  Bull. Chem. Soc. Jpn., 57, 97—102 (1984)

Studies of Bitter Peptides from Casein Hydrolyzate. VI.[1] Syntheses and Bitter Taste of BPIc (Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His) and Its Analogs and Fragments[2]

Hidenori KANEHISA
Research and Development Division, Sunstar Inc., Asahi-cho, Takatsuki, Osaka 569
(Received June 15, 1983)

In order to investigate the relationship between chemical structure and bitter taste, the bitter peptide BPIc (Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His) isolated from casein hydrolyzate by Minamiura *et al.* and its analogs and fragments were synthesized. BPIc, whose threshold value of bitter taste was 0.05 mM, was found to be one of the most bitter compounds, like quinine and phenylthiourea. However, [Gly^{5,6}]- and [Gly^{9,10}]-BPIc, and N-terminal octa- and heptapeptide fragments of BPIc possessed much weaker bitterness than BPIc. The results suggested that 5,6-proline and the basic nature of C-terminal are necessary for the strong bitterness exhibited by BPIc.

In a series of studies of bitter peptides, the first paper[3] described the synthesis of bitter peptide BPIa (H-Arg-Gly-Pro-Pro-Phe-Ile-Val-OH) isolated from enzymatic hydrolyzate of cow milk casein by Minamiura *et al.*[4] The peptide possessed an extremely bitter taste of the same level as well known bitter substances such as quinine and phenylthiourea. The threshold value of bitter taste of BPIa was found to be 0.05 mM (1 M=1 mol dm^{-3}). To elucidate the structure-taste relationship of BPIa, its numerous fragments and analogs were prepared and their tastes were measured.[1,5-7] All the fragments except N-terminal hexapeptide fragment exhibited a weaker bitterness than BPIa itself.[5] Des-Gly^2-BPIa was the same as BPIa,[5] whereas the analogs in which prolylproline residue was substituted for glycylglycine or D-prolyl-D-proline residues exhibited a weaker bitterness than BPIa.[6] The results of their CD examinations in water also suggested that at least six amino acid residues are necessary for the exhibition of strong bitterness and that the spatial structure of BPIa molecule attributed to L-proline residue at 3-position contributes to its bitter taste.[5,6] In order to clarify the nature of the N-terminal arginine residue, the strength of bitterness for Arg^1-substituted analogs were compared with that of des-Gly^2-BPIa.[1] When the position of arginine was filled by another basic amino acid, the analogs possessed a bitter taste. However, the strength of bitterness decreased with reducing of the side-chain length of the basic amino acid residues. On the other hand, the analogs in which the position of arginine was replaced by hydrophobic or bulky side chain amino acid still possessed a bitter taste. However its strength was slightly weaker than that of basic amino acid-substituted analogs. The C-terminal moiety in the sequence of BPIa was studied; the analog in which C-terminal tripeptide moiety of BPIa was substituted for triphenylalanine residue possessed the strongest bitterness in the series of BPIa analogs.[7] The results suggested that both basic amino acid residue in N-terminal position and hydrophobic amino acid residues in C-terminal moiety are necessary for an intense bitterness in BPIa. This finding is also supported from taste behavior results: that des-Arg^1-BPIa and the N-terminal pentapeptide fragment of BPIa exhibited a much weaker bitterness than BPIa.[5]

Minamiura *et al.* isolated a bitter decapeptide named BPIc from casein hydrolyzate in addition to BPIa and decided that its amino acid sequence was H-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His-OH.[4] It is interesting to compare the structure of BPIc with that of BPIa. They have several characteristic features in common. These peptides include prolylproline residue in the center of the molecules. Also, they include a basic amino acid residue (histidine or arginine) and some hydrophobic amino acid residues (valine, phenylalanine, isoleucine, and/or tyrosine) on both terminal positions in these molecules. Although BPIc is extremely bitter according to Minamiura *et al.*,[4] no study has made on the synthesis of BPIc.

In order to elucidate the relationship between chemical structure and bitterness of peptides, the author prepared BPIc. The author also prepared [Gly^{5,6}]-BPIc, in which prolylproline residue at 5,6-position was replaced by glycylglycine, and [Gly^{9,10}]-BPIc and N-terminal octa- and heptapeptide fragments, based on the findings in the synthetic studies of BPIa. The present paper describes the syntheses and bitter taste of BPIc and its analogs and fragments.

