Studies on Bitter Peptides from Casein Hydrolyzate. XIV.[1] Bitter Taste of Synthetic Analogs of Octapeptide, Arg–Gly–Pro–Phe–Pro–Ile–Ile–Val, Corresponding to the C-Terminal Portion of β-Casein[2]

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In order to elucidate the relationship between the chemical structure and bitter taste of the C-terminal portion of β-casein, some analogues, in which the phenylalanine residue was substituted by D-phenylalanine, lysine, glycine, glutamic acid or L-pyrenylalanine, were synthesized. Sensory analyses and CD spectra showed that the location of a hydrophobic amino acid with the L-configuration between the two proline residues should be important for this series of peptides to produce a strong bitterness.

It is well known that the enzymatic hydrolysis of casein produces many bitter peptide fragments. Hitherto, many workers have investigated the chemical properties of various bitter peptides. A bitter heptapeptide, Arg–Gly–Pro–Pro–Phe–Ile–Val (BPIa), was isolated by Minamiura et al. from casein hydrolyzate.[1] Our research group chemically synthesized BPIa and analogs in order to elucidate the relationship between the bitter taste and the chemical structure.[3—10] Synthetic BPIa produced a bitterness 20-times stronger than that of caffeine. Circular dichroism curves of BPIa and analogs showed that molecules could be folded by the Pro–Pro sequence to make the N-terminus basic portion and the C-terminus hydrophobic portion of the peptide to locate very close. Sensory analyses of BPIa and analogs supported the idea that the peptide produced a strong bitterness when the peptide had a folded structure.

In 1984, our research group synthesized the C-terminal portion of bovine β-casein (202—209, Ribadeau Dumas' formula),[11] Arg–Gly–Pro–Phe–Pro–Ile–Ile–Val. The octapeptide produced an extremely strong bitterness, which was 250-times stronger than that of caffeine.[12]

Although the amino acid contents and primary structure of the C-terminal octapeptide of bovine β-casein and BPIa were similar, the bitter taste potency of each peptide was quite different. In the octapeptide, phenylalanine is located between the two proline residues. On the other hand, BPIa possesses no amino acid between two Pro residues. This seems to be a key point for elucidating why the octapeptide produces such an extremely strong bitterness. In this paper, the preparation of several analogs of the octapeptide, in which phenylalanine residue was substituted by D-phenylalanine, lysine, glycine, glutamic acid or L-pyrenylalanine (Pya), is described. The relationship between the bitter potency and the structure of the synthesized peptides using their CD curves is also discussed.

Results and Discussion

The synthetic route for the D-Phe[4] analog, Arg–Gly–Pro–D-Phe–Pro–Ile–Ile–Val (**2**), is shown in Fig. 1. Syntheses of the intermediates (**7** and **13**) were reported in previous paper.[3,12] A water-soluble active ester, Boc–Pro–ODMSP,[13] was condensed with D-Phe in an aqueous solution to give compound **8**. Acyldipeptide (**8**) was then converted to the corresponding benzyl ester (**9**) by using HOBMCl. HOBMCl is a newly developed reagent that is suitable for amino acid or peptide benzyl esters.[15] Compound **9** was converted to hydrazide (**10**) to condense with **7** by the azide method. Fully protected hexapeptide (**11**) was treated with hydrogen chloride to give the hexapeptide ester hydrochloride (**12**), which was coupled with acyl dipeptide (**13**) to give acyl octapeptide ester (**14**). Hydrogenation of **14** gave the desired octapeptide (**2**). The other four analogs were prepared by a similar procedure. Details concerning the synthesis are described in the experimental section.

The results of sensory analyses of the synthetic peptides are listed in Table 1. Three analogs containing D-Phe (**2**), Lys (**3**), or Gly (**4**) produced a bitterness which was almost the same level as that of BPIa. The peptide in which Phe was substituted by Glu (**5**) produced a bitterness with a slight sweetness. Only Pya[4] analog (**6**) produced a strong bitterness that was as potent as that of the original peptide (**1**). Sensory analyses of these peptides showed the following two important factors for producing a strong bitterness: Any amino acid located between two Pro residues must have (a) the L-configuration and (b) a high hydrophobicity. Our previous studies on the model peptides indicated that the peptides produced a strong bitterness when their CD curves showed that they possessed folded structures.[14—17] It seems that the location of an L-hydrophobic amino acid between the two Pro residues has some effect on maintaining the folded structure of the peptide to produce an extremely strong bitterness.

