BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 43 2944—2948 (1970)

Studies of Peptide Antibiotics. XXI. Synthesis of Tyrocidine C[1)]

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(Received April 3, 1970)

A cyclic decapeptide, *cyclo*-(L-Trp-D-Trp-L-Asn-L-Gln-L-Tyr-L-Val-L-Orn-L-Leu-D-Phe-L-Pro-), having the amino acid sequence of natural tyrocidine C has been synthesized. Chemical and biological properties of this product have been described. An antibiological activity of the product toward Gram positive microorganisms was found to be nearly the same degree as that of tyrocidine B.

In 1952 Battersby and Craig found that a crystalline tyrocidine contained at least three components, tyrocidine A (TA), B (TB) and C (TC), using a countercurrent distribution.[2)] In 1965 Craig and his coworkers isolated a pure crystalline hydrochloride of TC and determined its structure as shown in Fig. 1 by analyses on partial hydrolyzate with hydrochloric acid.[3)] However, they did not describe a quantitative feature of its antibacterial activity and some physical properties. Furthermore, a complete synthesis of TC was not accomplished.

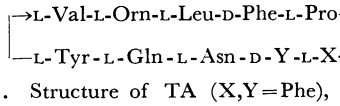

Fig. 1. Structure of TA (X,Y=Phe), TB (X=Trp, Y=Phe) and TC or IX(X, Y=Trp).

This paper will describe the synthesis of a cyclic decapeptide hydrochloride (IX·HCl) having an amino acid sequence of TC (Fig. 1), and the chemical and biological properties of a synthetic product.

Figure 2 indicates the scheme for synthesis of a

1) This work was presented at the 7th Symposium on Peptide Chemistry at the University of Tokyo, Tokyo, November 21 1969, and communicated briefly; K. Kuromizu and N. Izumiya, *Tetrahedron Lett.*, **1970**, 1471.

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

3) M. A. Ruttenberg, T. P. King and L. C. Craig, *Biochemistry*, **4**, 11 (1965).

