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Studies of Bitter Peptides from Casein Hydrolyzate. IX.[1] Syntheses and Bitter Taste of Bitter Peptide BPIa Dimer, (Arg-Gly-Pro-Pro-Phe-Ile-Val)$_2$, and Gly-Gly BPIa Derivatives[2]

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In order to elucidate the relationship between taste exhibition and chemical structure of bitter peptide BPIa, di-BPIa, Gly-Gly-BPIa, BPIa-Gly-Gly, and Gly-Gly-BPIa-Gly-Gly were synthesized. All of the peptides possessed a strong bitter taste of the same level as that of BPIa. In addition, the CD curves of the peptides were similar to that of BPIa. The results suggested that their spatial structures are essentially similar and that the whole molecular shape of BPIa contributes to its bitterness.

Minamiura *et al.* isolated a bitter heptapeptide named BPIa from cow milk casein hydrolyzate by bacterial proteinase and determined its amino acid sequence to be H-Arg-Gly-Pro-Pro-Phe-Ile-Val-OH.[3] We synthesized it and confirmed that synthetic BPIa was identical to Minamiura's natural one.[4] Synthetic BPIa possessed an extremely bitter taste, with a threshold value of 0.05 mM (1 M=1 mol dm^{-3}). To elucidate the relationship between chemical structure and bitterness of BPIa, various fragments and analogs which possessed several characteristic features of BPIa have been prepared in our laboratory. N-Terminal hexapeptide (des-Val^7-BPIa) exhibited the same bitterness as BPIa, whereas the bitterness of des-Arg^1-BPIa and of N-terminal pentapeptide fragment were much weaker than that of BPIa.[5] These results indicated that both arginine residue in the N-terminal position and hydrophobic amino acids in the C-terminal moiety are necessary for the intense bitter taste of BPIa. This finding was favored by the bitterness exhibited by both Arg^1- and C-terminal tripeptide-substituted analogs of BPIa.[6,7] On the other hand, the analogs in which prolylproline resiude was substituted for glycylglycine or D-prolyl-D-proline residues exhibited a weaker bitterness than BPIa, and their CD curves measured in water were different from that of BPIa.[8] The results suggested that the spatial structure of BPIa molecule attributed to prolylproline in the center also contributes to its bitter taste. In addition, retro-BPIa, which has the reverse sequence of BPIa, exhibited a strong bitter taste of the same level as that of BPIa. This indicated that it is possible for the N-terminal basic amino acid residue and C-terminal hydrophobic amino acids moiety in BPIa to change places with each other.[9] In the previous paper,[1] we reported that *cyclo*-BPIa, in which N-terminal arginine and C-terminal valine residues were combined, possessed the same bitternss as BPIa and that its CD curve measured in water was similar to that of BPIa. The findings of *cyclo*-BPIa suggested that the two bitter functional groups of both terminals of BPIa are situated close together in the molecule and that the bitterness of BPIa is caused by its characteristic spatial structure which is analogous to that of *cyclo*-BPIa.

The next subject in the investigations of BPIa is to examine the relationship of molecular size to bitter taste. We prepared a dimerized BPIa (di-BPIa, **1**) and ascertained whether or not the synergistic effect by molecular size for bitter taste was produced.

The synthesis of di-BPIa was carried out according to Fig. 1. Boc-Arg(NO_2)-Gly-OH was coupled with H-Pro-Pro-Phe-Ile-Val-OBzl·HCl, which was an intermediate in the synthesis of BPIa,[4] by the mixed anhydride method to yield the protected BPIa derivative (**12**). It was converted to the corresponding heptapeptide ester hydrochloride (**13**) by the action of hydrogen chloride in formic acid. Then **13** was coupled with the acid (**14**) derived from another intermediate in the synthesis of BPIa, Z-Arg(NO_2)-Gly-Pro-Pro-Phe-Ile-Val-OBzl, by the dicyclohexylcarbodiimide-1-hydroxybenzotriazole (DCC-HOBt) method to yield acyltetradecapeptide ester (**15**). The catalytic hydrogenation of **15** gave di-BPIa (**1**). The purity of final product and its intermediates was confirmed by thin-layer chromatography on two solvent systems and by elemental analyses.