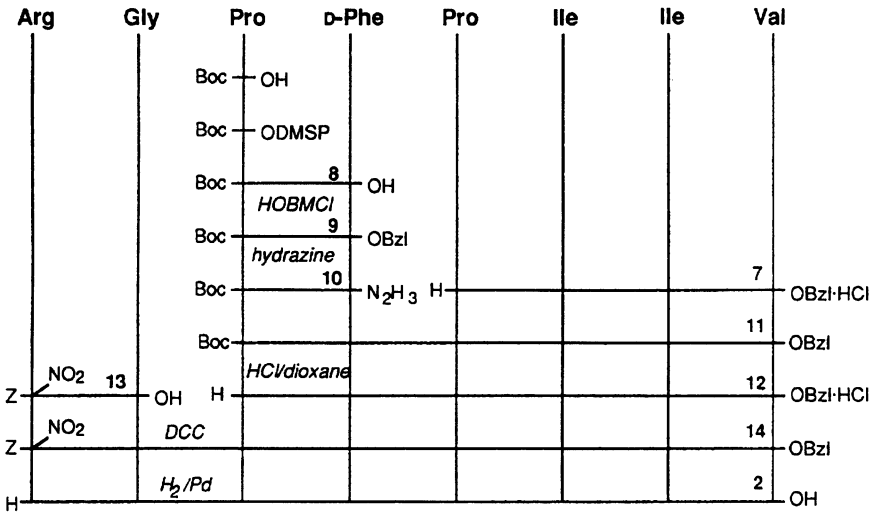

Fig. 1. Synthesis of D-Phe[4] analog (**2**).

Table 1. Threshold Value for Bitter Taste of Peptides Synthesized

Compound	Threshold value[a] / mol dm^{-3}	Rcaf[b]	Taste
Arg–Gly–Pro–Phe–Pro–Ile–Ile–Val (**1**)	0.004	250	Bitter
Arg–Gly–Pro–D-Phe–Pro–Ile–Ile–Val (**2**)	0.083	12	Bitter
Arg–Gly–Pro–Lys–Pro–Ile–Ile–Val (**3**)	0.016	12	Bitter
Arg–Gly–Pro–Gly–Pro–Ile–Ile–Val (**4**)	0.083	64	Bitter
Arg–Gly–Pro–Glu–Pro–Ile–Ile–Val (**5**)	0.031	32	Bitter>Sweet
Arg–Gly–Pro–L-Pya–Pro–Ile–Ile–Val (**6**)	0.004	250	Bitter
Arg–Gly–Pro–Pro–Phe–Ile–Ile–Val (BPIa)	0.05	20	Bitter
Caffeine	1.0	1	Bitter

a) Threshold value for bitter taste. b) Rcaf; ratio of caffeine.

In order to confirm these ideas, the CD spectra of the synthetic peptides were measured. The circular dichroism spectra measured in an aqueous solution are shown in Fig. 2. In the CD curves of **1**, the characteristic spectra was observed at 220 nm. A similar spectra was observed in the CD curve of **6**, which produced the same bitterness potency as that of **1**. On the other hand, the other peptides, which produced a weaker bitterness than that of **1** or **6**, did not show any characteristic spectra at around 220 nm. The results of the CD analysis indicate that **1** and **6** could maintain the turn-type structure, which is essential for producing a strong bitterness in an aqueous solution, while, **2**, **3**, **4**, and **5** could not.

The circular dichroism spectra of the peptides in 90% methanol were then measured. A characteristic spectra induced by the turn-type structure were observed in all of the peptides. For example, the CD curves of **2** in 90% methanol and water are shown in Fig. 3. Probably, peptides (**2**, **3**, **4**, and **5**) with a weaker bitter potency than **1** or **6** were not sufficiently stable to maintain their turn conformation, and their conformations were easily destroyed due to the hydrogen bond in an aqueous solution.

If the folded conformation of **1** was retained in an aqueous solution, the ^{1}H NMR spectra of the peptide could provide some information about its structure. Therefore, the ^{1}H COSY spectra of **1** (10 mM, M= mol dm^{-3}) were measured in D_2O (Fig. 4). Figure 4 shows that NOE's were observed between protons of the N-terminus Arg (γCH_2, δ=1.70; βH, δ=1.77—1.90) and the C-terminus peptide fragments (IleγCH_3, δ= 0.77—0.90; IleβH, δ = 1.90; IleαH, δ = 4.15—4.21; ValγCH_3, δ=0.77—0.90; ValβH, δ=2.07—2.12; ValαH, δ=4.08—4.12). This result definitely indicates that **1** possesses the turn-type structure, which makes the N-terminus moiety and the C-terminus moiety very close to each other. Because **1** and **6** both possess characteristic spectra at around 220 nm in the CD curves and produce the same bitter potency, the conformation of these two peptides in an aqueous solution should be similar.

In conclusion, any amino acid which is located between the two Pro residues should be of the L-form and hydrophobic in order to produce a strong bitterness, as described above. When one or both of these two factors are unsatisfied, the bitterness of any analogs of the octapeptide C-terminal portion of β-casein becomes

