Studies of Peptide Antibiotics. XXXIV.[1] Syntheses of Tyrocidine A and Its Analogs Containing Glycine

Kouji OKAMOTO,* Kazuhiko NONAKA, and Nobuo IZUMIYA

Laboratory of Biochemistry, Faculty of Science, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812

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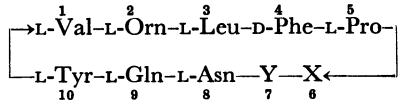
The synthesis of Tyrocidine A (TA), an antibiotic cyclic decapeptide, was achieved by a revised conventional method. Two analogs of TA, 6-glycine-TA and 7-glycine-TA, were synthesized by a similar method to investigate the contribution of L-phenylalanine[6] and D-phenylalanine[7] residues in TA to its antibacterial activity. The properties of synthetic TA were identical to natural TA. The antibacterial activities of the two analogs were weaker when compared with TA; the activity of 6-glycine-TA being weaker than 7-glycine-TA. The three cyclic peptides synthesized were subjected to droplet countercurrent chromatography (DCCC) and optical rotatory dispersion (ORD), and the elucidation of the difference in the activities between the two analogs was followed with DCCC and ORD.

Tyrocidine A (TA) is an antibiotic cyclic peptide isolated from *Bacillus brevis* and its structure has been proposed to be that of **18a** (Fig. 1). It is of interest to note that the pentapeptide sequence, L-Val–L-Orn–L-Leu–D-Phe–L-Pro, is found in gramicidin S (GS). In 1966, Ohno *et al.* synthesized a cyclic peptide corresponding to the sequence of TA by a conventional solution-phase method, and showed the synthetic peptide to be identical to natural TA.[2] Recently, we synthesized TA and its analogs by a solid-phase method.[3] As has been generally recognized for a solid-phase method, we observed that the procedure to synthesize the cyclic peptides, including TA, was easier compared with a solution-phase method; however the purity of the TA synthesized was inferior to that synthesized by the solution-phase method.[3] In the present study, therefore, we selected the solution-phase method for peptide synthesis.



	X	Y
TA (**18a**)	L-Phe	D-Phe
[Gly[6]]-TA (**18b**)	Gly	D-Phe
[Gly[7]]-TA (**18c**)	L-Phe	Gly

Fig. 1. Structure of TA and its analogs.

First we intended to synthesize pure TA through a revised route in a solution-phase method. In the previous paper,[2] Ohno *et al.* cyclized a decapeptide active intermediate, H–Phe[6]–D-Phe–Asn–Gln–Tyr–Val–Orn(Z)–Leu–D-Phe–Pro[5]–ONp,[4] as the key step. Recently, we carried out the cyclization reaction of a pentapeptide *via* several active intermediates and observed that the azide and *N*-hydroxysuccinimide intermediates were excellent ones in regards to high yield, experimental simplicity and absence of racemization.[5] By applying a mechanism of biosynthetic cyclization, Tanaka *et al.*[6] and Abe *et al.*[1] indicated that GS and its analogs could be obtained in excellent yields by the cyclization reaction of linear peptides. In *B. brevis*, TA is biosynthesized with the cyclization of a linear decapeptide (H–D-Phe[4]–Pro–Phe–D-Phe–Asn–Gln–Tyr–Val–Orn–Leu[3]–OH).[7] Considering the facts previously mentioned, a route of TA synthesis was chosen which would place a L-Leu[3] residue at the C-terminal and which would activate the carboxyl as an azide as shown in Fig. 2. As described later, the route was effective, giving pure TA in good yield.

Second, we intended to clarify the contribution of L-Phe[6] and D-Phe[7] residues in the TA molecule to its biological activity. For this purpose, we designed the following syntheses of two analogs, [Gly[6]]-TA and [Gly[7]]-TA (Fig. 1). The route for syntheses of these analogs was similar to that of TA synthesis shown in Fig. 2. The present paper reports the syntheses, and physicochemical and antibacterial properties of TA and its analogs.

For the syntheses of the three cyclic peptides (**18a—c**), a hexapeptide ester (**13**) was prepared by stepwise elongation from the carboxyl toward the amino end, and this same component (**13**) was used throughout the syntheses (Fig. 2). Boc-tetrapeptide–hydrazides

Table 1. Antibacterial activity of cyclic peptides (Minimum inhibitory concentration, μg/ml[a])

Compound	*Escherichia coli*	*Staphylcoccus aureus*	*Bacillus subtilis*	*Mycobacterium Takeo*
GS	>50 (>50)	5 (10)	5 (5)	>50 (>50)
Natural TA	>50 (>50)	20 (50)	10 (10)	>50 (>50)
Synthetic TA (**18a**)	>50 (>50)	20 (20)	10 (10)	>50 (>50)
[Gly[6]]-TA (**18b**)	>50 (>50)	>50 (>50)	50 (50)	>50 (>50)
[Gly[7]]-TA (**18c**)	>50 (>50)	50 (50)	20 (20)	>50 (>50)

a) The assays were carried out with a bouillon agar medium. Numbers in parentheses represent the concentration with a synthetic agar medium.

* Present address: Laboratory of Molecular Biophysics, University of Alabama, Birmingham, Alabama, U. S. A.