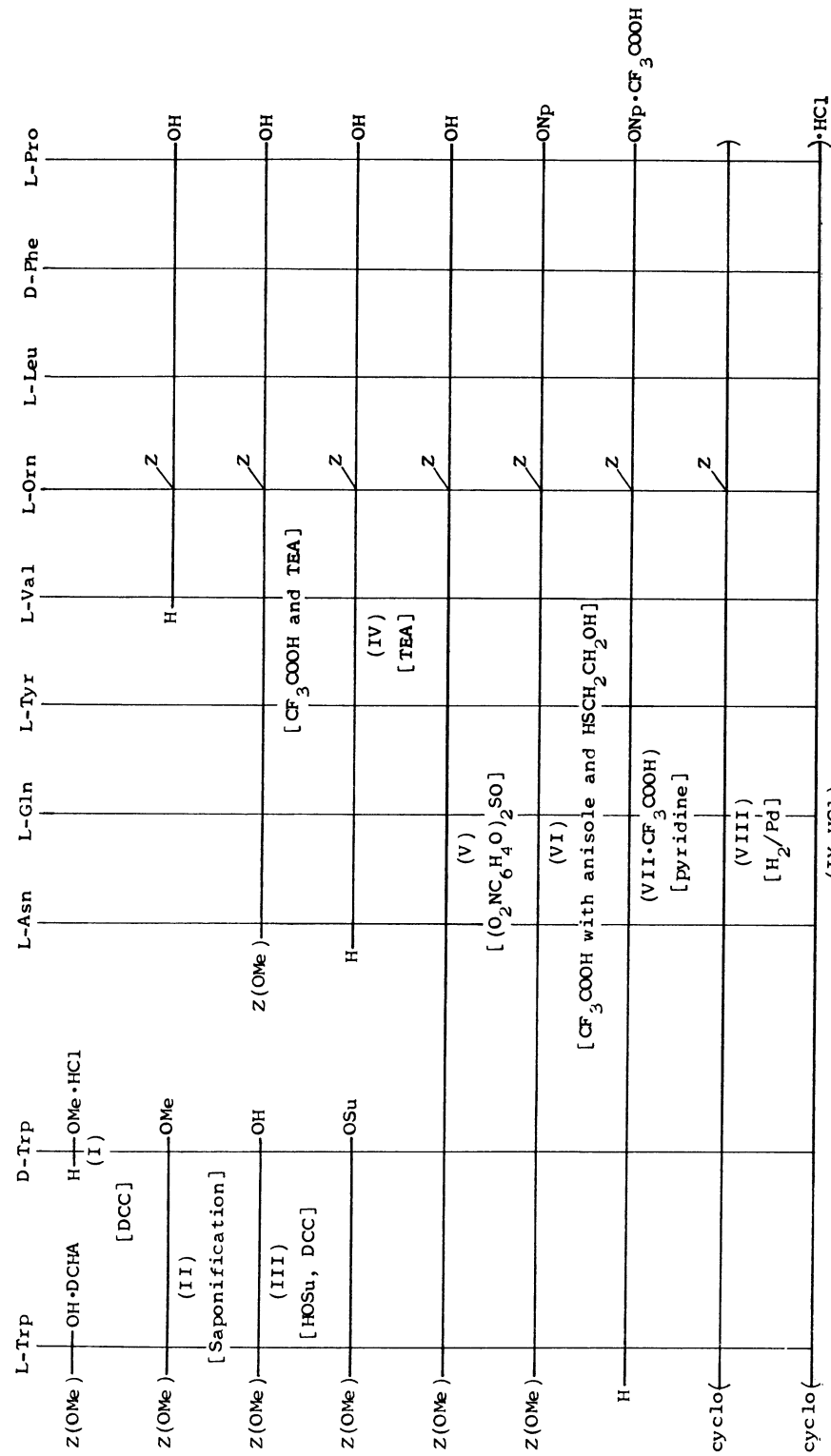

Fig. 2. Synthesis of a cyclic decapeptide hydrochloride (IX·HCl).

TABLE 1. 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*
IX	>100	6.25	>100	>100	>100	>100	>100	>100
TB	>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

desired decapeptide (IX).[4] Some peptide derivatives shown in the scheme had been prepared previously; a pentapeptide H-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH was prepared during our synthetic studies on TA[5] or tyrocidine E[6] and an octapeptide trifluoroacetate (IV·CF_3COOH) during our synthetic studies on TB.[7]

Strategy for synthesis of decapeptide (IX) is essentially similar as reported for TB synthesis.[7] *p*-Methoxybenzyloxycarbonyldipeptide acid (III) was coupled with a neutral octapeptide (IV) by a method using reagents of HOSu and DCC, and an acyldecapeptide acid (V) was obtained as a crystalline product. It was assumed that no racemization occurred on the D-Trp residue of -L-Trp-D-Trp-L-Asn- sequence in a decapeptide (V or IX) since we had observed[7] that the use of HOSu and DCC in the same condition in the present experiment caused no racemization by an assay technique developed in this laboratory.[8]

A desired decapeptide (IX) was prepared as follows. Acyldecapeptide acid (V) was transformed into a corresponding *p*-nitrophenyl ester (VI), and its *p*-methoxybenzyloxycarbonyl group was removed by trifluoroacetic acid. The cyclization reaction of a decapeptide ester (VII) by the usual way[5,7] gave a benzyloxycarbonyl-substituted cyclic decapeptide (VIII), its molecular weight determination demonstrating that molecular size of VIII corresponds to that of cyclic decapeptide. Hydrogenolysis of VIII in the presence of an equivalent of hydrogen chloride afforded a crystalline cyclic decapeptide hydrochloride (IX·HCl). Its homogeneity was ascertained by thin-layer and paper chromatography, paper electrophoresis and an amino acid analysis. Patterns of ultraviolet spectra on IX, synthetic TA and TB indicated that insertion of one (TB) or two (TC) Trp residues instead of Phe in a TA molecule increased intensities of absorbance at 280.5 mμ.