Taste of peptides was organoleptically determined by panel evaluation employing five people. Di-BPIa

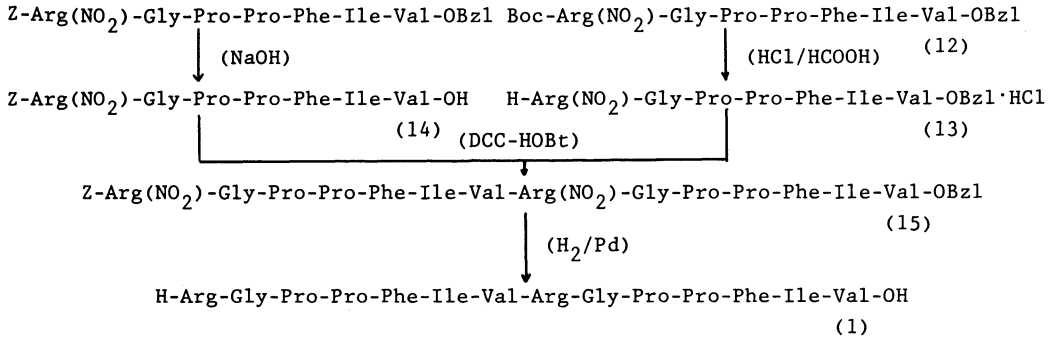

Fig. 1. Synthesis of di-BPIa.

Table 1. The threshold value for bitter taste of BPIa and its derivatives

Compound	Threshold value for bitter taste/mM
BPIa	0.05
di-BPIa (**1**)	0.04
Gly-Gly-BPIa (**2**)	0.08
BPIa-Gly-Gly (**3**)	0.05
Gly-Gly-BPIa-Gly-Gly (**4**)	0.04

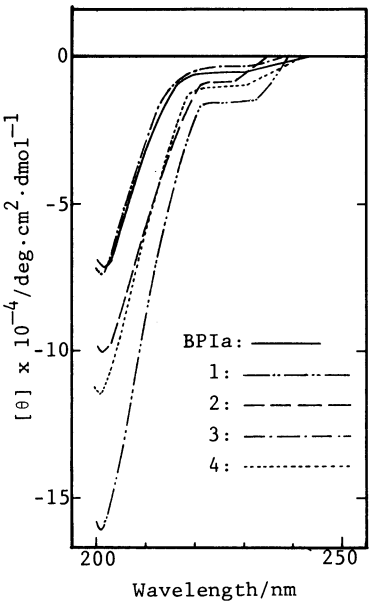

Fig. 2. CD curves of BPIa and its derivatives in water.

exhibited a strong bitter taste. Although we at first expected that di-BPIa would exhibit much stronger bitterness than BPIa, actually its threshold value was nearly equal to that of BPIa, as shown in the Table. This suggested that the bitterness of di-BPIa is caused by one of a pair of BPIa molecules and that the size of BPIa molecule is fit for a receptor of bitter taste in a taste bud. CD curves of di-BPIa and BPIa measured in water are presented in Fig. 2. The pattern of di-BPIa is similar to that of BPIa. It seems that the spatial structure of BPIa, which is important for showing the strong bitter taste, is kept in di-BPIa.

To further ascertain the relationship of the molecular shape of BPIa to its bitter taste, we prepared Gly-Gly-BPIa (**2**), BPIa-Gly-Gly (**3**), and Gly-Gly-BPIa-Gly-Gly (**4**), in which a tasteless glycylglycine was coupled to either the N-terminal or the C-terminal or both terminals of BPIa and examined whether both the strong bitter taste and CD pattern of BPIa were maintained in those BPIa derivatives in spite of the introduction of glycylglycine residue.