The sequence of reactions employed for the synthesis of BPIc is shown in Fig. 1. Acyldipeptide acid (**2**), derived from the corresponding methyl ester (**1**), was condensed with H-Pro-Phe-OEt·HCl (**4**) to yield the protected N-terminal tetrapeptide derivative (**5**) by means of the dicyclohexylcarbodiimide-1-hydroxybenzotriazole (DCC-HOBt) method. It was converted to the corresponding acid (**6**) by saponification with sodium hydroxide. Acyltripeptide ester (**8**) was prepared from Boc-Pro-Pro-OH (**7**) and H-Gly-OBzl·TsOH by the mixed anhydride (MA) method, and then the N-terminal protected group of **8** was removed by the action of hydrogen chloride in dioxane to yield the tripeptide ester hydrochloride (**9**).

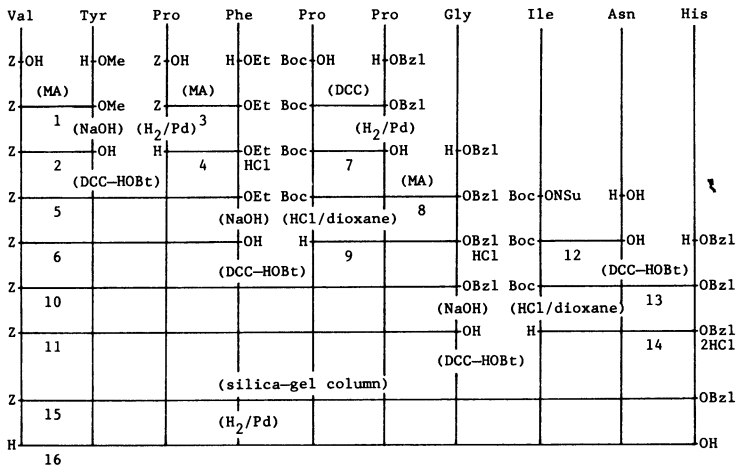

Fig. 1. Synthesis of BPIc.

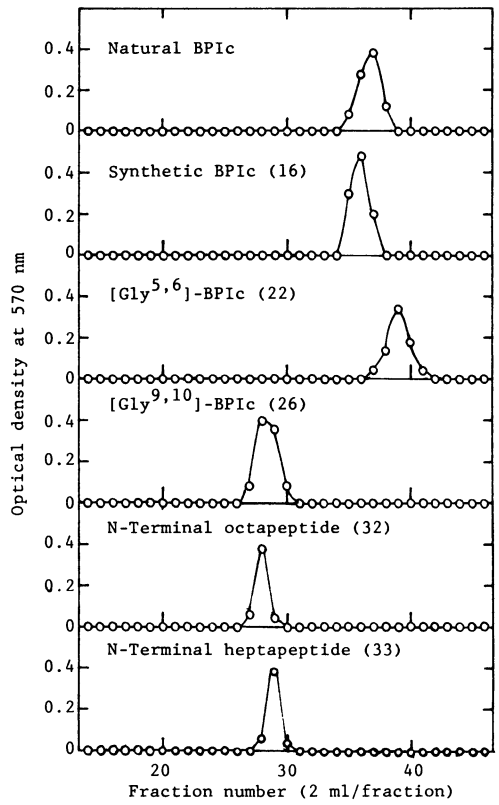

Fig. 2. Carboxymethylcellulose column chromatography of synthetic and natural BPIc. Solvent: 0.2 M pyridinium acetate, pH 5.0.

9 was coupled with **6** by the DCC-HOBt method and the resulting acylheptapeptide ester (**10**) was easily converted to the corresponding acid (**11**) by saponification. Asparagine was acylated to Boc-Ile-Asn-OH (**12**) with Boc-Ile-ONSu, then **12** and H-His-OBzl·2TsOH were coupled to yield acyltripeptide ester (**13**) by the DCC-HOBt method. The amino protection of **13** was removed by the action of hydrogen chloride in 98% formic acid. The compound **14** thus obtained and **11** were condensed by the DCC-HOBt method in DMF. The resulting acyldecapeptide ester (**15**) was purified with extraction of 1-butanol, and then the extract was placed on a silica-gel column and eluted with chloroform-methanol (5:1, v/v). The catalytic hydrogenation of pure **15** in methanol containing hydrogen chloride gave decapeptide BPIc (**16**).

