Studies of Bitter Peptides from Casein Hydrolyzate. VIII.[1] Bitter Taste of Cyclic Analog of BPIa (Arg-Gly-Pro-Pro-Phe-Ile-Val)[2]

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Synopsis. In order to elucidate the structure-taste relationship of bitter peptide BPIa (Arg-Gly-Pro-Pro-Phe-Ile-Val), *cyclo*-BPIa was synthesized. The result of both taste and CD examinations suggested that the bitterness of BPIa is caused by its spatial structure, which is analogous to that of *cyclo*-BPIa.

In our synthetic investigations of bitter peptide BPIa isolated from casein hydrolyzate by Minamiura *et al.*,[3] it has been confirmed that N-terminal arginine residue and C-terminal hydrophobic moiety are necessary for an intence bitterness of BPIa and that the spatial structure of whole molecule attributed to prolylproline in the center also contributes to its bitter taste. We also assume that N- and C-terminals of BPIa are situated close together by prolylproline. In order to confirm our assumption, we synthesized *cyclo*-BPIa, in which N-terminal arginine and C-terminal valine residues were combined, and compared its bitter taste with that of BPIa.

The synthetic route for *cyclo*-BPIa is shown in Fig. 1. Boc-Pro-Pro-Phe-Ile-Val-OBzl, which was an intermediate in the synthesis of BPIa,[4] was hydrogenated to yield the corresponding acid (**1**). It was condensed with H-Arg(NO_2)-Gly-OBzl·HCl (**3**) by the DCC-HOBt method and the resulting protected heptapeptide (**4**) was converted to the corresponding acid (**5**) by a saponification reaction. **5** was esterfied by the DCC-HONSu method to afford the corresponding active ester (**6**). After the amino protection of **6** was removed by the action of hydrogen chloride in 98% formic acid, the resulting heptapeptide active ester hydrochloride (**7**) was treated with pyridine under high-dilution conditions for cyclization. The reaction mixture yielded a clude N^G-nitro substituted cyclic heptapeptide (**8**), which was then purified by passing through columns of acidic and basic ion exchangers. **8** thus obtained was hydrogenated in the presence of palladium black to yield *cyclo*-BPIa (**9**). The homogeneity of the final product was confirmed by thin-layer chromatography, amino acid analysis, paper electrophoresis, and elemental analysis.

The taste of *cyclo*-BPIa was organoleptically determined by panel evaluation with four people. *cyclo*-BPIa possessed an extremely bitter taste: its threshold value was 0.02 mM (1 M=1 mol dm^{-3}). The value was of the same level as BPIa; the threshold value of BPIa was 0.05 mM. The CD curves of both *cyclo*-BPIa and BPIa measured in water are presented in Fig. 2. *cyclo*-BPIa has a curve with a negative trough at 202 nm; BPIa possesses a similarly shaped curve.

The results of both taste and CD examinations suggested that the bitter taste of BPIa is caused by its characteristic molecular shape, which is analogous to that of *cyclo*-BPIa.

Experimental

All the melting points are uncorrected. Thin-layer chromatography was carried out on Merck silica gel G with the solvent systems: R_f^1, *n*-BuOH-AcOH-pyridine-H_2O (4:1:1:2, v/v); R_f^2, $CHCl_3$-MeOH (5:1, v/v). Opical rotations were measured on a Union PM-101 polarimeter. Amino acid analysis in acid hydrolyzate with 6 M HCl at 110 °C for 72 h was performed with a Hitachi amino acid analyzer, KLA-5 type. Prior to analyses, the compounds were dried over phosphorus pentaoxide at 66 °C and 2 mmHg (1 mmHg= 133.332 Pa) for 2 h.

Boc-Pro-Pro-Phe-Ile-Val-OH (1). This was prepared from Boc-Pro-Pro-Phe-Ile-Val-OBzl[4] (7.62 g, 10 mmol) in

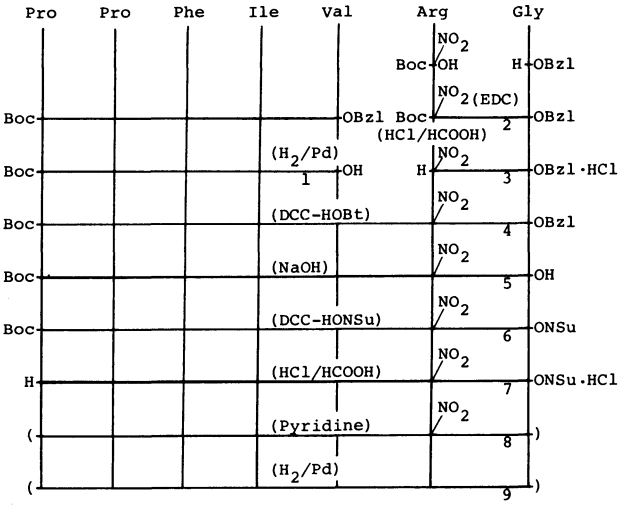

Fig. 1. Synthesis of *cyclo*-BPIa.