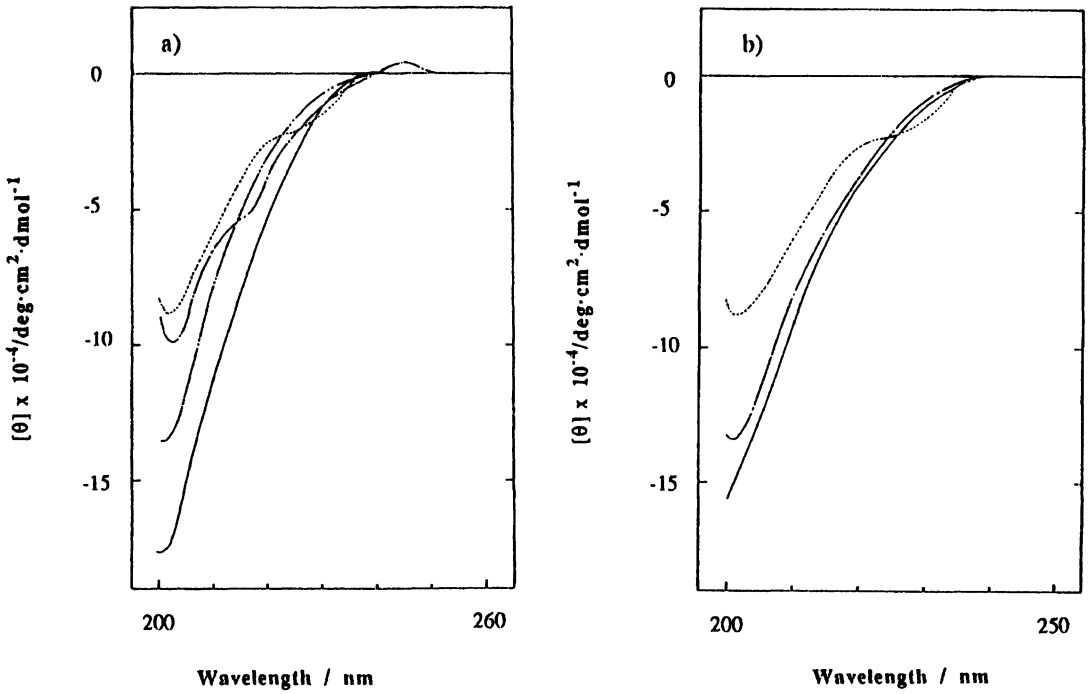

Fig. 2. CD curves of **1**—**6** in water. a) Compound **1** (····), Compound **2** (——), Compound **4** (–··–), Compound **6** (–·–). b) Compound **1** (····), Compound **3** (——), Compound **5** (–·–).

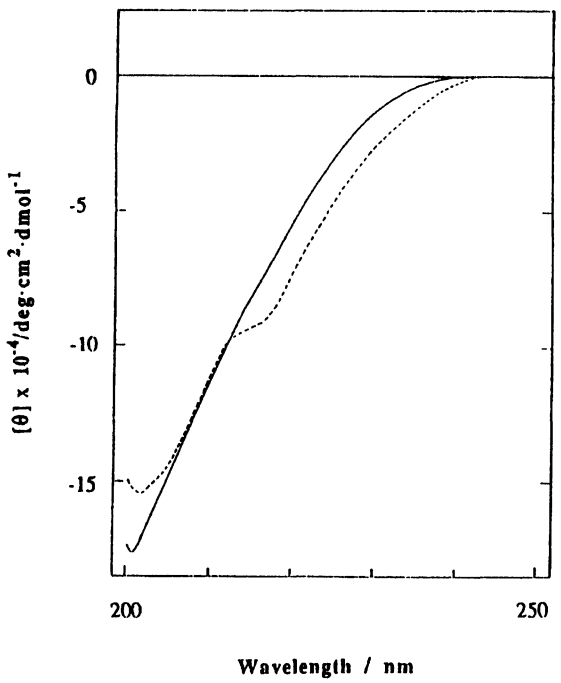

Fig. 3. CD curves of **2** in water (——) and in 90% methanol (- - -).

weak. Comparing the CD curves, it is suggested that their spatial structure in water is very important for producing a strong bitterness. It seems that an amino acid which locates between two Pro residues plays an important role regarding the C-terminus hydrophobic portion of the peptide. Although we have been studying the relationship between the bitterness and secondary structure of peptides, it was very difficult to elucidate the relationship between the bitterness and structure when we studied oligopeptides, due to their flexibility. The octapeptide of the C-terminal portion of β-casein and its Pya^4 analog can be good tools for elucidating the relationship between the bitterness and structure of the oligopeptide, since they were found to retain a rigid structure in an aqueous solution.

Experimental

General. All of the melting points were uncorrected. The optical rotations were measured on a Union PM-101 polarimeter. TLC was carried out on a Merck Silicagel G with 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 the TLC plate were detected by spraying with ninhydrin; those of the amino-group blocked materials were detected by spraying with 25% HBr in acetic acid, and then ninhydrin. Samples for amino acid analysis were hydrolyzed with 6 M HCl in sealed tubes at 110 °C.

Boc–Pro–D-Phe–OH (8). To an ice-cooled solution of D-Phe (1.82 g, 11 mmol) and Et_3N (1.54 ml, 11 mmol) in water (40 ml) was added Boc–Pro–ODMSP (4.64 g, 10 mmol). The mixture was stirred at 0 °C for 12 h and extracted with ethyl acetate (100 ml). The organic layer was successively washed with 4% citric acid and brine, dried over anhydrous sodium sulfate, and evaporated. The residue was crystallized with ether. It was recrystallized from methanol–ether–petroleum ether giving 2.39 g (66%) of **8**; mp 76—78 °C; $[\alpha]_D^{25}$ −17° (*c* 1.0, DMF); R_f^1 0.72; R_f^2 0.86. Found: C, 62.60; H, 7.36; N, 7.64%. Calcd for $C_{19}H_{26}O_5N_2$: C, 62.96;