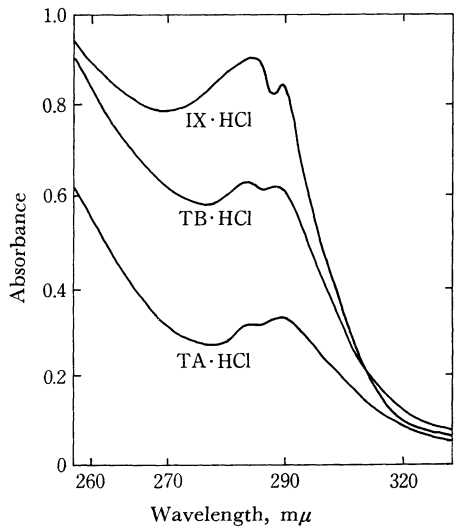

Fig. 3. Ultraviolet spectra of TA, TB and IX in 0.1 N NaOH-EtOH (1:1 v/v). Concentrations of TA·HCl, TB·HCl and IX·HCl; 9.04×10^{-5} M, 9.14×10^{-5} M and 7.05×10^{-5} M.

Antibacterial activities of IX toward several microorganisms are listed in Table 1, that of TA, TB and gramicidin S (GS) as reference compounds also being indicated. It was found that the degree of the activities of decapeptide (IX) was nearly the same as that of TA toward Gram positive (*Staph. aureus* and *B. subtilis*) and Gram negative microorganisms (*E. coli* and others) except for a microorganism, *Candida albicans*. The results indicate that both L- and D-Phe residues in a TA molecule can be replaced by L- and D-Trp residues, respectively, without a remarkable decrease in activity.

Experimental

Thin-layer chromatography was performed on Merck silica gel G with the following solvent systems: $R_f{}^1$, *n*-

4) Abbreviations; Z, benzyloxycarbonyl; Z(OMe), *p*-methoxybenzyloxycarbonyl; ONp, *p*-nitrophenyl ester; HOSu, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; TEA, triethylamine; DMF, dimethylformamide. Amino acid symbols denote L configuration otherwise noted.

5) 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 (1966).

6) N. Mitsuyasu and N. Izumiya, *Experientia*, **26**, 476 (1970); This Bulletin, **43**, 1829 (1970).

7) K. Kuromizu and N. Izumiya, *Experientia*, **26**, 587 (1970); This Bulletin, **43**, 2199 (1970).

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

butanol - acetic acid - pyridine - water 4 : 1 : 1 : 2 *v/v*; R_f^2, chloroform - methanol 5 : 1 *v/v*. Paper chromatography was performed with the following solvent system: R_f^1, the same solvent used for R_f^1.

H-D-Trp-OMe·HCl (I). D-Tryptophan (30 g, 14.7 mmol) and thionyl chloride (22 m*l*) in methanol (300 m*l*) were treated following the same procedure as for the preparation of L-isomer.[9] Yield, 40.8 g (92%); mp 212°C (decomp.); $[\alpha]_D^{20}$ −17.5° (*c* 1, MeOH). Reported values for the L-isomer; mp 216°C (decomp.)[9] and 214°C (decomp.).[10]

Z(OMe)-Trp-D-Trp-OMe (II). To a solution of Z(OMe)-Trp-OH dicyclohexylamine salt[7] (5.5 g, 10 mmol) and I (2.55 g, 10 mmol) in chloroform (40 m*l*) was added DCC (2.06 g, 10 mmol) at 0°C. After stirring had been continued for 3 hr at 0°C, the mixture was left to stand for overnight at room temperature. The mixture was evaporated *in vacuo*, and ethyl acetate was added to the residue. The filtrate from dicyclohexylurea was washed successively with 8% citric acid, 4% sodium bicarbonate solution and water, and dried over sodium sulfate. Evaporation of the filtrate yielded glass-like powder; 5.04 g (*ca.* 90%); mp 85—87°C; R_f^2 0.55; $[\alpha]^{20}$ −7.2° (*c* 1, MeOH).

Z(OMe)-Trp-D-Trp-OH (III). To a solution of II (2.54 g, 4.4 mmol) in methanol (16 m*l*) and dioxane (16 m*l*) was added N sodium hydroxide (8.8 m*l*). The solution was allowed to stand for 1 hr at room temperature. After addition of water (10 m*l*), the solution was concentrated *in vacuo* to remove organic solvent at 10—15°C, and acidified with 0.5 M citric acid under cooling. The oily product was extracted with ethyl acetate and the extract was dried over sodium sulfate. The filtrate was evaporated and the residue was crystallized by addition of ether and petroleum ether. It was recrystallized from ethyl acetate-ether-petroleum ether; yield, 2.10 g (86%); mp 115—116°C; R_f^2 0.34; $[\alpha]_D^{20}$ −21.6° (*c* 0.5, MeOH).