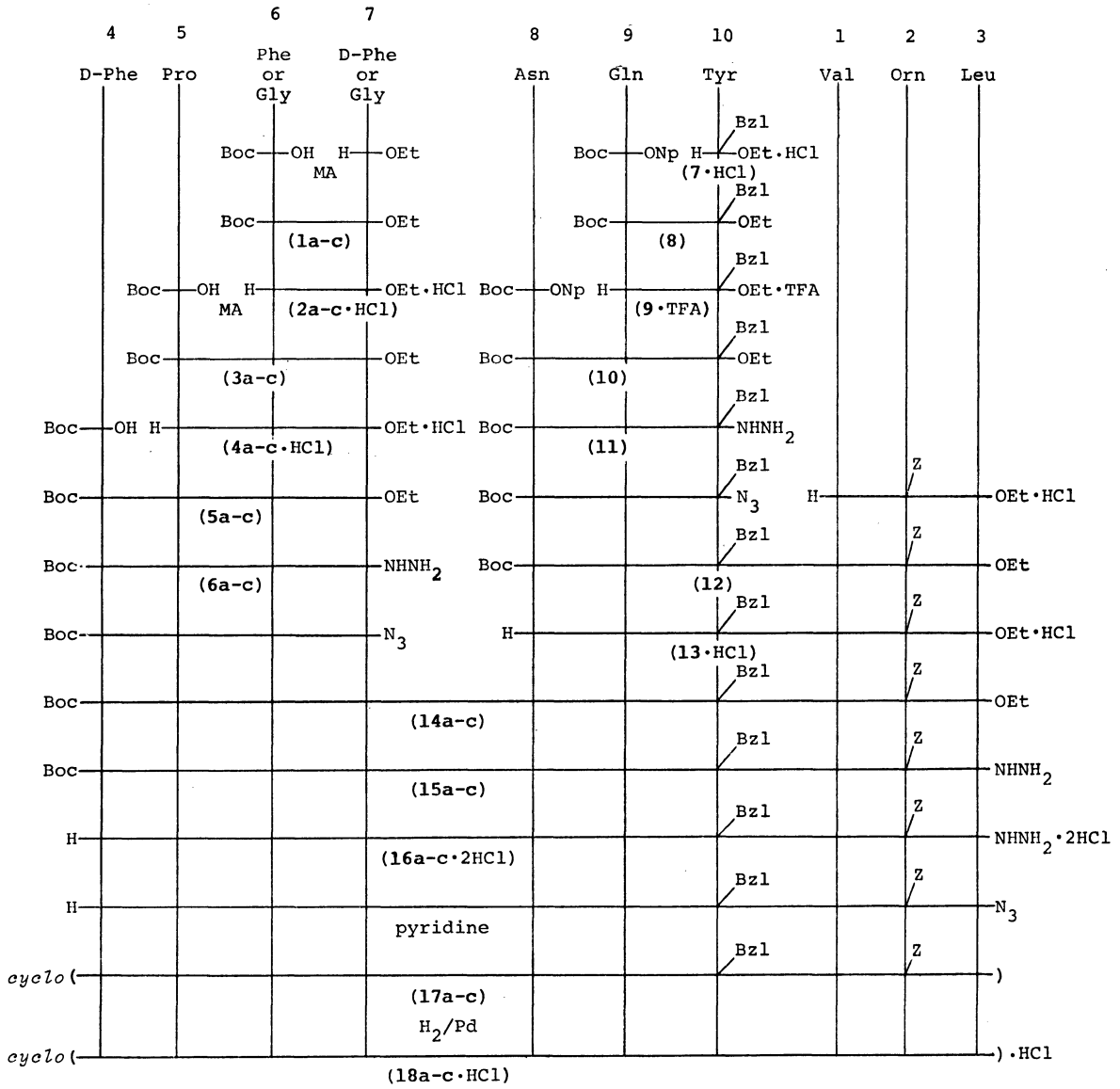


Fig. 2. Synthesis of TA and its analogs. **a**, -Phe^6-D-Phe^7-; **b**, -Gly^6-D-Phe^7-; **c**, -Phe^6-Gly^7-.

(**6a**—**c**) were also prepared by stepwise elongation. Boc-decapeptide ester (**14a**—**c**) was prepared by the coupling of **13** and each azide derived from **6a**—**c**; and a protected cyclic decapeptide (**17a**—**c**) was prepared by the cyclization reaction of each decapeptide azide derived from **16a**—**c**.

For the syntheses of TA (**18a**) and its analogs (**18b**—**c**), **17a**—**c** were subjected to hydrogenolysis, and the cyclic peptides were obtained as crystalline hydrochlorides (**18a**—**c**·HCl). In a previous paper,[8] we indicated that droplet countercurrent chromatography (DCCC) was very effective in the isolation of a desired peptide in a mixture of compounds of similar structures. Here, we applied DCCC for the detection of possible impurities in synthetic TA and the analogs. As shown in Fig. 3, each cyclic peptide (**18a**—**c**) gave a single peak without any additional peaks due to impurities.[9] The synthetic TA gave a single peak at the same position as natural TA. It is of interest to note that **18c** eluted faster than **18b** because **18c** is more hydrophobic in the organic phase in DCCC in spite of the fact that **18b** or **18c** is an analog which replaced only one Phe residue (position 6 or 7) with a Gly residue. The homogeneity of **18a**—**c** was further ascertained by paper and thin-layer chromatographies, paper electrophoresis, and elemental and amino acid analyses.

