}$ −5.5° (*c* 1, DMF); R_f^1 0.84.

Found: C, 55.49; H, 6.11; N, 11.02%. Calcd for $C_{29}H_{37}O_{10}N_5 \cdot \frac{1}{2}H_2O$: C, 55.75; H, 6.13; N, 11.21%.

Z(OMe)-Asn-Gln-Tyr-NHNH$_2$ (VII). A solution of VI (3.55 g, 5.8 mmol) and hydrazine hydrate (5.58 ml, 115 mmol) in DMF (30 ml) was allowed to stand at room temperature for 2 days. The solution was then concentrated *in vacuo* in order to remove excess hydrazine, after which water (300 ml) was added to the residual solution; the resulting product was collected by filtration. It was recrystallized from DMF - ether - petroleum ether; yield, 3.3 g (95%); mp 243°C (decomp.); $[\alpha]_D^{20}$ −11.0° (*c* 1, DMSO); R_f^1 0.68.

Found: C, 53.19; H, 6.00; N, 16.50%. Calcd for $C_{27}H_{35}O_9N_7 \cdot 1/2H_2O$: C, 53.10; H, 5.94; N, 16.06%.

Z-Leu-D-Phe-OBzl (VIII). Z-L-Leu-OH·DCHA (8.95 g, 20 mmol) and II (8.55 g, 20 mmol) in chloroform (80 ml) were treated with DCC (4.14 g, 20 mmol) as described for the preparation of III. The product was recrystallized from ethanol - ether; yield, 8.22 g (79%); mp 131°C; R_f^1 0.98, R_f^2 0.78; $[\alpha]_D^{20}$ −2.0° (*c* 0.5, methanol).

Found: C, 71.72; H, 6.95; N, 5.54%. Calcd for $C_{30}H_{34}O_5N_2$: C, 7.69; H, 6.82; N, 5.57%.

Z-Leu-D-Phe-NHNH$_2$ (IX). A solution of VIII (8.25 g, 1.57 mmol) and hydrazine hydrate (15 ml) in DMF (90 ml) was treated as described for the preparation of VII. The product was recrystallized from DMF - ether - petroleum ether; yield, 6.00 g (90%); mp 162—163°C; R_f^2 0.57; $[\alpha]_D^{20}$ +3.4° (*c* 1, DMF) (Found: C, 64.72; H, 7.18; N, 13.29%). Erlanger *et al.* prepared IX from Z-Leu-D-Phe-OMe and hydrazine; mp 166°C.[20]

Z-Leu-D-Phe-Pro-OEt (X). To a solution of IX (5.34 g, 12 mmol) dissolved in a mixture of DMF (5 ml) - acetic acid (50 ml) - N hydrochloric acid (13.2 ml) were added 2N sodium nitrite (6.6 ml) and N hydrochloric acid (13.2 ml) at −5°C. After it had stood for 5 min, cold water (900 ml) was added to the solution. The azide precipitated was collected by filtration and washed with cold 3% sodium bicarbonate solution and water. After it had been dried in a vacuum desiccator, the azide was added to a chilled solution of H-Pro-OEt·TsOH (3.94 g, 12.5 mmol) dissolved in a mixture of triethylamine (1.7 ml, 12.5 mmol) and DMF (50 ml). After it had been stirred for 2 days at 0°C, the solution was evaporated *in vacuo* and the residue was dissolved in ethyl acetate (80 ml). The solution was washed with 2% hydrochloric acid, 3% sodium bicarbonate solution and water, dried over sodium sulfate, and evaporated to dryness; yield of oil, 4.93 g (76%); R_f^2 0.81. This compound was used for the preparation of H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH·HCl (XI·HCl) as described in literature.[21]

Z(OMe)-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH (XII). To a solution of VII (0.722 g, 1.2 mmol) in a mixture of DMF (5 ml) and DMSO (10 ml) were added N ethanolic hydrogen chloride (2.4 ml) and isoamyl nitrite[22] (0.162 ml, 1.2 mmol) at −5°C. After 5 min, triethylamine (0.336 ml, 2.4 mmol) was added to this solution. To the solution containing the azide was added a mixture of XI·HCl (0.978 g, 1.25 mmol) and triethylamine (0.344 ml, 2.5 mmol) in DMF (8 ml). The reaction mixture was stirred for 3 days at 0°C and evaporated *in vacuo* to a small volume (about 10 ml). After the addition of N ethanolic hydrogen chloride (1.2 ml) and water (150 ml), the percipitate was collected and recrystallized from DMF - ether - petroleum ether; yield, 1.35 g (87%); mp 226—227°C; R_f^1 0.85; $[\alpha]_D^{25}$ −22.2° (*c* 1, DMF).