The sequence of reactions employed for the synthesis of Gly-Gly-BPIa is shown in Fig. 3. Condensation of Z-Gly-Gly-OH with H-Arg(NO_2)-Gly-OBzl·HCl by the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) method gave Z-Gly-Gly-Arg(NO_2)-Gly-OBzl (**5**). Saponification of **5** gave the corresponding acid (**6**). Then **6** was coupled with H-Pro-Pro-Phe-Ile-Val-OBzl·HCl by DCC-HOBt method to yield acylnonapeptide ester (**7**). It was hydrogenated in the

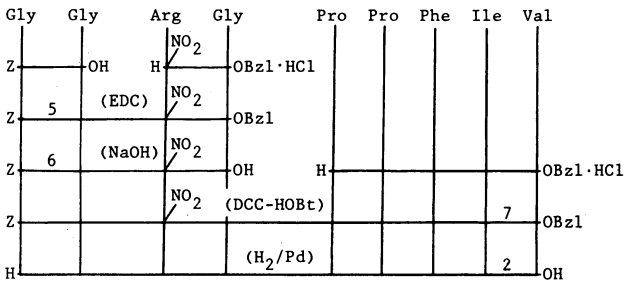

Fig. 3. Synthesis of Gly-Gly-BPIa.

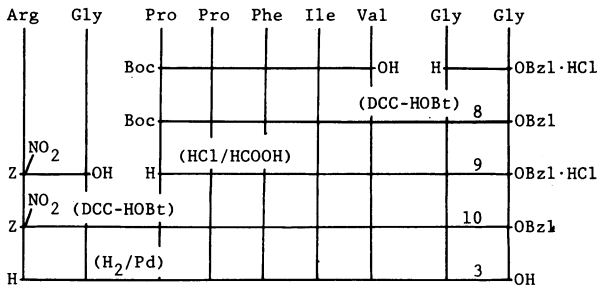

Fig. 4. Synthesis of BPIa-Gly-Gly.

presence of palladium black to yield Gly-Gly-BPIa (**2**).

The synthetic route for BPIa-Gly-Gly is shown in Fig. 4. Boc-Pro-Pro-Phe-Ile-Val-OH, which was an intermediate in the synthesis of *cyclo*-BPIa,[1] was coupled with H-Gly-Gly-OBzl·HCl by DCC-HOBt method to yield the protected heptapeptide (**8**). It was converted to the corresponding heptapeptide ester hydrochoride (**9**) by the action of hydrogen chloride in formic acid. Condensation of Z-Arg(NO_2)-Gly-OH with **9** by DCC-HOBt method afforded the fully protected BPIa-Gly-Gly (**10**). The catalytic hydrogenation of **10** yielded BPIa-Gly-Gly (**3**). Gly-Gly-BPIa-Gly-Gly (**4**) was yielded by hydrogenation of Z-Gly-Gly-Arg(NO_2)-Gly-Pro-Pro-Phe-Ile-Val-Gly-Gly-OBzl (**11**) derived from **6** and **9** by DCC-HOBt method.

The results of sensory tests are listed in the Table. All the Gly-Gly introduced BPIa derivatives possessed a strong bitter taste of the same level as BPIa. The results indicated that glycylglycine residue coupled not only to the N-termial but also to the C-terminal of BPIa did not affect the bitterness of BPIa. The CD curves of these peptides in water are shown in Fig. 2. These indicated similarly shaped curves with nagative troughs at 202 nm to that of BPIa. The results of CD measurements indicated that the spatial structure of BPIa is kept in those BPIa derivatives and that the structure causes the bitterness of 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, 1-butanol-acetic acid-pyridine-water (4:1:1:2, v/v); R_f^2, chloroform-methanol (5:1, v/v). Spots of materials possessing a free amino group on a thin layer plate were detected by spraying ninhydrin, and those of amino group blocked materials by spraying 25% hydrogen bromide in acetic acid and then ninhydrin. The optical

rotations were measured on a Union PM-101 polarimeter. Amino acid analyses in acid hydrolyzate with 6 M hydrochloric acid at 110°C for 72 h were 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.