The antibacterial activities of **18a**—**c** toward several microorganisms were tested, the results shown in Table 1. It was proved that the specific activity of synthetic TA was identical to that of natural TA. Both analogs (**18b**—**c**) exhibited weak activity against *S. aureus* and *B. subtilis*; the results indicating that the aromatic side chain of Phe residue at position 6 and 7 was important for full activity, but not quite essential. At the beginning of this study, we expected that [Gly^7]-TA (**18c**) which the D-Phe^7 residue is replaced might possess weaker activity than [Gly^6]-TA (**18b**) which the L-Phe^6 is replaced because we assumed that the D-configuration at position 7 was more meaningful than L at position 6. Contrary to our expectation, the activity of **18c** was

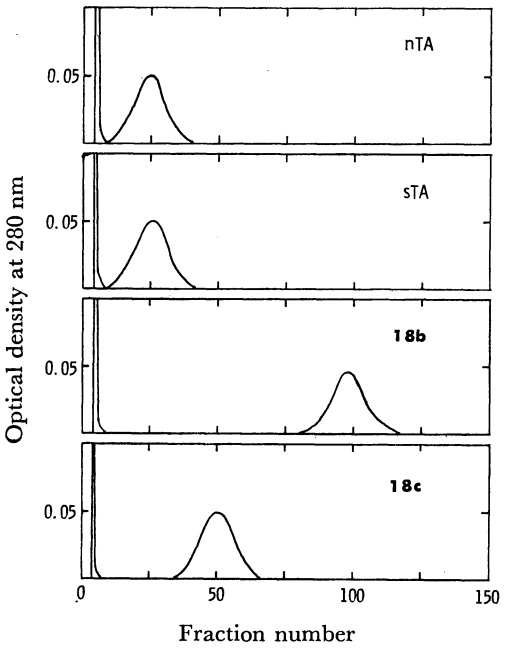


Fig. 3. Droplet countercurrent chromatography of the cyclic peptides. nTA, natural TA; sTA, synthetic TA (**18a**).

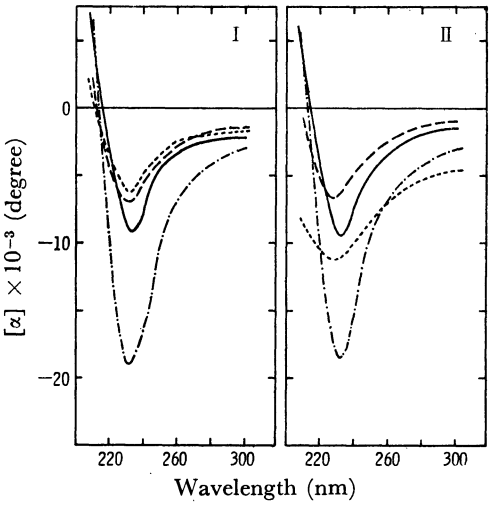


Fig. 4. ORD curves of the cyclic peptides. Solvent: I, 50% EtOH; II, 6 M urea in 50% EtOH. ——, nTA and sTA; – – –, [Gly⁶]-TA (**18b**); ------, [Gly⁷]-TA (**18c**); ·---·, GS.

slightly greater than **18b** (Table 1). We can give no definite explanation for the difference of specific activities between **18b** and **18c** from the standpoint of molecular structure, but we can show by the DCCC results that hydrophobicity of **18c** is greater than **18b** and is close to TA (Fig. 3).

The ORD curves of the peptides in 50% ethanol are shown in Fig. 4-I. The curve of synthetic TA was identical with that of natural TA. The negative troughs of the two analogs were shallower than that of TA, but the position at 233 nm was similar to TA and GS. In a solution of 6 M urea, causing denaturation of some polypeptides, the position of the troughs of the two analogs shifted slightly to 229 nm while that of TA and GS remained constant. We showed that conformations of the analogs are different (Fig. 4-I) from TA and more flexible (Fig. 4-II) than TA, and consequently the biological activities of the two analogs are lower than TA.

Experimental

Melting points were uncorrected. TLC was performed on Merck silica gel G with the following solvent systems: R_f^1, BuOH–AcOH–pyridine–H_2O (15:3:10:12, v/v); R_f^2, $CHCl_3$–MeOH (5:1, v/v); R_f^3, BuOH–AcOH–H_2O (4:1:5, v/v). Paper chromatography was performed on Toyo Roshi No. 52 with the following solvent systems: R_f^4, same solvent as that used for R_f^1; R_f^5, BuOH–formic acid–H_2O (15:3:2, v/v). Optical rotations were determined with a Union high sensitivity polarimeter PM-71.

*Boc–Phe–D-Phe–OEt (**1a**).* To a chilled solution of Boc–Phe–OH (3.98 g, 15 mmol) and Et_3N (2.1 ml, 15 mmol) in THF (22 ml) was added isobutyl chloroformate (1.97 ml, 15 mmol) at −5 °C. After 10 min, a chilled solution of H–D–Phe–OEt·HCl (3.45 g, 15 mmol) and Et_3N (2.1 ml, 15 mmol) in $CHCl_3$ (22 ml) was added. The mixture was left to stand at room temperature overnight, evaporated *in vacuo*, and the oily residue was dissolved in AcOEt. The solution was washed successively with 4% $NaHCO_3$, 10% citric acid and water, dried (Na_2SO_4), and evaporated. The resulting solid was recrystallized from EtOH–ether–petroleum ether; yield, 4.82 g (73%); mp 91—93 °C; $[\alpha]_D^{25}$ +1.6° (*c* 1, MeOH); R_f^1 0.98, R_f^2 0.90.

Found: C, 68.34; H, 7.35; N, 6.35%. Calcd for $C_{25}H_{32}O_5N_2$: C, 68.16; H, 7.32; N, 6.36%.

*Boc–Gly–D-Phe–OEt (**1b**).* This was prepared from Boc–Gly–OH (3.50 g) and H–D–Phe–OEt·HCl (4.59 g) as described for the preparation of **1a**; yield of oil, 6.42 g (92%); R_f^1 0.89, R_f^2 0.69.

*Boc–Phe–Gly–OEt (**1c**).* This was prepared from Boc–Phe–OH (3.90 g) and H–Gly–OEt·HCl (2.05 g) as described for the preparation of **1a**; yield, 3.64 g (71%); mp 100—101 °C (lit, mp 89.5—90 °C,[10] 88—89.5 °C[11]); $[\alpha]_D^{25}$ −5.0° (*c* 2, EtOH) (lit, $[\alpha]_D^{25}$ −4.3°,[10] −4.2°[11]); R_f^1 0.86, R_f^2 0.62.