The preparation of [Gly^{5,6}]-BPIc was described as follows. **6** was condensed with triglycine ethyl ester (**18**) by the DCC-HOBt method and the obtained heptapeptide (Z-Val-Tyr-Pro-Phe-Gly-Gly-Gly-OEt, **19**) was easily saponified to yield the corresponding acid (**20**), which was made to condense with **14** by the DCC-HOBt method. The Z-Val-Tyr-Pro-Phe-Gly-Gly-Gly-Ile-Asn-His-OBzl (**21**) thus obtained was hydrogenated in the presence of palladium black to give [Gly^{5,6}]-BPIc (**22**).

[Gly^{9,10}]-BPIc was prepared as described below. Boc-Ile-OH was coupled with H-Gly-Gly-OBzl·HCl by the MA method to give Boc-Ile-Gly-Gly-OBzl (**23**). It was converted to the corresponding tripeptide ester hydrochloride (**24**) by the treatment of hydrogen chloride in dioxane. Then **24** and **11** were condensed by the DCC-HOBt method to yield Z-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Gly-Gly-OBzl (**25**). It was hydrogenated to give [Gly^{9,10}]-BPIc (**26**).

N-Terminal octapeptide fragment of BPIc was synthesized by the following procedures. Boc-Gly-OH and H-Ile-OBzl·TsOH were coupled by the MA method to give Boc-Gly-Ile-OBzl (**27**). This was converted to the corresponding dipeptide ester hydrochloride (**28**) by the action of hydrogen chloride in dioxane. **28** and **7** were condensed by the MA method to yield Boc-Pro-Pro-Gly-Ile-OBzl (**29**). This was converted to the corresponding tetrapeptide ester hydrochloride (**30**) by the action of hydrogen chloride in dioxane. **30** and **6** were then condensed by the DCC-HOBt method to give Z-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-OBzl (**31**). Catalytic hydrogenation of **31** gave N-terminal octapeptide fragment of BPIc (**32**). The N-terminal heptapeptide fragment of BPIc, H-Val-Tyr-Pro-Phe-Pro-Pro-Gly-OH (**33**), was obtained from **9** by hydrogenation.

The purity of synthetic BPIc, its analogs and fragments, and their intermediates was confirmed by thin-layer examinations on two solvent systems, and by elemental analyses. The homogeneity of the final products was also confirmed by paper electrophoresis, carboxymethylcellulose column chromatography, and amino acid analysis.

The taste of final products was organoleptically determined by panel evaluation employing several persons. The results are listed in the Table. All the synthetic peptides possessed a bitter taste. BPIc, whose threshold value of bitterness was 0.05 mM, was the same as BPIa. However, the strength of bitterness of BPIc analog **22** was *ca.* 1/5 of that of BPIc. This means that 5,6-proline residues are necessary

Table. The threshold values for bitter taste of BPIc and its analogs and fragments

Compound	Threshold values for bitter taste/mM
Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His (BPIc, **16**)	0.05
Val-Tyr-Pro-Phe-Gly-Gly-Gly-Ile-Asn-His (**22**)	0.23
Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Gly-Gly (**26**)	0.30
Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile (**32**)	0.15
Val-Tyr-Pro-Phe-Pro-Pro-Gly (**33**)	0.30
Ile-Asn-His[8]	25.00
Asn-His[8]	100.00

for the strong bitterness of BPIc. The analog **26** was one-sixth as bitter as BPIc. Also, bitterness of BPIc fragments (**32** and **33**) was weaker than that of BPIc. Further, H-Ile-Asn-His-OH and H-Asn-His-OH were nearly tasteless. These results indicate that the basic nature of C-terminal position in BPIc, in addition to the hydrophobic nature of its N-terminal contributes to its bitter taste. The findings obtained from BPIc are equivalent to those from BPIa. Therefore, BPIc can be regarded as a retro form of BPIa possessing a reverse sequence of BPIa; in bitter peptides contain prolylproline residue in the center of the molecule and both basic and hydrophobic amino acid residues in N- and C-terminal positions respectively, it is possible for N- and C-terminal amino acid residues to change places with each other. Further studies of this problem are in progress.