Found: C, 59.03; H, 6.88; N, 11.67%. Calcd for $C_{65}H_{85}O_{17}N_{11} \cdot 2H_2O$: C, 58.77; H, 6.75; N, 11.60%.

The homogeneity of this product was established further by paper chromatography and paper electrophoresis after hydrogenolysis; R_f^I 0.78 and R_f 0.99× GS.[23]

H-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH·CF$_3$COOH (XIII·CF$_3$COOH). To a mixture of XII (1.55 g, 1.2 mmol) and anisole (1 ml) was added trifluoroacetic acid (8 ml) at −5°C. When swirled, the reaction mixture changed to a clear solution within some 10 min. After 25 min, the solution was evaporated *in vacuo* at 0°C and the oily residue was triturated with ether. The solid formed was collected by filtration and washed with ether; yield, 1.45 g (97%). The homogeneity of this product was confirmed by paper chromatography in which only one spot was detected; R_f^I 0.85. The material was used in the next reaction without further purification.

Z(OMe)-Trp-D-Phe-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH (XIV). To a chilled solution of IV (0.805 g, 1.56 mmol) in ethyl acetate (10 ml) were added HOSu (0.207 g, 1.8 mmol) and DCC (0.297 g, 1.44 mmol) at 0°C. After the mixture had been stirred for 3 hr at 0°C, dicyclohexylurea precipitated was

19) E. Schröder and E. Klieger, *Ann.*, **673**, 208 (1964).

20) B. F. Erlanger, H. Sachs and E. Brand, *J. Amer. Chem. Soc.*, **76**, 1806 (1954).

21) M. Waki and N. Izumiya, *ibid.*, **89**, 1278 (1967); This Bulletin, **40**, 1687 (1967).

22) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

23) Paper electrophoresis was carried out for 2.5 hr under the following conditions: solvent, formic acid - acetic acid - methanol - water (1 : 3 : 6 : 10 v/v, pH 1.8); voltage gradient, 20 V/cm.

filtered off and washed with cold ethyl acetate. The filtrate was evaporated to dryness *in vacuo* at 0°C and the residue was dissolved in cold DMF (5 ml). To the solution was added a mixture of XIII·CF_3COOH (1.45 g, 1.27 mmol) in DMF (10 ml) and triethylamine (0.356 ml) at 0°C. After the stirring of the reaction mixture had been continued for 5 hr at 0°C and for additional 2 hr at room temperature, the mixture was concentrated *in vacuo* to a small volume and the residue was treated with 0.5M citric acid (30 ml). The product was collected by filtration and recrystallized from DMF-ethyl acetate - ether; yield, 1.35 g (70%); mp 215—216°C (decomp.); $[\alpha]_D^{20}$ +33.1° (*c* 1, DMF); R_f^1 0.79.

Found: C, 61.26; H, 6.42; N, 11.85%. Calcd for $C_{85}H_{104}O_{19}N_{14}\cdot 2H_2O$: C, 61.42; H, 6.55; N, 11.80%.

The homogeneity of this material was further established by paper chromatography and paper electrophoresis after hydrogenolysis, in which only one spot was detected; R_f^1 0.69 and R_f 1.1×GS.[23]

Detection of Possible Racemization in the Coupling by HOSu-DCC Reagents. The conditions (reaction times, temperatures, mole ratios of reagents, and others) for the coupling reaction were the same as described above. Z-Gly-L-Ala-OH (1.2 equiv.) in DMF was transformed to the corresponding hydroxysuccinimide ester with HOSu (1.4 equiv.) and DCC (1.1 equiv.). To the hydroxysuccinimide ester was added H-L-Leu-OBzl (1 equiv.) in DMF. After evaporation, the residue was dissolved in ethyl acetate. The solution was washed with 2% hydrochloric acid and 4% sodium bicarbonate solution, and dried over sodium sulfate. The filtrate was subjected to hydrogenolysis and a part of the hydrogenated material was analyzed by the amino acid analyzer as described previously,[13] the extent of racemization was calculated as less than 0.1%.