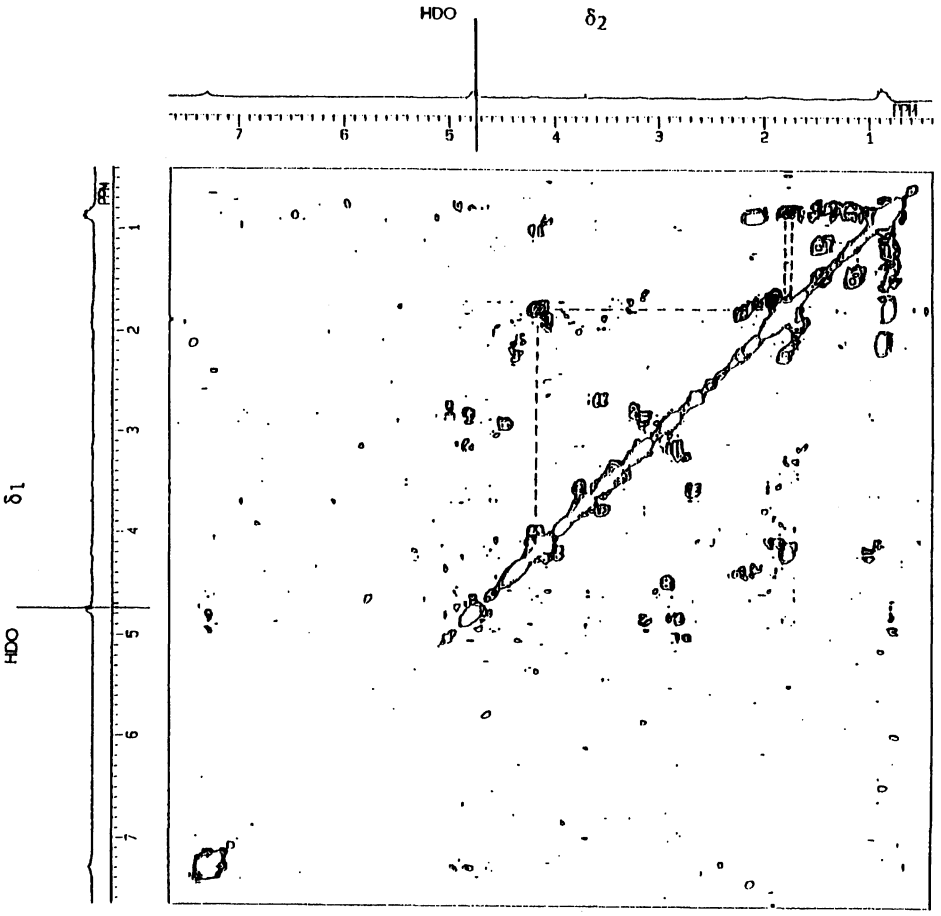

Fig. 4. ^{1}H COSY spectra of Compound **1** (10 mM) in D_2O.

H, 7.23; N, 7.73%.

Boc–Pro–D-Phe–OBzl (9). To a solution of **8** (1.81 g, 5 mmol) and K_2CO_3 (0.7 g, 5 mmol) in DCM (10 ml) was added HOBMCl (2.0 g, 7.5 mmol). The mixture was stirred at room temperature overnight and filtered. The filtrate was concentrated in vacuo and the residue was dissolved in toluene. The organic layer was washed with 0.5 M NaOH and brine, dried over anhydrous sodium sulfate, and evaporated. Colorless oil of the product (0.96 g, 53%) was obtained; $[\alpha]_D^{25}$ −18° (*c* 1.0, DMF); R_f^1 0.86; R_f^2 0.91. Found: C, 68.42; H, 7.12; N, 5.97%. Calcd for $C_{26}H_{32}O_5N_2$: C, 69.00; H, 7.13; N, 6.19%.

Boc–Pro–D-Phe–N_2H_3 (10). To a solution of **9** (4.53 g, 10 mmol) in MeOH (20 ml) was added $N_2H_4 \cdot H_2O$ (2.5 ml, 50 mmol). The mixture was allowed to stand at room temperature for 2 d and evaporated. The residue was crystallized from ether–petroleum ether. It was recrystallized from methanol–ether–petroleum ether giving 2.90 g (77%) of the product; mp 153—155 °C; $[\alpha]_D^{25}$ −25° (*c* 1.0, DMF); R_f^1 0.81; R_f^2 0.80. Found: C, 60.29; H, 7.51; N, 14.84%. Calcd for $C_{19}H_{28}O_4N_4$: C, 60.62; H, 7.50; N, 14.88%.