Z-Gly-Gly-Arg(NO_2)-Gly-OBzl (5). To a solution of Z-Gly-Gly-OH[10] (2.66 g, 10 mmol), H-Arg(NO_2)-Gly-OBzl·HCl[1] (4.0 g, 10 mmol), and NMM (1.1 ml, 10 mmol) in DMF (20 ml), EDC (2.12 ml, 15 mmol) was added at 0°C with stirring. The reaction mixture was stirred for 3 h at 0°C, then at room temperature overnight. A large amount of water was poured into the mixture. The obtained precipitate was filtered, and washed with 4% sodium hydrogencarbonate, 0.5 M hydrochloric acid, and water successively. It was recrystallized from hot methanol: yield 4.68 g (76%); mp 101—103°C; $[\alpha]_D^{20}$ −8° (*c* 2, DMF); R_f^1 0.86 and R_f^2 0.57.

Found: C, 51.00; H, 5.54; N, 17.85%. Calcd for $C_{27}H_{34}O_9N_8 \cdot H_2O$: C, 51.25; H, 5.74; N, 17.71%.

Z-Gly-Gly-Arg(NO_2)-Gly-OH (6). Compound **5** (1.83 g, 3 mmol) was saponified with 1 M sodium hydroxide (3.6 ml, 3.6 mmol) in methanol (15 ml) for 1.5 h at room temperature. After evaporation, the aqueous solution was acidified to pH 3 with 1 M hydrochloric acid, and extracted with 1-butanol. The extract was washed with water and dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo* and the residue was crystallized with ether. It was recrystallized from methanol-ether: yield 0.94 g (60%); mp 139°C; $[\alpha]_D^{20}$ −15° (*c* 1, DMF); R_f^1 0.62 and R_f^2 0.07.

Found: C, 45.70; H, 5.35; N, 21.59%. Calcd for $C_{20}H_{28}O_9N_8$: C, 45.80; H, 5.38; N, 21.37%.

Z-Gly-Gly-Arg(NO_2)-Gly-Pro-Pro-Phe-Ile-Val-OBzl (7). To a solution of **6** (0.53 g, 1 mmol) and H-Pro-Pro-Phe-Ile-Val-OBzl·HCl[4] (0.70 g, 1 mmol) and NMM (0.11 ml, 1 mmol) in DMF (8 ml), HOBt (0.15 g, 1.1 mmol), and DCC (0.25 g, 1.2 mmol) were added at 0°C with stirring. The reaction mixture was stirred for 3 h at 0°C and then at room temperature overnight. DCUrea was filtered off and the filtrate was dissolved in 1-butanol. The solution was washed with 4% sodium hydrogencarbonate, 0.5 M hydrochloric acid, and water successively, then dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo* and the residue was crystallized with ether. It was recrystllized from methanol-ether: yield 0.78 g (67%); mp 125°C (decomp); $[\alpha]_D^{20}$ −51° (*c* 1, DMF); R_f^1 0.81 and R_f^2 0.70.

Found: C, 56.98; H, 6.79; N, 15.17%. Calcd for $C_{57}H_{77}O_{14}N_{13} \cdot 2H_2O$: C, 56.86; H, 6.78; N, 15.12%.

H-Gly-Gly-Arg-Gly-Pro-Pro-Phe-Ile-Val-OH·AcOH (2). Compound **7** (0.47 g, 0.4 mmol) was dissolved in a mixture of methanol (5 ml) and acetic acid (5 ml), and hydrogenated in the presence of palladium black for 24 h at room temperature. The filtrate from catalyst was evaporated *in vacuo* and the residual oil was solidified by the aid of acetone. It was recrystallized from methanol-ether: yield 0.31 g (80%); $[\alpha]_D^{20}$ −122° (*c* 0.5, H_2O); R_f^1 0.65 and R_f^2 0.00. Amino acid ratios in acid hydrolyzate: Arg 0.90, Gly 2.65, Pro 2.03, Phe 1.07, Ile 1.00, Val 1.00.