Z(OMe)-Trp-D-Phe-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-ONp (XV). To a solution of XIV (1.05 g, 0.65 mmol) dissolved in DMF (5 ml) and pyridine (5 ml) was added di-*p*-nitrophenyl sulfite (2.10 g, 6.5 mmol). The faintly yellow solution was allowed to stand for 18 hr at room temperature and then evaporated *in vacuo*. The oily residue was triturated several times with a mixture of ether and petroleum ether (1 : 1 v/v). The solid was collected by filtration and washed with a mixture of ether and petroleum ether until the yellow color could not be discerned upon the addition of N sodium hydroxide to the filtrate. The *p*-nitrophenyl ester content of this product (yield, 1.123 g) was estimated to be 105% measuring the optical density at 412 mμ with the solvent of DMF and N sodium hydroxide (1 : 1 v/v).[24] The product was used for the next reaction without further purification.

H-Trp-D-Phe-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-ONp·CF_3COOH (XVI·CF_3COOH). To a mixture of XV (1.12 g), anisole (1.2 ml) and 2-mercaptoethanol (0.12 ml) was added trifluoroacetic acid (12 ml) at −5°C. When swirled, the suspension turned to a clear solution within 10 min. The solution was evaporated *in vacuo* at 0°C and the oily residue was triturated with ether. The solid was collected by filtration and washed with ether (yield, 1.092 g). This was used as such in the next step.

***cyclo*-(Trp-D-Phe-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-) (XVII).** XVI·CF_3COOH (1.092 g) was dissolved in a mixture of DMF (15 ml) and acetic acid (0.5 ml). The solution was stirred drop by drop into pyridine (200 ml) kept at 55—60°C over 5 hr, stirring being continued for additional 3 hr at the same temperature. After the solvent had been removed, the residue was dissolved in 100 ml of a mixture of methanol - dioxane - water (1 : 2 : 1 v/v). This solution was passed successively through columns (1.5×12 cm) of Amberlite IRC-50 (H^+ form) and Amberlite IR45 (OH^- form). The columns were washed with the same solvent. The filtrate and washings were combined (total 300 ml) and then evaporated to dryness *in vacuo*. The residual product was collected by filtration with the aid of water and recrystallized from DMF-ether; yield, 0.715 g (43% from XIV); mp 198—200°C; $[\alpha]_D^{20}$ −86.9° (*c* 0.5, methanol); R_f^1 0.98, R_f^2 0.62; amino acid ratio in acid hydrolysate, Phe 1.99, Asp 0.98, Glu 1.02, Tyr 0.91, Val 0.97, Orn 1.00, Leu 1.00, Pro 0.99.[25]

Found: C, 61.79; H, 6.58; N, 13.05%; mol wt 1450.[26] Calcd for $C_{76}H_{94}O_{15}N_{14}\cdot 2H_2O$: C, 61.68; H, 6.68; N, 13.25%; mol wt, 1479.

***cyclo*-(Trp-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-D-Phe-Pro-) Hydrochloride (XVIII·HCl).** A solution of XVII (144 mg, 0.1 mmol) dissolved 0.02N methanolic hydrogen chloride (6 ml) was subjected to hydrogenolysis in the presence of palladium black. After 3.5 hr, the filtrate from the catalyst was evaporated to dryness *in vacuo* and ether (4 ml) was added to yield crystals (yield, 120 mg). Recrystallization from methanol - ether - petroleum ether gave 111 mg (78%) as the desiccator-dried product (XVIII·HCl·$5H_2O$); mp 236—237° (decomp.); $[\alpha]_D^{20}$ −93.0° (*c* 0.5, methanol).