Found: C, 63.06; H, 5.78; N, 9.36%. Calcd for $C_{31}H_{28}O_6N_4 \cdot 2H_2O$: C, 63.25; H, 5.48; N, 9.52%.

H-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH (IV). To a mixture of Z(OMe)-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH[7] (0.65 g, 0.5 mmol) and anisole (0.5 m*l*) was added trifluoroacetic acid (3 m*l*) at −5°C. When swirled, the reaction mixture turned to a clear solution within some 10 min. After 15 min, the solution was evaporated *in vacuo* at 0°C and the oily residue was dissolved in DMF (5 m*l*). TEA (0.21 m*l*, 1.5 mmol) was added, the solution was evaporated *in vacuo*, and water (20 m*l*) was added to the residue. The precipitate collected was recrystallized from DMF - ethyl acetate - ether; yield, 0.453 g (80%); mp 285—287°C (decomp.); R_f^1 0.74; $[\alpha]_D^{20}$ −19.7° (*c* 0.8, DMF).

Found: C, 60.09; H, 6.86; N, 13.55%. Calcd for $C_{56}H_{77}O_{14}N_{11}$: C, 59.81; H, 6.88; N, 13.66%.

In a previous paper, we reported the preparation of IV trifluoroacetate as powder which was used for the coupling reaction with Z(OMe)-Trp-D-Phe-OH.[7]

Z(OMe)-Trp-D-Trp-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OH (V). To a chilled solution of III (0.276 g, 0.5 mmol) in ethyl acetate (3 m*l*) were added HOSu (0.067 g, 0.58 mmol) and DCC (0.095 g, 0.46 mmol) at 0°C. After the mixture had been stirred for 3 hr at 0°C, dicyclohexylurea precipitated was 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 (3 m*l*). To the solution was added a mixture of IV (0.48 g, 0.385 mmol) and TEA (0.055 m*l*, 0.39 mmol) in DMF (3 m*l*) at 0°C. After stirring 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 0.5 M citric acid solution (10 m*l*). The precipitate collected was recrystallized from DMF - ether; yield, 0.436 g (68%); R_f^1 0.82; mp 208—209°C (decomp.); $[\alpha]_D^{20}$ −23.6° (*c* 1, DMF).

Found: C, 61.89; H, 6.28; N, 12.40%. Calcd for $C_{87}H_{105}O_{19}N_{15} \cdot H_2O$: C, 62.08; H, 6.41; N, 12.48%.

Z(OMe)-Trp-D-Trp-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-ONp (VI). To a solution of V (0.333 g, 0.2 mmol) dissolved in DMF (3 m*l*) and pyridine (3 m*l*) was added di-*p*-nitrophenyl sulfite (0.65 g, 2 mmol). The solution was allowed to stand for 48 hr at room temperature and then evaporated *in vacuo*. After the oily residue was treated with a mixture of ether and petroleum ether, the powder was collected by filtration. The *p*-nitrophenyl ester content of this product (yield, 0.356 g) was estimated to be 104% measuring the optical density at 412 mμ.[7] This product was used for the next reaction without further purification.

***cyclo*-(Trp-D-Trp-Asn-Gln-Tyr-Val-Orn(δ-Z)-Leu-D-Phe-Pro-) (VIII).** To a mixture of VI (0.355 g) and 2-mercaptoethanol (0.05 m*l*)[11] in anisole (0.5 m*l*) was added trifluoroacetic acid (5 m*l*) at −5°C. After 10 min, the solution was evaporated at 0°C and the oily residue was triturated with ether. The powder was collected by filtration and washed with ether (yield of VII·CF_3COOH, 0.556 g). This was dissolved in a mixture of DMF (4 m*l*) and acetic acid (0.2 m*l*). The solution was added to pyridine (50 m*l*) kept at 55—60°C over 5 hr, stirring being continued for additional 3 hr at the same temperature. After evaporation, the residue was dissolved in 15 m*l* of a mixture of methanol - dioxane - water (1 : 2 : 1). The solution was passed successively through columns (0.9 × 12 cm) of Amberlite IRC-50 (H^+ form) and Amberlite IR-45 (OH^- form), the columns being washed with the same solvent. After the filtrate and washings (total 50 m*l*) were evaporated, the residual product was collected by filtration with the aid of water and recrystallized from DMF - ether; yield, 0.176 g (59% from V); mp 225—228°C (decomp.); R_f^1 0.82; $[\alpha]_D^{20}$ −36.1° (*c* 0.5, MeOH).