Found: C, 53.42; H, 7.42; N, 16.54%. Calcd for $C_{42}H_{66}O_{10}N_{12} \cdot CH_3COOH \cdot 2H_2O$: C, 53.10; H, 7.50; N, 16.89%.

Boc-Pro-Pro-Phe-Ile-Val-Gly-Gly-OBzl (8). To a solution of Boc-Pro-Pro-Phe-Ile-Val-OH[1] (2.02 g, 3 mmol), H-Gly-Gly-OBzl·HCl[11] (1.16 g, 4.5 mmol) and NMM (0.50 ml, 4.5 mmol) in DMF (10 ml), HOBt (0.61 g, 4.5 mmol) and DCC (0.93 g, 4.5 mmol) were added at 0°C with stirring. The reaction mixture was stirred for 3 h at 0°C and kept in a refrigerator overnight. DCUrea was filtered off and the filtrate was dissolved in 1-butanol. The solution was washed with 4% sodium hydrogencarbonate, 4% citric acid, and water successively, then dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo* and the residue was crystallized with ether: yield 2.45 g (93%); mp 193—196°C; $[\alpha]_D^{20}$ −36° (*c* 1, DMF); R_f^1 0.86 and R_f^2 0.76.

Found: C, 62.87; H, 7.86; N, 11.28%. Calcd for $C_{46}H_{65}O_{10}N_7$: C, 63.06; H, 7.50; N, 11.19%.

H-Pro-Pro-Phe-Ile-Val-Gly-Gly-OBzl·HCl (9). Compond **8** (1.75 g, 2 mmol) was dissolved in 98% formic acid (5 ml) and 3.5 M hydrogen chloride in dioxane (10 ml) at 0°C. After 30 min, the solvent was removed by evaporation and the oily residue was solidified by the aid of ether: yield 1.60 g (98%); mp 160—162°C; $[\alpha]_D^{20}$ −39° (*c* ,1 DMF); R_f^2 0.77 and R_f^2 0.44.

Found: C, 58.12; H, 7.17; N, 11.69%. Calcd for $C_{41}H_{57}O_8N_7 \cdot 2H_2O$: C, 58.04; H, 7.37; N, 11.56%.

Z-Arg(NO_2)-Gly-Pro-Pro-Phe-Ile-Val-Gly-Gly-OBzl (10). This compound was prepared from Z-Arg(NO_2)-Gly-OH[4] (0.50 g, 1 mmol) and **9** (0.81 g, 1 mmol) as described for the preparation of **7**: yield 0.97 g (83%); mp 145°C (decomp); $[\alpha]_D^{20}$ −41° (*c* 1, DMF); R_f^1 0.84 and R_f^2 0.70.

Found: C, 58.80; H, 6.70; N, 15.35%. Calcd for $C_{57}H_{77}O_{14}N_{13}$: C, 58.60; H, 6.64; N, 15.59%.

H-Arg-Gly-Pro-Pro-Phe-Ile-Val-Gly-Gly-OH·AcOH (3). Compound **10** (0.47 g, 0.4 mmol) was treated with hydrogen as described for the preparation of **2**: yield 0.32 g (83%); $[\alpha]_D^{20}$ −90° (*c* 0.5, H_2O); R_f^1 0.62 and R_f^2 0.00. Amino acid ratios in acid hydrolyzate: Arg 0.95, Gly 3.38, Pro 1.84, Phe 1.04, Ile 1.00, Val 0.94.