Boc–Pro–D-Phe–Pro–Ile–Ile–Val–OBzl (11). Compound **10** (1.51 g, 4 mmol) was dissolved in 1 M HCl (10 ml) and acetic acid (27 ml), and cooled at −15 °C. To this solution was added $NaNO_2$ (0.30 g, 4.4 mmol). The solution was allowed to stand at −15 °C for 5 min; chilled water was added, and stirred at the same temperature for 30 min. The solution was extracted with EtOAc, the organic layer was washed with chilled 10% sodium hydrogen carbonate, dried over anhyrdrous sodium sulfate, and filtered. The filtrate was added to a precooled solution of **7** (2.27 g, 4 mmol) in DMF (40 ml). The mixture was stirred below 0 °C for 3 d and evaporated. The residue was dissolved in EtOAc, washed with 4% potassium hydrogen sulfate, 4% sodium hydrogen carbonate and water, dried over anhydrous sodium sulfate, and evaporated. The oily residue was crystallized from ether-petroleum ether to give 2.73 g (78%) of the product; mp 115—117 °C; $[\alpha]_D^{25}$ −107° (*c* 1.0, DMF); R_f^1 0.95; R_f^2 0.91. Found: C, 65.28; H, 8.03; N, 9.64%. Calcd for $C_{48}H_{70}O_9N_6$: C, 65.88; H, 8.06; N, 9.60%.

H–Pro–D-Phe–Pro–Ile–Ile–Val–OBzl·HCl (12). Compound **11** (1.58 g, 1.8 mmol) was dissolved in 4 M hydrogen chloride in dioxane (10 ml). The solution was allowed to stand for 1 h at room temperature and evaporated. The residue was crystallized from ether giving 1.39 g (95%) of **12**; mp 150—152 °C; $[\alpha]_D^{25}$ −132° (*c* 1.0, DMF); R_f^1 0.74; R_f^2 0.83. Found: C, 63.59; H, 7.92; N, 10.15%. Calcd for $C_{43}H_{63}O_7N_6$: C, 63.64; H, 7.83; N, 10.36%.

Z-Arg(NO_2)–Gly–Pro–D-Phe–Pro–Ile–Ile–Val–OBzl (14). To an ice-cooled solution of **12** (0.68 g, 1.65 mmol), **13** (1.22 g, 1.5 mmol), and NMM (165 μl, 1.5 mmol) in DMF (3 ml) and chloroform (3 ml) was added DCC (0.34 g, 1.65 mmol). The mixture was stirred at 0 °C for 2 h, then at room temperature overnight. After removing DCurea, the filtrate was evaporated. The residue was dissolved in EtOAc, washed with 0.5 M HCl, 4% sodium

hydrogen carbonate, dried over anhydrous sodium sulfate, and evaporated. An oily residue was collected by the aid of ether and applied to a column of silica gel (2×60 cm). Flash column chlomatography was carried out using chloroform–methanol (20 : 1 v/v), and fractions containing the product were collected. Compound **14** (0.37 g, 64%) was solidified by evaporation; mp 131—133 °C; $[\alpha]_D^{25}$ −95° (*c* 1.0, DMF); R_f^1 0.81; R_f^2 0.74. Found: C, 58.30; H, 7.20; N, 13.90%. Calcd for $C_{59}H_{82}O_{13}N_{12}\cdot 2H_2O$: C, 58.87; H, 7.22; N, 13.97%.

H-Arg–Gly–Pro–D-Phe–Pro–Ile–Ile–Val–OH·2HCl (2). Compound **14** (0.47 g, 0.4 mmol) was dissolved in MeOH (3 ml) and acetic acid (3 ml). The mixture was hydrogenated in the presence of 10% palladium on carbon at room temperature for 72 h. The filtrate from the catalyst was evaporated, and an oily residue was solidified by the aid of ether to give a hygroscopic acetate form. It was dissolved in methanol and 4 M hydrogen chloride in dioxane was added. The solution was evaporated and residue was solidified from acetate and ether; yield 0.29 g (79%); $[\alpha]_D^{25}$ −54° (*c* 1.0, DMF); R_f^1 0.62; R_f^2 0.00. Found: C, 50.35; H, 7.66; N, 15.08%. Calcd for $C_{44}H_{73}O_9N_{11}Cl_2\cdot 4H_2O$: C, 50.65; H, 7.84; N, 14.77%. Amino acid analysis (6 M HCl, 110 °C, 48 h): Arg, 0.91; Gly, 1.00; Pro, 2.07; D-Phe, 1.02; Ile, 2.00; Val, 1.00.

Boc–Pro–Lys(Z)–OH (15). Compound **15** was prepared from Boc–Pro–ODMSP (2.32 g, 5 mmol) and Lys(Z) (1.54 g, 5.5 mmol), as described regarding the preparation of **8**. The product was obtained as hygroscopic powders; yield 1.56 g (65%); $[\alpha]_D^{25}$ −32° (*c* 1.0, DMF); R_f^1 0.79; R_f^2 0.70. Found: C, 60.28; H, 7.48; N, 10.22%. Calcd for $C_{24}H_{35}O_7N_3$: C, 60.36; H, 7.39; N, 10.29%.

Boc–Pro–Lys(Z)–Pro–Ile–Ile–Val–OBzl (16). To an ice-cooled solution of **15** (1.43 g, 3 mmol), **7** (1.70 g, 3 mmol), Et_3N (0.42 ml, 3 mmol) and HOBt (0.49 g, 3.6 mmol) was added DCC (0.68 g, 3.3 mmol). The mixture was stirred at 0 °C for 2 h, then at room temperature overnight. After removing DCurea, the filtrate was concentrated in vacuo. The residue was dissolved in EtOAc, washed with 10% citric acid, 4% sodium hydrogen carbonate and water, dried over anhydrous sodium sulfate, and evaporated. The oily residue was solidified from ether and petroleum ether giving 2.18 g (73%) of the product; mp 127—129 °C; $[\alpha]_D^{25}$ −64° (*c* 1.0, DMF); R_f^1 0.95; R_f^2 0.93. Found: C, 64.82; H, 8.21; N, 8.81%. Calcd for $C_{53}H_{79}O_{11}N_6$: C, 65.19; H, 8.17; N, 8.61%.