*H–Phe–D-Phe–OEt·HCl (**2a**·HCl).* Compound **1a** (4.41 g, 10 mmol) was dissolved in 0.1 M hydrogen chloride in formic acid (120 ml). After being left to stand at room temperature for 20 min, the solution was evaporated to dryness, and the resulting solid was collected by filtration with the aid of ether–petroleum ether. The product was recrystallized from EtOH–ether–petroleum ether; yield, 3.56 g (94%); mp 103—106 °C; $[\alpha]_D^{25}$ +14.0° (*c* 0.5, MeOH); R_f^1 0.87, R_f^2 0.86.

Found: C, 63.40; H, 6.72; N, 7.37%. Calcd for $C_{20}H_{25}O_3N_2Cl$: C, 63.74; H, 6.69; N, 7.43%.

*H–Gly–D-Phe–OEt·HCl (**2b**·HCl).* Compound **1b** (3.68 g, 10.5 mmol) was treated with 0.1 M hydrogen chloride in formic acid (126 ml) as described for the preparation of **2a**·HCl; yield, 2.60 g (86%); mp 131—134 °C; $[\alpha]_D^{25}$ −11.2° (*c* 1, MeOH); R_f^1 0.72, R_f^2 0.24.

Found: C, 54.25; H, 6.71; N, 9.62%. Calcd for $C_{13}H_{19}O_3N_2Cl$: C, 54.45; H, 6.68; N, 9.77%.

*H–Phe–Gly–OEt·HCl (**2c**·HCl).* This was prepared from **1c** (3.50 g) as described for the preparation of **2a**·HCl; yield of oil, 2.80 g (98%); R_f^1 0.75, R_f^2 0.25.

*Boc–Pro–Phe–D-Phe–OEt (**3a**).* Boc–Pro–OH (1.94 g, 9 mmol) and **2a**·HCl (3.39 g, 9 mmol) were coupled by the mixed anhydride method as described for the preparation of **1a**. The product was recrystallized from AcOEt–ether–petro-

leum ether; yield, 2.95 g (61%); mp 103—105 °C; $[\alpha]_D^{25}$ −48.0° (*c* 1, MeOH): R_f^1 0.96, R_f^2 0.81.

Found: C, 66.83; H, 7.40; N, 7.86%. Calcd for $C_{30}H_{39}O_6N_3$: C, 67.02; H, 7.31; N, 7.82%.

Boc–Pro–Gly–D-Phe–OEt (3b). This was prepared from Boc–Pro–OH (1.72 g) and **2b**·HCl (2.29 g) as described above; yield, 2.81 g (78%); mp 117—119 °C; $[\alpha]_D^{25}$ −45.0° (*c* 1, MeOH); R_f^1 0.88, R_f^2 0.76.

Found: C, 62.12; H, 7.43; N, 9.42%. Calcd for $C_{23}H_{33}O_6N_3$: C, 61.72; H, 7.43; N, 9.39%.

Boc–Pro–Phe–Gly–OEt (3c). This was prepared from Boc–Pro–OH (2.0 g) and **2c**·HCl (2.66 g); yield, 2.88 g (69%); mp 109—111 °C; $[\alpha]_D^{25}$ −54.4° (*c* 0.25, MeOH); R_f^1 0.82, R_f^2 0.50.

Found: C, 61.42; H, 7.22; N, 9.32%. Calcd for $C_{23}H_{33}O_6N_3$: C, 61.72; H, 7.43; N, 9.39%.

H–Pro–Phe–D-Phe–OEt·HCl (4a·HCl). Compound **3a** (2.16 g, 4.02 mmol) was dissolved in 3.5 M hydrogen chloride in dioxane (34.3 ml). After being left to stand at room temperature for 2 h, the solution was evaporated, and the resulting hygroscopic solid was collected by filtration with the aid of ether–petroleum ether. The product was recrystallized from EtOH–ether–petroleum ether; yield, 1.37 g (72%); mp 93 °C; $[\alpha]_D^{25}$ −23.0° (*c* 0.5, MeOH); R_f^1 0.72, R_f^2 0.52.

Found: C, 63.74; H, 7.07; N, 8.56%. Calcd for $C_{25}H_{32}O_4N_3Cl$: C, 63.35; H, 6.81; N, 8.87%.

H–Pro–Gly–D-Phe–OEt·HCl (4b·HCl). Compound **3b** (2.51 g, 5.6 mmol) was treated with 3.5 M hydrogen chloride in dioxane (48.0 ml) as described above; yield of oil, 2.0 g (93%); R_f^1 0.78, R_f^2 0.30.

H–Pro–Phe–Gly–OEt·HCl (4c·HCl). This was prepared from **3c** (2.73 g) as described above; yield of oil, 1.96 g (84%); R_f^1 0.79, R_f^2 0.52.

Boc–D-Phe–Pro–Phe–D-Phe–OEt (5a). Boc–D-Phe–OH (0.76 g, 2.86 mmol) and **4a**·HCl (1.36 g, 2.86 mmol) were coupled by the mixed anhydride method as described for the preparation of **1a**; yield of oil, 1.81 g (92%); R_f^1 0.94, R_f^2 0.70.

Boc–D-Phe–Pro–Gly–D-Phe–OEt (5b). This was prepared from Boc–D-Phe–OH (1.38 g) and **4b**·HCl (2.0 g) as described above. The product was recrystallized from AcOEt–ether–petroleum ether; yield, 2.08 g (67%); mp 128—130 °C; $[\alpha]_D^{25}$ −36.2° (*c* 1, MeOH); R_f^1 0.82, R_f^2 0.53.