Found: C, 52.78; H, 7.36; N, 16.83%. Calcd for $C_{42}H_{66}O_{10}N_{12} \cdot CH_3COOH \cdot 2H_2O$: C, 53.10; H, 7.50; N, 16.89%.

Z-Gly-Gly-Arg(NO_2)-Gly-Pro-Pro-Phe-Ile-Val-Gly-Gly-OBzl (11). This compound was prepared from **6** (0.53 g, 1 mmol) and **9** (0.81 g, 1 mmol) as described for the preparation of **7**: yield 0.96 g (75%); mp 149°C (decomp); $[\alpha]_D^{20}$ −41° (*c* 1, DMF); R_f^1 0.85 and R_f^2 0.59.

Found: C, 57.18; H, 6.64; N, 16.54%. Calcd for $C_{61}H_{83}O_{16}N_{15}$: C, 57.13; H, 6.52; N, 16.38%.

H-Gly-Gly-Arg-Gly-Pro-Pro-Phe-Ile-Val-Gly-Gly-OH·AcOH (4). Compound **11** (0.39 g, 0.3 mmol) was hydrogenated as described for the preparation of **2**: yield 0.23 g (71%); $[\alpha]_D^{20}$ −100° (*c* 0.5, H_2O); R_f^1 0.59 and R_f^2 0.00. Amino acid ratios in acid hydrolyzate: Arg 1.15, Gly 5.28, Pro 2.02, Phe 1.02, Ile 1.00, Val 0.95.

Found: C, 53.16; H, 7.42; N, 17.84%. Calcd for $C_{46}H_{72}O_{12}N_{14} \cdot CH_3COOH \cdot H_2O$: C, 52.83; H, 7.12; N, 17.97%.

Boc-Arg(NO_2)-Gly-Pro-Pro-Phe-Ile-Val-OBzl (12). Boc-Arg(NO_2)-Gly-OBzl[1] (1.41 g, 3 mmol) was saponified with 1 M sodium hydroxide (3.6 ml, 3.6 mmol), as described for the preparation of **6**, except for acidification. Aqueous 10% citric acid solution was used instead of 1 M hydrochloric acid: yield of hygroscopic Boc-Arg(NO_2)-Gly-OH, 0.74 g (67%); R_f^1 0.69 and R_f^2 0.08. Then this compound was coupled with H-Pro-Pro-Phe-Ile-Val-OBzl·HCl[4] to yield **12**. To a solution of Boc-Arg(NO_2)-Gly-OH (0.74 g, 2 mmol) and NMM (0.22 ml, 2 mmol) in DMF (6 ml), ethyl chloroformate (0.2 ml, 2 mmol) was added at −5°C with stirring. After 10 min, a precooled solution of H-Pro-Pro-Phe-Ile-Val-OBzl·HCl[4] (1.40 g, 2 mmol) and NMM (0.22 ml, 2 mmol) in chloroform (4 ml) was added to it. The reaction mixture was stored in an ice bath for 1 h, then at room temperature overnight. The mixture was evaporated *in vacuo* and the residue was dissolved in ethyl acetate. The solution was washed with 4% sodium hydrogencarbonate, 4% citric acid, and water successively, then dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo* and the residual oil was crystallized with ether; yield 1.45 g (71%); mp 130°C (decomp); $[\alpha]_D^{20}$ −49° (*c* 1, DMF); R_f^1 0.86 and R_f^2 0.69.

Found: C, 58.66; H, 7.27; N, 15.25%. Calcd for $C_{50}H_{73}O_{12}N_{11}$: C, 58.86; H, 7.21; N, 15.10%.

H-Arg(NO_2)-Gly-Pro-Pro-Phe-Ile-Val-OBzl·HCl (13). Compound **12** (1.02 g, 1 mmol) was treated with hydrogen

chlorid as described for the preparation of **9**: yield 0.84 g (88%); mp 157—160 °C; $[\alpha]_D^{20}$ −47° (*c* 1, DMF); R_f^1 0.78 and R_f^2 0.31.