Experimental

All melting points are uncorrected. Thin layer chromatography was carried out on Merck silica-gel G. Developing solvents commonly used were (1) 1-butanol-acetic acid-pyridine-water (4:1:1:2, v/v), and (2) chloroform-methanol (5:1, v/v). Materials possessing free amino groups on a thin layer plate were detected by spraying with ninhydrin. Compounds with blocked amino groups were detected by spraying with 25% hydrogen bromide in acetic acid and then with ninhydrin. Optical rotations were measured on a JASCO automatic polarimeter, DIR-SL type. Amino acid analyses were performed with a Hitachi amino acid analyzer, KLA-5 type. Prior to analysis, compounds were dried over phosphorus pentaoxide at 66 °C and 2 mmHg (1 mmHg≈133.332 Pa) for 2 h.

Synthesis of BPIc. *Z-Val-Tyr-OMe (1)*: A solution of Z-Val-OH[9] (10.05 g, 40 mmol) and NMM (4.4 ml, 40 mmol) in THF (100 ml) was chilled to −5 °C, then ECF (4 ml, 40 mmol) was added to it. After 10 min, a precooled solution of H-Tyr-OMe·HCl[10] (14.64 g, 40 mmol) and NMM (4.4 ml, 40 mmol) in DMF (100 ml) was added to it. The reaction mixture was refrigerated for 1 h and then allowed to stand overnight at room temperature. The mixture was evaporated *in vacuo*, and the oily residue was dissolved in ethyl acetate (400 ml). The solution was washed successively with 4% sodium hydrogencarbonate, 0.5 M hydrochloric acid and water, and then dried over anhydrous sodium sulfate. The solution was concentrated to an oily residue which was crystallized by addition of ether; yield 15.13 g (88%); mp 152—153 °C (lit,[11] 150 °C); $[\alpha]_D^{20}$ +14.0° (*c* 1, pyridine) (lit,[11] +12.1° (*c* 1, pyridine)); R_f^1 0.98 and R_f^2 0.69.

Found: C, 64.30; H, 6.74; N, 6.53%. Calcd for $C_{23}H_{28}O_6N_2$: C, 64.47; H, 6.59; N, 6.54%.

Z-Val-Tyr-OH (2): To a solution of **1** (12.9 g, 30 mmol) in methanol (40 ml), 2 M sodium hydroxide (31 ml) was added. The reaction mixture was allowed to stand for 1 h at room temperature and then diluted with water (40 ml). The mixture was then extracted with ether. The aqueous layer was acidified to pH 2 with 2 M hydrochloric acid. The oily product was extracted with ethyl acetate (400 ml). The organic layer was washed with water and dried with anhydrous sodium sulfate. The filtrate was evaporated *in vacuo*, the oily residue was crystallized by ether and petroleum ether; yield 8.60 g (69%); mp 163—164 °C (lit,[12] 161—163 °C); $[\alpha]_D^{20}$ +26.5° (*c* 1, pyridine) (lit,[12] +26.0° (*c* 1, pyridine)); R_f^1 0.85 and R_f^2 0.24.

Found: C, 63.91; H, 6.52; N, 6.73%. Calcd for $C_{22}H_{26}O_6N_2$: C, 63.75; H, 6.32; N, 6.76%.

Z-Pro-Phe-OEt (3): This compound was obtained from Z-Pro-OH[13] (9.95 g, 40 mmol) and H-Phe-OEt·TsOH[14] (16.45 g, 45 mmol), as described for the preparation of **1**; yield 12.68 g (75%) mp 67—68 °C (lit,[15] 65—68 °C); $[\alpha]_D^{20}$ −39.8° (*c* 1, methanol) (lit,[15] −33° (alcohol)); R_f^1 0.96 and R_f^2 0.92.

Found: C, 67.79; H, 6.65; N, 6.62%. Calcd for $C_{24}H_{28}O_5N_2$: C, 67.90; H, 6.65; N, 6.60%.

H-Pro-Phe-OEt·HCl (4): Compound **3** (11.46 g, 27 mmol) was hydrogenated in the presence of palladium black and 0.3 M methanolic hydrogen chloride (100 ml). The filtrate from catalyst was evaporated *in vacuo*, and the residue was crystallized from ether. It was recrystallized from ethanol-ether; yield 8.35 g (94%); 149—150 °C; $[\alpha]_D^{20}$ −41.5° (*c* 1, water) (lit,[16] −40.9° (*c* 2, water)); R_f^1 0.79 and R_f^2 0.66.