Found: C, 63.86; H, 7.02; N, 9.39%. Calcd for $C_{32}H_{42}O_7N_4 \cdot 1/2\ H_2O$: C, 63.66; H, 7.18; N, 9.28%.

Boc–D-Phe–Pro–Phe–Gly–OEt (5c). This was prepared from Boc–D-Phe–OH (1.35 g) and **4c**·HCl (1.96 g) as described above; yield, 2.28 g (75%); mp 139—141 °C; $[\alpha]_D^{25}$ −72.6° (*c* 1, MeOH); R_f^1 0.86, R_f^2 0.66.

Found: C, 63.29; H, 7.29; N, 9.35%. Calcd for $C_{32}H_{42}O_7\ N_4 \cdot 1/2H_2O$: C, 63.66; H, 7.18; N, 9.28%.

Boc–D-Phe–Pro–Phe–D-Phe–NHNH$_2$ (6a). A solution of **5a** (0.685 g, 1 mmol) and hydrazine hydrate (2.9 ml, 60 mmol) in MeOH (12 ml) was allowed to stand at 36 °C for 12 h. The solution was evaporated, water was added, and the resulting solid was collected. The product was recrystallized from MeOH–water; yield, 0.302 g (45%); mp 117—120 °C; $[\alpha]_D^{25}$ −67.0° (*c* 0.2, MeOH); R_f^1 0.89, R_f^2 0.83.

Found: C, 64.20; H, 6.85; N, 11.93%. Calcd for $C_{37}H_{46}O_6N_6 \cdot H_2O$: C, 64.51; H, 7.02; N, 12.20%.

Boc–D-Phe–Pro–Gly–D-Phe–NHNH$_2$ (6b). Compound **5b** (0.357 g) was treated with hydrazine hydrate (0.58 ml) in MeOH (5 ml) as described above. The solution was evaporated, and the residue was dissolved in AcOEt. The solution was washed with a small volume of water, dried (Na_2SO_4), and evaporated. The residual oil was crystallized by the addition of ether–petroleum ether; yield, 0.264 g (76%); mp 100—102 °C; $[\alpha]_D^{25}$ −6.0° (*c* 1, MeOH); R_f^1 0.87, R_f^2 0.74.

Found: C, 60.39; H, 6.97; N, 14.32%. Calcd for $C_{30}H_{40}O_6N_6 \cdot H_2O$: C, 60.18; H, 7.07; N, 14.04%.

Boc–D-Phe–Pro–Phe–Gly–NHNH$_2$ (6c). This was prepared from **5c** (0.357 g) and hydrazine hydrate (0.58 ml) as described above; yield, 0.284 g (82%); mp 110—113 °C; $[\alpha]_D^{25}$ −73.0° (*c* 1, MeOH); R_f^1 0.94, R_f^2 0.69.

Found: C, 60.01; H, 6.92; N, 13.89%. Calcd for $C_{30}H_{40}O_6N_6 \cdot H_2O$: C, 60.18; H, 7.07; N, 14.04%.

H–Tyr(Bzl)–OEt·HCl (7·HCl). To a solution of H–Tyr(Bzl)–OH[12] (2.71 g, 10 mmol) in EtOH (20 ml) was added $SOCl_2$ (0.872 ml, 12 mmol). The reaction mixture was stirred at 40 °C for 0.5 h and at room temperature overnight, and evaporated. The resulting oil was crystallized by the addition of ether–petroleum ether; yield, 2.34 g (70%); mp 190—192 °C; $[\alpha]_D^{25}$ +7.1° (*c* 1, MeOH); R_f^1 0.75, R_f^2 0.74.

Found: C, 63.95; H, 6.68; N, 4.17%. Calcd for $C_{18}H_{22}O_3NCl$: C, 64.37; H, 6.60; N, 4.17%.

Boc–Gln–Tyr(Bzl)–OEt (8). To a solution of **7**·HCl (2.02 g, 6 mmol) and Et_3N (0.924 ml, 6.6 mmol) in DMF (70 ml) were added Boc–Gln–ONp (2.20 g, 6 mmol) and 1-hydroxybenzotriazole (0.081 g, 0.6 mmol). After being left to stand at room temperature for 12 h, several drops of 1-(2-aminoethyl)piperazine[13] were added. The solution was diluted with water (2 l), and the resulting solid was collected, washed with 10% citric acid, 4% $NaHCO_3$, and water; yield, 2.82 g (89%); mp 138—141 °C; $[\alpha]_D^{25}$ −3.9° (*c* 1, DMF); R_f^1 0.96, R_f^2 0.80.

Found: C, 63.56; H, 7.16; N, 7.85%. Calcd for $C_{28}H_{37}O_7N_3$: C, 63.74; H, 7.07; N, 7.97%.

H–Gln–Tyr(Bzl)–OEt·TFA (9·TFA). A solution of **8** (1.58 g, 3 mmol) in TFA (30 ml) was allowed to stand at room temperature for 10 min and evaporated. The resulting oil was solidified by the addition of ether–petroleum ether. The product was recrystallized from EtOH–ether–petroleum ether; yield, 1.48 g (91%); mp 112—116 °C; $[\alpha]_D^{25}$ +14.6° (*c* 1, MeOH), R_f^1 0.80, R_f^2 0.57.

Found: C, 55.37; H, 5.75; N, 7.52%. Calcd for $C_{25}H_{30}O_7N_3F_3$: C, 55.45; H, 5.58; N, 7.76%.