H-Pro–Lys(Z)–Pro–Ile–Ile–Val–OBzl·HCl (17). Compound **16** (1.89 g, 2 mmol) was treated, as described regarding the preparation of **12**; yield 1.55 g (88%); mp 134—136 °C; $[\alpha]_D^{25}$ −55° (*c* 1.0, DMF); R_f^1 0.75; R_f^2 0.62. Found: C, 62.08; H, 7.88; N, 10.45%. Calcd for $C_{48}H_{72}O_9N_7Cl$: C, 62.21; H, 7.85; N, 10.58%.

Z-Arg(NO₂)–Gly–Pro–Lys(Z)–Pro–Ile–Ile–Val–OBzl (18). Compound **18** was prepared by the condensation of **13** (0.68 g, 1.65 mmol) and **17** (1.33 g, 15 mmol) by the similar manner, as described regarding the preparation of **14**: yield 1.47 g (75%); mp 115—117 °C; $[\alpha]_D^{25}$ −63° (*c* 1.0, DMF); R_f^1 0.89; R_f^2 0.67. Found: C, 58.53; H, 7.21; N, 13.67%. Calcd for $C_{64}H_{91}O_{15}N_{13}\cdot H_2O$: C, 59.10; H, 7.22; N, 14.00%.

H-Arg–Gly–Pro–Lys–Pro–Ile–Ile–Val–OH·3HCl (3). Compound **18** (0.45 g, 0.35 mmol) was hydrogenated, as described regarding the preparation of **2**; yield 0.29 g (90%); $[\alpha]_D^{25}$ −135° (*c* 0.5, H_2O); R_f^1 0.40; R_f^2 0.00. Found: C, 46.27; H, 7.39; N, 16.28%. Calcd for $C_{41}H_{77}O_9N_{12}Cl_3\cdot 4H_2O$: C, 46.62; H, 7.95; N, 16.17%. Amino acid analysis (6 M HCl, 110 °C, 48 h): Arg, 0.90; Gly, 1.00; Pro, 2.02; Lys, 0.98; Ile, 1.96; Val, 1.03.

Boc–Pro–Gly–OH (19). Compound **19** was prepared from Boc–Pro–ODMSP (4.64 g, 10 mmol) and Gly (0.83 g, 10 mmol), as described regarding the preparation of **8**; yield 1.86 g (68%); mp 165—166 °C; $[\alpha]_D^{25}$ −63° (*c* 1.0, DMF); R_f^1 0.74; R_f^2 0.89. Found: C, 52.73; H, 7.72; N, 9.98%. Calcd for $C_{12}H_{20}O_5N_2$: C, 52.91; H, 7.52; N, 10.11%.

Boc–Pro–Gly–Pro–Ile–Ile–Val–OBzl (20). Compound **19** (0.82 g, 3 mmol) and NMM (0.33 ml, 3 mmol) was dissolved in THF (6 ml); the solution was cooled at −15 °C. To this solution was added ECF (0.3 ml, 3 mmol) and the mixture was stirred at −15 °C for 5 min. A solution of **7** (1.70 g, 3 mmol) and NMM (0.33 ml, 3 mmol) in DCM (6 ml) was added and the mixture was stirred at −15 °C for 2 h, then at room temperature overnight, and evaporated. The oily residue was dissolved in EtOAc, washed with 4% potassium hydrogen sulfate, 4% sodium hydrogen carbonate and water, dried over anhydrous sodium sulfate, and evaporated. The oily residue was solidified from ether–petroleum ether to give 1.95 g (83%) of the product; mp 193—194 °C; $[\alpha]_D^{25}$ −75° (*c* 1.0, DMF); R_f^1 0.85; R_f^2 0.78. Found: C, 61.94; H, 8.40; N, 10.33%. Calcd for $C_{40}H_{64}O_9N_5$: C, 62.15; H, 8.35; N, 10.87%.

H-Pro–Gly–Pro–Ile–Ile–Val–OBzl·HCl (21). Compound **20** (1.57 g, 2 mmol) was treated with hydrogen chloride, as described regarding the preparation of **12**; yield 1.43 g (99%); mp 240 °C<; $[\alpha]_D^{25}$ −77° (*c* 1.0, DMF); R_f^1 0.78; R_f^2 0.43. Found: C, 59.85; H, 8.03; N, 11.66%. Calcd for $C_{36}H_{57}O_7N_6Cl$: C, 59.93; H, 7.98; N, 11.65%.

Z-Arg(NO₂)–Gly–Pro–Gly–Pro–Ile–Ile–Val–OBzl (22). This was prepared from **13** (0.68 g, 1.65 mmol) and **21** (1.08 g, 1.5 mmol), as described regarding the preparation of **14**; yield 1.03 g (64%); mp 140—142 °C; $[\alpha]_D^{25}$ −72° (*c* 0.5, H_2O); R_f^1 0.88; R_f^2 0.65. Found: C, 56.46; H, 7.20; N, 14.83%. Calcd for $C_{37}H_{66}O_9N_{11}\cdot 2H_2O$: C, 56.09; H, 7.26; N, 15.10%.