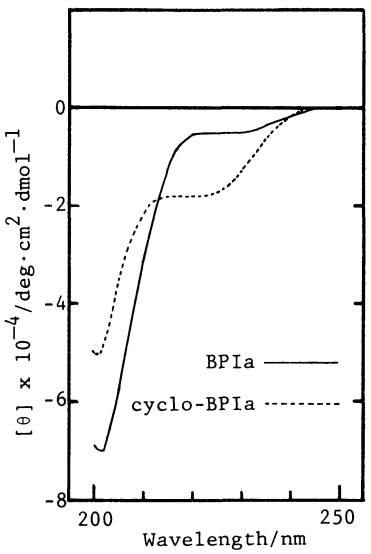

Fig. 2. CD curves of *cyclo*-BPIa and BPIa.

MeOH (30 ml) by hydrogenation in the presence of Pd black; yield 5.95 g (89%); mp 169—171 °C; $[\alpha]_D^{20}$ −96° (*c* 1, MeOH); R_f^1 0.86 and R_f^2 0.73.

Found: C, 62.34; H, 8.16; N, 10.26%. Calcd for $C_{35}H_{53}O_8N_5$: C, 62.57; H, 7.95: N, 10.43%.

Boc-Arg(NO$_2$)-Gly-OBzl (2). This was prepared from Boc-Arg(NO$_2$)-OH (9.60 g, 30 mmol) and H-Gly-OBzl·TsOH (12.12 g, 36 mmol) in acetonitrile (100 ml) by the EDC method;[5] yield 10.85 g (76%); mp 96 °C; $[\alpha]_D^{20}$ −11° (*c* 1, MeOH); R_f^1 0.91 and R_f^2 0.77

Found: C, 51.61; H, 6.31; N, 17.88%. Calcd for $C_{20}H_{30}O_7N_6$: C, 51.49; H, 6.48; N, 18.02%.

H-Arg(NO$_2$)-Gly-OBzl·HCl (3). This was prepared from **2** (4.66 g, 10 mmol) by the action of hydrogen chloride in 98% formic acid. The product was obtained as a hygroscopic solid; yield 4.02 g (100%); R_f^1 0.76 and R_f^2 0.31.

Boc-Pro-Pro-Phe-Ile-Val-Arg(NO$_2$)-Gly-OBzl (4). This was prepared from **1** (5.38 g, 8 mmol) and **3** (4.02 g, 10 mmol) in DMF (20 ml) by the DCC-HOBt method.[6] The product was recrystallized from hot MeOH; yield 5.14 g (63%); mp 231—232 °C; $[\alpha]_D^{20}$ −41.5° (*c* 1, DMF); R_f^1 0.91 and R_f^2 0.76.

Found: C, 58.59; H, 7.31; N, 15.03%. Calcd for $C_{50}H_{73}O_{12}N_{11}$: C, 58.86; H, 7.21; N. 15.10%.

Boc-Pro-Pro-Phe-Ile-Val-Arg(NO$_2$)-Gly-OH (5). Compound **4** (5.1 g, 5 mmol) was saponified with 1 M NaOH (7.5 ml) in MeOH (10 ml). The purification was done by the extraction with *n*-BuOH: yield 4.42 g (97%); mp 105 °C (decomp); $[\alpha]_D^{20}$ −32.3° (*c* 1, DMF); $R_f^1$0.56 and R_f^2 0.21.

Found: C, 54.11; H, 7.27; N, 16.15%. Calcd for $C_{43}H_{67}O_{12}N_{11}\cdot H_2O$: C, 54.47; H, 7.34; N, 16.25%.