Boc–Asn–Gln–Tyr(Bzl)–OEt (10). Boc–Asn–ONp (0.897 g) was coupled with **9**·TFA (1.38 g), Et_3N (0.391 ml), and 1-hydroxybenzotriazole (0.034 g) as described for the preparation of **8**. The product was recrystallized fromDMF–ether; yield, 1.31 g (80%); mp 240—241 °C (dec); $[\alpha]_D^{25}$ −22.0° (*c* 0.6, DMF); R_f^1 0.91, R_f^2 0.72.

Found: C, 59.70; H, 6.88; N, 10.91%. Calcd for $C_{32}H_{43}O_9N_5$: C, 59.89; H, 6.75; N, 10.91%.

Boc–Asn–Gln–Tyr(Bzl)–NHNH$_2$ (11). A solution of **10** (1.28 g, 3 mmol) and hydrazine hydrate (1.94 ml, 40 mmol) in DMF (40 ml) was allowed to stand at room temperature overnight. The solution was evaporated, water (1000 ml) was added, and the resulting solid was collected; yield, 1.09 g (87%); mp 215—216 °C (dec); $[\alpha]_D^{25}$ −41.0° (*c* 1, DMF); R_f^1 0.72, R_f^2 0.16.

Found: C, 56.54; H, 6.70; N, 15.35%. Calcd for $C_{30}H_{41}O_8N_7 \cdot 1/2H_2O$: C, 56.59; H, 6.65; N, 15.40%.

Boc–Asn–Gln–Tyr(Bzl)–Val–Orn(Z)–Leu–OEt (12). To a solution of **11** (1.06 g, 1.69 mmol) in DMF (15 ml) was added 3.5 M hydrogen chloride in dioxane (1.49 ml) and isopentyl nitrite[14] (0.262 ml, 1.86 mmol) at −50 °C. After being left to stand at −20 °C for 10 min, the solution was cooled to −60 °C and neutralized with Et_3N (0.731 ml, 5.22 mmol). To this solution was added a chilled solution of H–Val–Orn(Z)–Leu–OEt·HCl (1.10 g, 2.02 mmol)[1] and Et_3N (0.283 ml, 2.02 mmol) in DMF (8 ml). The reaction mixture was allowed to stir at 0 °C for 3 days and evaporated. After the addition of 0.02 M citric acid (300 ml), the solid was

collected and washed with water; yield, 1.59 g (85%); mp 279—281 °C (dec); $[\alpha]_D^{25}$ −41.0° (*c* 0.2, DMF); R_f^1 0.84, R_f^2 0.67.

Found: C, 60.37; H, 7.30; N, 11.28%. Calcd for $C_{56}H_{79}O_{14}N_9 \cdot 1/2H_2O$: C, 60.52; H, 7.26; N, 11.34%.

H-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-OEt·HCl (**13**·HCl). Compound **12** (1.70 g, 1.54 mmol) was treated with 0.1 M hydrogen chloride in formic acid (23 ml) as described for the preparation of **2a**·HCl; yield, 1.48 g (92%); mp 265—267 °C (dec); $[\alpha]_D^{25}$ −29.0° (*c* 0.2, DMF); R_f^1 0.81, R_f^2 0.34.

Found: C, 58.01; H, 6.95; N, 11.78%. Calcd for $C_{51}H_{72}O_{12}N_9Cl \cdot H_2O$: C, 57.97; H, 7.06; N, 11.93%.

Boc-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-OEt (**14a**). Compound **6a** (0.257 g, 0.383 mmol) and **13**·HCl (0.437 g, 0.421 mmol) were coupled by the azide method as described for the preparation of **12**; yield, 0.522 g (83%); mp 243—245 °C (dec); $[\alpha]_D^{25}$ −29.0° (*c* 0.5, AcOH); R_f^1 0.98, R_f^2 0.58.

Found: C, 61.33; H, 6.80; N, 10.54%. Calcd for $C_{88}H_{113}O_{18}N_{13} \cdot 4H_2O$: C, 61.70; H, 7.12; N, 10.63%.

Boc-D-Phe-Pro-Gly-D-Phe-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-OEt (**14b**). This was prepared from **6b** (0.222 g) and **13**·HCl (0.418 g) as described above; yield, 0.482 g (81%); mp 253—255 °C (dec); $[\alpha]_D^{25}$ −27.0° (*c* 1, AcOH); R_f^1 0.98, R_f^2 0.63.

Found: C, 60.26; H, 6.92; N, 11.36%. Calcd for $C_{81}H_{107}O_{18}N_{13} \cdot 3H_2O$: C, 60.62; H, 7.10; N, 11.35%.

Boc-D-Phe-Pro-Phe-Gly-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-OEt (**14c**). This was prepared from **6c** (0.222 g) and **13**·HCl (0.418 g); yield, 0.505 g (85%); mp 252—254 °C (dec); $[\alpha]_D^{25}$ −43.0° (*c* 0.5, AcOH); R_f^1 0.98, R_f^2 0.64.

Found: C, 61.26; H, 7.01; N, 11.51%. Calcd for $C_{81}H_{107}O_{18}N_{13} \cdot 2H_2O$: C, 61.31; H, 7.05; N, 11.47%.

Boc-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-NHNH$_2$ (**15a**). Compound **14a** (0.523 g, 0.319 mmol) was treated with hydrazine hydrate (1.55 ml, 31.9 mmol) as described for the preparation of **11**; yield, 0.42 g (81%); mp 252—254 °C (dec); $[\alpha]_D^{25}$ −28.0° (*c* 0.5, AcOH); R_f^1 0.91, R_f^2 0.41.

Found: C, 60.51; H, 6.81; N, 12.22%. Calcd for $C_{86}H_{111}O_{17}N_{15} \cdot 4H_2O$: C, 60.79; H, 7.06; N, 12.37%.