H-Arg–Gly–Pro–Gly–Pro–Ile–Ile–Val–OH·2HCl (4). Compound **4** was prepared from **22** (0.215 g, 0.2 mmol), as described regarding the preparation of **12**; yield 1.43 g (99%); $[\alpha]_D^{25}$ −96° (*c* 0.5, H_2O); R_f^1 0.49; R_f^2 0.00. Found: C, 46.38; H, 8.04; N, 16.09%. Calcd for $C_{37}H_{67}O_9N_{11}Cl_2\cdot 4H_2O$: C, 46.64; H, 7.95; N, 16.17%. Amino acid analysis (6 M HCl, 110 °C, 48 h): Arg, 0.89; Gly, 2.03; Pro, 2.04; Ile, 1.92; Val, 1.00.

Boc–Pro–Glu(OBzl)–OH·DCHA (23). To a solution of Glu(OBzl) (1.30 g, 5.5 mmol) and Et_3N (0.77 ml, 5.5 mmol) in THF (7.5 ml) and water (7.5 ml) was added Boc–Pro–OSu (1.56 g, 5 mmol). The solution was stirred at room temperature overnight and evaporated. Water was added to the residue, and the pH of the aqueous solution was adjusted at pH 3 with 20% citric acid. The aqueous layer was extracted with EtOAc and the organic layer was washed with 4% citric acid and water, dried over anhydrous sodium sulfate, and evaporated. The residual oil was dissolved in ether and DCHA was added. The resulting precipitate of the DCHA salt was collected; yield 2.73 g (89%); mp 104—106 °C; $[\alpha]_D^{25}$ −20° (*c* 1.0, DMF); R_f^1 0.86; R_f^2 0.82. Found: C, 66.23; H, 8.66; N, 6.80%. Calcd for $C_{34}H_{53}O_7N_3$: C,

66.31; H, 8.68; N, 6.82%.

Boc–Pro–Glu(OBzl)–Pro–Ile–Ile–Val–OBzl (24). Compound **23** (1.85 g, 3 mmol) was treated with 10% citric acid to remove DCHA salt. The DCHA-free acyl dipeptide was condensed with **7** (1.70 g, 3 mmol) in the same manner, as described regarding the preparation of **16**; yield 2.26 g (80%); mp 160—162 °C; $[\alpha]_D^{25}$ −73° (*c* 1.0, DMF); R_f^1 0.97; R_f^2 0.94. Found: C, 64.35; H, 8.05; N, 8.75%. Calcd for $C_{51}H_{74}O_{11}N_6$: C, 64.66; H, 7.89; N, 8.87%.

H- Pro- Glu(OBzl)- Pro- Ile- Ile- Val- OBzl·HCl (25). Compound **24** (1.89 g, 2 mmol) was treated with hydrogen chloride, as described regarding the preparation of **12**; yield 1.64 g (93%); mp 152—154 °C; $[\alpha]_D^{25}$ −62° (*c* 1.0, DMF); R_f^1 0.77; R_f^2 0.67. Found: C, 62.34; H, 7.86; N, 9.48%. Calcd for $C_{46}H_{67}O_9N_6Cl$: C, 62.52; H, 7.66; N, 9.51%.

Z- Arg(NO$_2$)- Gly- Pro- Glu(OBzl)- Pro- Ile- Ile- Val–OBzl (26). Compound **26** was prepared by the condensation of **13** (0.68 g, 1.65 mmol) with **25** (1.33 g, 1.5 mmol), as described regarding the preparation of **14**; yield 0.14 g (73%); mp 139—141 °C; $[\alpha]_D^{25}$ −63° (*c* 1.0, DMF); R_f^1 0.88; R_f^2 0.77. Found: C, 58.89; H, 7.18; N, 12.88%. Calcd for $C_{62}H_{86}O_{15}N_{12}\cdot 2H_2O$: C, 58.37; H, 7.13; N, 13.18%.

H-Arg–Gly–Pro–Glu–Pro–Ile–Ile–Val–OH·2HCl (5). Compound **26** (0.43 g, 0.35 mmol) was hydrogenated, as described regarding the preparation of **2**; yield 0.30 g (94%); $[\alpha]_D^{25}$ −134° (*c* 0.5, H_2O); R_f^1 0.45; R_f^2 0.00. Found: C, 45.33; H, 7.15; N, 14.14%. Calcd for $C_{40}H_{71}O_{11}N_{11}Cl_2\cdot 6H_2O$: C, 45.27; H, 6.95; N, 14.52%. Amino acid analysis (6 M HCl, 110 °C, 48 h): Arg, 0.90; Gly, 1.00; Pro, 2.04; Glu, 0.98; Ile, 1.96; Val, 1.03.