Found: C, 56.71; H, 6.82; N, 13.62%. Calcd for $C_{68}H_{88}O_{13}N_{14}\cdot HCl\cdot 5H_2O$: C, 56.87; H, 6.95; N, 13.66%.

This desiccator-dried sample lost 6.4% of its weight after being dried for 4 hr at 110°C, 2 mmHg. Calcd for $5H_2O$: 6.3%.

Determination of Homogeneity of XVIII. The homogeneity of the compound XVIII·HCl·$5H_2O$ was ascertained further as follows. Data for TA or gramicidin S dihydrochloride (GS·2HCl) as the reference peptides are also indicated. *(a) TLC.* One spot with the com-

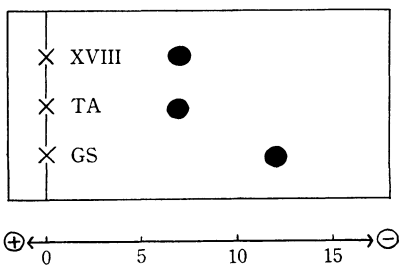

Fig. 3. Paper electrophoreses of XVIII·HCl, TA·HCl and GS·2HCl.

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

25) We are indebted to Mr. K. Noda in this laboratory for the amino acid analysis.

26) The molecular weight was determined on a Hitachi Osmometer, type 115, using methanol as a solvent.

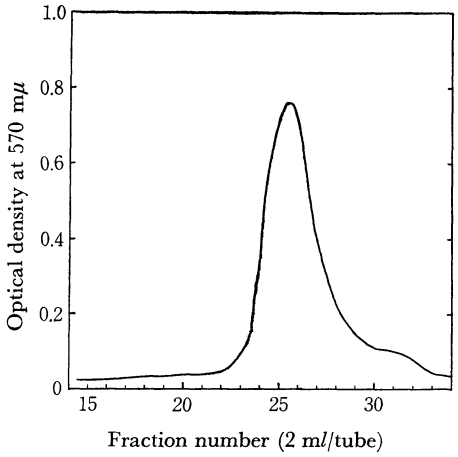

Fig. 4. CMC column chromatography of XVIII·HCl.

pound was detected; R_f^1 0.78 and R_f^2 0.02. That of TA·HCl; R_f^1 0.78 and R_f^2 0.02. *(b) Paper Chromatography.* R_f^{I} 0.98 and R_f^{II} 0.87 for the compound, R_f^{I} 0.98 and R_f^{II} 0.85 for TA·HCl. *(c) Paper Electrophoresis.*[23] The patterns of three compounds are shown in Fig. 3. The mobility of XVIII was found to be nearly the same as that of TA. *(d) CMC Column Chromatography.* A sample (0.5 mg) was dissolved in 0.4 ml of 0.2M pyridinium acetate buffer containing methanol (75% v/v, pH 5.0) and the solution was applied to a column (0.9×100 cm) with CMC. The mixture was developed with the same solvent and 2 ml fractions were collected at a flow rate of 12 ml per hr. The components were determined by the Yemm-Cocking method[27] after alkaline hydrolysis described previously.[8] From the chromatogram shown in Fig. 4, it was confirmed that XVIII was homogeneous. *(e) Amino Acid Analysis.* Amino acid ratios[25] in acid hydrolysate were as follows; Phe 1.86, Asp 1.01, Glu 1.02, Tyr 1.07, Val 0.97, Orn 1.00, Leu 0.97, Pro 0.96 and NH_3 2.20. Molar ratios of Trp and Tyr were determined in conformity with the extinction at 280 mμ and 294.4 mμ in ultraviolet spectrum of XVIII using a solvent of 0.1N soium hydroxide - ethanol (1 : 1 v/v) as in literature;[28] Trp 1.04, Tyr 1.05.

Microbiological Assays. The microorganisms employed are listed in Table 1. The minimum amount of the compounds necessary for the complete inhibition of growth was determined by a dilution method. TA and GS were examined as reference compounds. Table 1 indicates that the degree of the activities of synthetic XVIII toward the Gram positive microorganisms (*Staph. aureus* and *B. subtilis*) was nearly the same as that of TA except a microorganism, *Candida albicans*.

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27) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).

28) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).