Found: C, 60.67; H, 6.63; N, 13.82%; mol wt 1550. Calcd for $C_{87}H_{95}O_{15}N_{15} \cdot 3H_2O$: C, 60.95; H, 6.62; N, 13.68%; mol wt 1537.[12]

***cyclo*-(Trp-D-Trp-Asn-Gln-Tyr-Val-Orn-Leu-D-Phe-Pro-) hydrochloride (IX·HCl).** A solution of VIII (76.8 mg, 0.05 mmol) in 0.02 N methanolic hydrogen chloride (2.5 m*l*) was hydrogenated in the presence of palladium black. After 4 hr, the filtrate from

9) R. A. Boissonnas, St. Guttmann, R. L. Huguenin, P. A. Jaquenoud and Ed. Sandrin, *Helv. Chim. Acta*, **41**, 1867 (1958).

10) E. Abderhalden and M. Kempe, *Z. Physiol. Chem.*, **52**, 207 (1907).

11) J. Blake and C. H. Li, *J. Amer. Chem. Soc.*, **90**, 5882 (1968).

12) Molecular weight was determined on a Hitachi Osmometer, type 115, using methanol as a solvent.

the catalyst was evaporated to dryness *in vacuo* and ether (4 m*l*) was added. Crystals collected were recrystallized from methanol - ethyl acetate - ether; yield of an air-dried product (IX·HCl·$6H_2O$), 59.7 mg (82%); mp 218—220°C (decomp.); $[\alpha]_D^{20}$ −84.3° (*c* 0.5, MeOH).

Found: C, 56.35; H, 6.86; N, 13.83%. Calcd for $C_{70}H_{89}O_{13}N_{15}$·HCl·$6H_2O$: C, 56.30; H, 6.88; N, 14.07%.

Ultraviolet spectra of TA·HCl, TB·HCl and IX·HCl with a Hitachi EPS-2 spectrophotometer are shown in Fig. 3. From the spectra, the characteristic features on each compound are observed; insertion of one (TB) or two (TC) Trp residues in a TA molecule increased intensities of absorbance in reasonable ratio.

Determination of Homogeneity of IX. Homogeneity of compound IX·HCl·$6H_2O$ was ascertained further as follows. Data of TA·HCl, TB·HCl and GS·2HCl as reference peptides are also indicated.

(a) Thin-layer Chromatography. One spot was detected on IX; 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^1 0.96 for IX, R_f^1 0.96 for TA·HCl and TB·HCl.

(c) Paper Electrophoresis.[13] Mobility of IX was found to be nearly the same as that of TA and TB; R_f, 0.60 × GS.

(d) Amino Acid Analysis.[14] Amino acid ratios in acid hydrolyzate were as follows; Asp 0.97, Glu 1.05, Tyr 0.82, Val 1.00, Orn 1.04, Leu 1.07, Phe 0.99, Pro 1.06 and NH_3 2.30. Molar ratios of Trp and Tyr were determined from ultraviolet spectrum of IX in a similar manner as described previously;[7] Trp 1.80, Tyr 1.05.

Microbiological assays.[15] The microorganisms employed are listed in Table 1. In addition to IX, a series of TA, TB and GS were examined as reference compounds. Table 1 indicates that the degree of activities of synthetic IX toward Gram positive (*Staph. aureus* and *B. subtilis*) and Gram negative (*E. coli* and others) microorganisms is nearly the same as that of TB, and TA except for a microorganism, *Candida albicans*.

13) Paper electrophoresis was carried out 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.

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

15) We are indebted to Meiji Seika Co., Ltd. for microbiological assays.