Boc–Pro–Pya–OH (27). Compound **27** was prepared by the condensation of Pya[19] (0.96 g, 3.3 mmol) and Boc–Pro–OSu (0.94 g, 3 mmol), as described regarding the preparation of **23**; yield 0.30 g (61%); mp 106—108 °C (decomp); $[\alpha]_D^{25}$ −58° (*c* 1.0, DMF); R_f^1 0.72; R_f^2 0.75. Found: C, 71.55; H, 6.22; N, 5.82%. Calcd for $C_{29}H_{30}O_5N_2$: C, 71.57; H, 6.23; N, 5.76%.

Boc–Pro–Pya–Pro–Ile–Ile–Val–OBzl (28). To an ice-cooled solution of **27** (0.98 g, 2 mmol), **7** (1.13 g, 2 mmol), Et_3N (0.28 g, 2 mmol), and HOBt (0.32 g, 2 mmol) in DCM (10 ml) was added EDC·HCl (0.42 g, 2.2 mmol). The mixture was stirred at 0 °C for 2 h, then at room temperature overnight, and evaporated. The residue was dissolved in EtOAc, washed with 4% potassium hydrogen sulfate, 4% sodium hydrogen carbonate and water, dried over anhydrous sodium sulfate, and evaporated. The oily residue was solidified from ether-petroleum ether; yield 1.70 g (85%); mp 130—132 °C (decomp); $[\alpha]_D^{25}$ −61° (*c* 1.0, DMF); R_f^1 0.98; R_f^2 0.96. Found: C, 69.47; H, 7.56; N, 8.55%. Calcd for $C_{58}H_{74}O_9N_6$: C, 69.70; H, 7.48; N, 8.41%.

H- Pro- Pya- Pro- Ile- Ile- Val- OBzl·HCl (29). Compound **28** (1.50 g, 1.5 mmol) was treated with hydrogen chloride, as described regarding the preparation of **12**; yield 1.28 g (91%); mp 176—178 °C (decomp); $[\alpha]_D^{25}$ −108° (*c* 0.5, MeOH); R_f^1 0.82; R_f^2 0.75. Found: C, 67.96; H, 7.11; N, 8.66%. Calcd for $C_{53}H_{67}O_7N_6Cl$: C, 68.03; H, 7.23; N, 8.98%.

Z-Arg(NO$_2$)–Gly–Pro–Pya–Pro–Ile–Ile–Val–OBzl (30). Compound **30** was prepared by the condensation of **13** (0.45 g, 1.1 mmol) with **29** (0.94 g, 1 mmol), as described regarding the preparation of **14**; yield 0.74 g (58%); mp 143—146 °C (decomp); $[\alpha]_D^{25}$ −68° (*c* 0.5, DMF); R_f^1 0.92; R_f^2 0.85. Found: C, 63.11; H, 6.88; N, 12.68%. Calcd for $C_{69}H_{86}O_{13}N_{12}\cdot H_2O$: C, 63.32; H, 6.65; N, 12.85%.

H-Arg–Gly–Pro–Pya–Pro–Ile–Ile–Val–OH·2HCl (6). Compound **30** (0.39 g, 0.3 mmol) was hydrogenated, as described regarding the preparation of **2**; yield 0.22 g (70%); $[\alpha]_D^{25}$ −88° (*c* 0.5, H_2O); R_f^1 0.59; R_f^2 0.00. Found: C, 49.51; H, 7.72; N, 11.75%. Calcd for $C_{54}H_{77}O_9N_{11}Cl_2\cdot 4H_2O$: C, 49.46; H, 7.23; N, 11.75%. Amino acid analysis (6 M HCl, 110 °C, 48 h): Arg, 1.00; Gly, 0.98; Pro, 2.10; Pya, 1.01; Ile, 1.96; Val, 0.94.

CD Measurements. The CD spectra were recorded on a JASCO J-500A spectropolarimeter equipped with a Taiyo thermo supplier EZ-100 using a quartz cell of 1 mm pathlength. The spectra in water or in 90% MeOH was measured at a peptide concentration of 100 mM.

NMR Measurements. The ^{1}H COSY spectra were measured in D_2O on a JEOL JNM-EX270 spectrometer. The chemical shifts are given in δ values (ppm) from tetramethylsilane as an international standard.

Sensory Test. Sensory analyses of the synthetic peptides were carried out according to the method of Ishibashi et al.[20] The results of sensory analyses are listed in Table 1.

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2) Abbreviations used are according to IUPAC-IUB Commissions, *Eur. J. Biochem.*, **138**, 9 (1984). Amino acid symbols except glycine denoted L-configuration. Additional abbreviations: Pya, L-pyrenylalanine; AH: electron positive group(s); X: hydrophobic group(s); BPIa, bitter peptide Ia; Boc, *t*-butoxycarbonyl; HODMSP, (*p*-hydroxyphenyl) dimethylsulfonium sulfate; HOBMCl, (*p*-hydroxyphenyl) benzylmethylsulfonium chloride; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxy benzotriazole; Bzl, benzyl; DCHA, dicyclohexylamine; Rcaf, ratio of caffeine; Et_3N, triethylamine; DMF, *N,N*-dimethylformamide; DCM, dichloromethane; DCurea, *N,N'*-dicyclohexylurea; NMM, *N*-methylmorpholine; THF, tetrahydrofuran; ECF, ethyl chloroformate; Su, succinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

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