Found: C, 55.32; H, 6.89; N, 15.60%. Calcd for $C_{45}H_{66}O_{10}N_{11}Cl \cdot H_2O$: C, 55.46; H, 7.03; N, 15.81%.

Z-Arg(NO$_2$)-Gly-Pro-Pro-Phe-Ile-Val-OH (14). To a solution of Z-Arg(NO$_2$)-Gly-Pro-Pro-Phe-Ile-Val-OBzl[4] (1.05 g, 1 mmol) in methanol (5 ml), 1 M sodium hydroxide (2 ml, 2 mmol) was added. After 6 h at room temperature, the mixture was evaporated *in vacuo* and diluted with water. The solution was extracted with ethyl acetate. The aqueous layer was acidified to pH 3 with 1 M hydrochloric acid and extracted with 1-butanol. The extract was washed with water and dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo* and the product was crystallized with ether. It was recrystllized from methanol-ether: yield 0.81 g (84%); mp 185 °C (decomp); $[\alpha]_D^{20}$ −43° (*c* 1, DMF); R_f^1 0.69 and R_f^2 0.18.

Found: C, 56.98; H, 6.61; N, 16.04%. Calcd for $C_{46}H_{65}O_{12}N_{11}$: C, 57.30; H, 6.80; N, 15.98%.

Z-Arg(NO$_2$)-Gly-Pro-Pro-Phe-Ile-Val-Arg(NO$_2$)-Gly-Pro-Pro-Phe-Ile-Val-OBzl (15). This compound was prepared from **13** (0.57 g, 0.6 mmol) and **14** (0.58 g, 0.6 mmol) as described for the preparation of **7**: yield 0.84 g (75%); mp 158 °C (decomp); $[\alpha]_D^{20}$ −59° (*c* 1, DMF); R_f^1 0.82 and R_f^2 0.58.

Found: C, 61.88; H, 6.99; N, 16.49%. Calcd for $C_{98}H_{134}O_{21}N_{22} \cdot H_2O$: C, 61.88; H, 7.31; N, 16.20%.

H-Arg-Gly-Pro-Pro-Phe-Ile-Val-Arg-Gly-Pro-Pro-Phe-Ile Val-OH·2AcOH (1). Compound **15** (0.37 g, 0.2 mmol) was treated with hydrogen for 48 h as described for the preparation of **2**: yield 0.30 g (90%); $[\alpha]_D^{20}$ −84° (*c* 1, methanol); R_f^1 0.44 and R_f^2 0.00. Amino acid ratios in acid hydrolyzate: Arg 1.67, Gly 2.00, Pro 3.97, Phe 2.32, Ile 2.19, Val 2.07.

Found: C, 54.62; H, 7.40; N, 16.06%. Calcd for $C_{76}H_{118}O_{15}N_{20} \cdot 2CH_3COOH \cdot 5H_2O$: C, 54.53; H, 7.78; N, 15.90%.

The air-dried product lost 4.9% of its weight after drying or 2 h at 110 °C and 2 mmHg. Calcd for $5H_2O$: 5.1%.

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. Patterns in water are presented in Fig. 2.

Sensory Test. Taste of the peptides was organoleptically determined by panel evaluation employing four people. A series of solutions of decreasing concentration, each half as strong as the preceding one, were prepared. Before tasting the sample, the mouth was thoroughly rinsed with deionized water. The sample solution was held in the mouth for *ca.* 10 s and then spit out, and the threshold value was determined. The results are listed in the Table.

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2) Theabbreviations recommended by the IUPAC-IUB commission of Biochemical Nomenclature (*J. Biol. Chem.*, **247**, 977 (1972)) have been used. Amino acid symbols except glycine denote L-configuation. Additional abbreviations: DCC, dicycloheylcarbodiimide; DCUrea, *N,N'*-dicyclohexylurea; DMF, *N,N*-dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxyenzotriazole; NMM, *N*-methylmorpholine.

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