Boc-D-Phe-Pro-Gly-D-Phe-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-NHNH$_2$ (**15b**). This was prepared from **14b** (0.183 g) and hydrazine hydrate (0.572 ml) as described above; yield, 0.162 g (90%); mp 254—255 °C (dec); $[\alpha]_D^{25}$ −27.0° (*c* 1, AcOH); R_f^1 0.90, R_f^2 0.30.

Found: C, 59.41; H, 6.93; N, 13.55%. Calcd for $C_{79}H_{105}O_{17}N_{15} \cdot 3H_2O$: C, 59.65; H, 7.03; N, 13.21%.

Boc-D-Phe-Pro-Phe-Gly-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-NHNH$_2$ (**15c**). This was prepared from **14c** (0.476 g) and hydrazine hydrate (1.49 ml); yield, 0.437 g (93%); mp 254—256 °C (dec); $[\alpha]_D^{25}$ −29.0° (*c* 0.5, AcOH); R_f^1 0.92, R_f^2 0.36.

Found: C, 60.02; H, 6.86; N, 13.38%. Calcd for $C_{79}H_{105}O_{17}N_{15} \cdot 2H_2O$: C, 60.33; H, 6.99; N, 13.36%.

H-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-NHNH$_2$·2HCl (**16a**·2HCl). Compound **15a** (0.163 g, 0.1 mmol) was treated with 0.1 M hydrogen chloride in formic acid (1.5 ml) as desctibed for the preparation of **2a**·HCl; yield, 0.147 g (92%); mp 235—237 °C (dec); $[\alpha]_D^{25}$ −35.0° (*c* 0.5, AcOH); R_f^1 0.85, R_f^2 0.38.

Found: C, 59.81; H, 6.75; N, 12.83%. Calcd for $C_{81}H_{105}O_{15}N_{15}Cl_2 \cdot H_2O$: C, 60.14; H, 6.67; N, 12.99%.

H-D-Phe-Pro-Gly-D-Phe-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-NHNH$_2$·2HCl (**16b**·2HCl). This was prepared from **15b** (0.141 g) as described above; yield, 0.129 g (93%); mp 237—240 °C (dec); $[\alpha]_D^{25}$ −15.0° (*c* 0.2, AcOH); R_f^1 0.83, R_f^2 0.38.

Found: C, 57.99; H, 6.72; N, 13.37%. Calcd for $C_{74}H_{99}O_{15}N_{15}Cl_2 \cdot H_2O$: C, 58.18; H, 6.66; N, 13.75%.

H-D-Phe-Pro-Phe-Gly-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-NHNH$_2$·2HCl (**16c**·2HCl). This was prepared from **15c** (0.17 g) as described above; yield, 0.148 g (88%); mp 238—241 °C (dec); $[\alpha]_D^{25}$ −63.0° (*c* 0.5, AcOH); R_f^1 0.87, R_f^2 0.44.

Found: C, 58.12; H, 6.65; N, 13.40%. Calcd for $C_{74}H_{99}O_{15}N_{15}Cl_2 \cdot H_2O$: C, 58.18; H, 6.66; N, 13.75%.

cyclo(-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-) (**17a**). Compound **16a**·2HCl (146 mg, 0.091 mmol) was dissolved in a mixture of DMF (4 ml), AcOH (0.2 ml) and 1 M HCl (0.19 ml). To the solution at −10 °C was added 1 M $NaNO_2$ (0.096 ml). After being left to stand at −10 °C for 15 min, the solution was added into pyridine (100 ml) at −5 °C. The solution was allowed to stir at −5 °C for 3 h and at 0 °C for 45 h, and evaporated. The residue was dissolved in a mixture (100 ml) of MeOH–dioxane–water (5: 3: 1), an insoluble material being removed by filtration. The filtrate was passed successively through columns (1.5×12 cm) of Amberlite IRC-50 (H^+ form) and Amberlite IR-45 (OH^- form). The effluent (250 ml) was evaporated, and the resulting solid was collected by filtration with the aid of water; yield, 68 mg (50%); mp 245—248 °C (dec); $[\alpha]_D^{25}$ −95° (*c* 0.1, DMF); R_f^1 0.98, R_f^2 0.70, R_f^3 0.74.

Found: C, 62.57; H, 6.78; N, 12.01%; mol wt, 1493.[15] Calcd for $C_{81}H_{99}O_{15}N_{13} \cdot 3H_2O$: C, 62.81; H, 6.83; N, 11.76%; mol wt, 1549.

cyclo(-D-Phe-Pro-Gly-D-Phe-Asn-Gln-Tyr(Bzl)-Val-Orn(Z)-Leu-) (**17b**). This was prepared from **16b**·2HCl (133 mg) as described above; yield, 65 mg (52%); mp 236—239 °C (dec); $[\alpha]_D^{25}$ −83° (*c* 0.1, DMF); R_f^1 0.95, R_f^2 0.64, R_f^3 0.74.

Found: C, 60.90; H, 6.92; N, 12.32%; mol wt, 1475. Calcd for $C_{74}H_{93}O_{15}N_{13} \cdot 3H_2O$: C, 60.93; H, 6.84; N, 12.48%; mol wt, 1459.

cyclo(-D-Phe-Pro-Phe-Gly-Asn-Gln-Tyr(Bzl)-Val-Orn-(Z)-Leu-) (**17c**). This was prepared from **16c**·2HCl (156 mg); yield, 83 mg (57%); mp 232—235 °C (dec); $[\alpha]_D^{25}$ −85° (*c* 0.1, DMF); R_f^1 0.98, R_f^2 0.64, R_f^3 0.79.