Boc-Pro-Pro-Phe-Ile-Val-Arg(NO$_2$)-Gly-ONSu (6). To a solution of **5** (1.37 g, 1.5 mmol) and HONSu (0.35 g, 3 mmol) in DMF (5 ml), DCC (0.62 g, 3 mmol) was added with stirring at 0 °C. The reaction mixture was stirred for 3 h at 0 °C, then at room temperature overnight. DCUrea was filtered off and a large amount of ether was poured into the filtrate. The precipitate thus obtained was collected by filtration. It was recrystallized from acetonitrile; yield 1.26 g (82%); mp 193 °C; $[\alpha]_D^{20}$ −46.1° (*c* 0.5, DMF); R_f^1 0.91 and R_f^2 0.81.

Found: C, 54.58; H, 7.15; N, 15.90%. Calcd for $C_{47}H_{70}O_{14}N_{12}\cdot 1/2H_2O$: C, 54.47; H, 6.91; N, 16.22%.

cyclo*(-Arg(NO$_2$)-Gly-Pro-Pro-Phe-Ile-Val-) (8).* Compound **6** (0.51 g, 0.5 mmol) was dissolved in 98% formic acid and 3.5 M hydrogen chloride in dioxane (10 ml) at 0 °C. After 30 min, the solution was evaporated *in vacuo* and the oily residue was solidified with ether (yield 0.44 g). The active ester hydrochloride (**7**) thus obtained was dissolved in DMF (5 ml) and the solution was added dropwise to pyridine (250 ml) with stirring. The reaction mixture was stirred for 24 h at room temperature and evaporated *in vacuo*. The residual oil was dissolved in a mixture of dioxane (50 ml) and water (10 ml). The solution was passed successively through columns (1.2 cm×12.5 cm) of Amberlite CG-120 (H^+ form) and Amberlite CG-400 (OH^- form). The columns were washed with the same solvent and the collected effluent was evaporated *in vacuo*; then the product was collected by the aid of water (yield 51 mg). It was recrystallized from MeOH-ether; yield 41 mg (10%); mp 221 °C (decomp); $[\alpha]_D^{20}$ −56.3° (*c* 0.5, MeOH); R_f^1 0.83 and R_f^2 0.65.

Found: C, 52.51; H, 6.98; N, 17.52%. Calcd for $C_{38}H_{57}O_9N_{11}\cdot 3H_2O$: C, 52.70; H, 7.33; N, 17.79%.

cyclo*(-Arg-Gly-Pro-Pro-Phe-Ile-Val-) (9).* Compound **8** (40 mg, 0.005 mmol) was dissolved in MeOH (2 ml) and AcOH (2 ml) and hydrogenated in the presence of Pd black for 24 h. The filtrate from catalyst was evaporated *in vacuo* and the oily residue was crystallized by the aid of ether. It was recrystallized from water-acetone; yield 40 mg (97%); mp 198 °C (decomp); $[\alpha]_D^{20}$ −31.4° (*c* 0.5, MeOH); R_f^1 0.57 and R_f^2 0.05. Amino acid ratios in acid hydrolyzate: Arg 1.11, Gly 0.97, Pro 2.01, Phe 1.09, Ile 1.00, Val 0.95.

Found: C, 53.38; H, 7.70; N, 15.30%. Calcd for $C_{38}H_{58}O_7N_{10}\cdot CH_3COOH\cdot 4H_2O$: C, 53.44; H, 7.85; N, 15.58%.

Paper Electrophoresis. This was carried out under the conditions previously reported.[7] *cyclo*-BPIa migrated toward the cathode and revealed a single spot by spraying Sakaguchi reagent; ninhydrin gave the same result. The R_{Arg} value[8] of *cyclo*-BPIa was 0.58.

CD Measurement. This was performed with a JASCO J-20A. A cell of path length 0.2 mm was used and runs were made at ambient temperature.

Sensory Test. Taste of cyclo-BPIa was organoleptically determined in the same manner as described in the previous pepers.[1,4,7]

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References

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2) The abbreviations recommended by IUPAC-IUB commission of Biochemical Nomenclature (*J. Biol. Chem.*, **247**, 977 (1972)) have been used. Amino acid symbols except glycine denoted L-configuration. Additional abbreviations: DCC, dicyclohexylcarbodiimide: DCUrea, *N,N'*-dicyclohexylurea; DMF, *N,N*-dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt; 1-hydroxybenzotriazole; HONSu, *N*-hydroxysuccinimide.

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8) The electrophoretic mobility was recorded as R_{Arg}, the ratio of the distance the compound moved to that which a standard arginine spot moved on the same electrophoreogram.