Found: C, 61.23; H, 6.69; N, 12.50%; mol wt, 1445. Calcd for $C_{74}H_{93}O_{15}N_{13} \cdot 3H_2O$: C, 60.93; H, 6.84; N, 12.48%; mol wt, 1459.

cyclo(-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-)·HCl (**18a**·HCl). A solution of **17a** (60 mg, 0.04 mmol) in 0.43 M hydrogen chloride (0.14 ml) in MeOH was hydrogenated in the presence of Pd black. After 20 h, the filtrate was evaporated, and the resulting crystals were collected by filtration with the aid of ether; yield of air-dried product (**18a**·HCl·7H$_2$O), 48 mg (92%); mp 240—243 °C (dec); $[\alpha]_D^{25}$ −111° (*c* 0.14, 50% EtOH) (lit. $[\alpha]_D^{25}$ −111°,[16] −112°[8]); R_f^1 0.78, R_f^2 0.03, R_f^4 0.98, R_f^5 0.87. Amino acid ratios in acid hydrolysate; Asp 1.02, Glu 1.00, Pro 0.93, Val 0.99, Leu 1.02, Tyr 0.92, Phe 3.19, Orn 0.96.

Found: C, 55.78; H, 6.79; N, 12.25%. Calcd for $C_{66}H_{87}O_{13}N_{13} \cdot HCl \cdot 7H_2O$: C, 55.32; H, 7.17; N, 12.71%.

cyclo(-D-Phe-Pro-Gly-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-)·HCl (**18b**·HCl). This was prepared from **17b** (56 mg) as described above; yield of air-dried product (**18b**·HCl·8H$_2$O), 46 mg (94%); mp 232—234 °C (dec); $[\alpha]_D^{25}$ −95° (*c* 0.17, 50% EtOH); R_f^1 0.77, R_f^2 0.03, R_f^4 0.96, R_f^5 0.85. Amino acid ratios in acid hydrolysate; Asp 1.00, Glu 1.00, Pro 0.97, Gly 1.03, Val 1.05, Leu 1.04, Tyr 1.04, Phe 1.93, Orn 1.02.

Found: C, 51.74; H, 6.96; N, 13.74%. Calcd for $C_{59}H_{81}$-

$O_{13}N_{13}\cdot HCl\cdot 8H_2O$: C, 52.07; H, 7.26; N, 13.38%.

cyclo(-D-Phe-Pro-Phe-Gly-Asn-Gln-Tyr-Val-Orn-Leu-)·HCl (18c·HCl). This was prepared from **17c** (70 mg); yield of air-dried product (**18c**·HCl·$7H_2O$), 56 mg (92%); mp 230—232 °C (dec); $[\alpha]_D^{25}$ −98° (*c* 0.14, 50% EtOH); R_f^1 0.97, R_f^2 0.03, R_f^4 0.97, R_f^5 0.85. Amino acid ratios in acid hydrolysate; Asp 1.03, Glu 1.00, Pro 1.06, Gly 1.06, Val 1.11, Leu 1.09, Tyr 1.00, Phe 1.93, Orn 1.06.

Found: C, 52.31; H, 6.93; N, 13.95%. Calcd for $C_{59}H_{81}O_{13}N_{13}\cdot HCl\cdot 7H_2O$: C, 52.76; H, 7.21; N, 13.56%.

Droplet Countercurrent Chromatography. An apparatus made by Seikagaku Kogyo Co., Tokyo, was used. It consists of 300 columns of glass tubing (0.24×60 cm) connected by teflon tubing (I. D., 0.05 cm). The solvent used was $CHCl_3$–MeOH–0.1 M HCl (19: 19: 12, v/v).[17] The upper phase of the solvent was loaded into 150 glass columns as the stationary phase. The 5 mg samples each of **18a—c** and natural TA were dissolved in the lower phase (1 ml) and placed at the top of first column. The lower phase, as the moving phase, was pumped with nitrogen pressure through the top of the first column at a flow rate of 15 ml/h. Fractions (4 g) from the last column were collected and their absorbances were determined at 280 nm. The results are shown in Fig. 3.

Paper Electrophoresis. This was carried out with Toyo Roshi No. 52 and a solvent system of formic acid–ACOH–MeOH–water (1:3:6:10, v/v; pH 1.8) for 2.5 h at 500 v/30 cm. Fig. 5. showed that each of **18a—c** revealed a single spot.

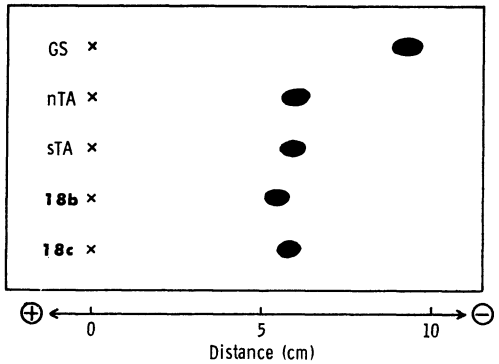


Fig. 5. Paper electrophoresis of the cyclic peptides.

Microbiological Assays,[18] *ORD Measurements.* These were carried out as described in a previous paper,[1] the results being shown in Table 1 and Fig. 4.

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17) Natural TA used in this study was prepared following Ref. 8. In Ref. 8, we reported the use of a solvent system, benzene–$CHCl_3$–MeOH–0.1 M HCl=11: 5: 10: 4, for the separation of gramicidin A and tyrocidine family, and recognized a mistake of the description after the publication of Ref. 8. We wish to correct that the solvent system used was benzene–$CHCl_3$–MeOH–0.1 M HCl=5: 10: 11: 4.

18) We are indebted to the staff of Takeda Chemical Industries, Ltd. for the assay.