Found: C, 58.69; H, 7.16; N, 8.56%. Calcd for $C_{16}H_{23}O_3N_2Cl$: C, 58.77; H, 7.09; N, 8.56%.

Z-Val-Tyr-Pro-Phe-OEt (5): To a chilled solution of **2** (9.95 g, 24 mmol), **4** (8.17 g, 25 mmol), NMM (2.75 ml, 25 mmol) and HOBt (3.38 g, 25 mmol) in THF (100 ml), DCC (4.95 g, 24 mmol) was added. The reaction mixture was held overnight at 0 °C and the DCUrea which formed was filtered off. The filtrate was evaporated *in vacuo*, and the residual oil was dissolved in ethyl acetate. The solution was washed with 4% sodium hydrogencarbonate, 0.5 M hydrochloric acid and water, and dried over sodium sulfate. The filtrate was evaporated *in vacuo*, and the residue was crystallized by addition of ether and petroleum ether; yield 14.42 g (88%); mp 122—124 °C; $[\alpha]_D^{20}$ −67.0° (*c* 1, methanol); R_f^1 0.97 and R_f^2 0.64.

Found: C, 66.20; H, 6.73; N, 8.25%. Calcd for $C_{38}H_{46}O_8N_4$: C, 66.45; H, 6.75; N, 8.16%.

Z-Val-Tyr-Pro-Phe-OH (6): Compound **5** (13.73 g, 20 mmol) was saponified by 2 M sodium hydroxide (21 ml) and methanol (50 ml) for 1 h. Purification of the product

was done by the procedure used for the preparation of **2**; yield 9.17 g (70%); mp 121—124 °C; $[\alpha]_D^{20}$ −52.8° (*c* 1, methanol); R_f^1 0.88 and R_f^2 0.46.

Found: C, 65.81; H, 6.45; N, 8.47%. Calcd for $C_{36}H_{42}O_8N_4$: C, 65.64; H, 6.43; N, 8.51%.

Boc-Pro-Pro-OH (7): To a chilled solution of Boc-Pro-OH[17] (4.26 g, 20 mmol), H-Pro-OBzl·HCl[18] (5.06 g, 22 mmol) and NMM (2.4 ml, 22 mmol) in acetonitrile (50 ml), DCC (4.12 g, 20 mmol) was added. The reaction mixture was held overnight at 0 °C, then DCUrea was separated by filtration. The filtrate was evaporated *in vacuo*, the residue was dissolved in ethyl acetate and washed successively with 4% sodium hydrogencarbonate, 4% citric acid and water, and then dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo*, and the residue was dissolved in methanol (100 ml). It was subjected to hydrogenolysis in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo*, and the residue was crystallized with ether. It was recrystallized from hot ethyl acetate; yield 3.74 g (60%); mp 181—182 °C (lit,[19] 187—187.5 °C); $[\alpha]_D^{20}$ −118.0° (*c* 1, methanol); R_f^1 0.77 and R_f^2 0.40.

Found: C, 57.58; H, 7.90; N, 8.92%. Calcd for $C_{15}H_{24}O_5N_2$: C, 57.67; H, 7.74; N, 8.97%.

Boc-Pro-Pro-Gly-OBzl (8): This was prepared from **7** (4.69 g, 15 mmol) and H-Gly-OBzl·TsOH[20] (5.07 g, 15 mmol) as described for the preparation of **1**; yield 4.76 g (70%); mp 107—109 °C; $[\alpha]_D^{20}$ −127.8° (*c* 1, methanol); R_f^1 0.90 and R_f^2 0.74.

Found: C, 62.57; H, 7.28; N, 9.18%. Calcd for $C_{24}H_{33}O_6N_3$: C, 62.72; H, 7.24; N, 9.14%.

H-Pro-Pro-Gly-OBzl·HCl (9): Compound **8** (4.60 g, 10 mmol) was dissolved in 4 M hydrogen chloride in dioxane (25 ml) and held for 1 h at room temperature. The solution was evaporated *in vacuo*, dissolved in dioxane (20 ml), and evaporated again. A precipitate was obtained by the addition of ether. This precipitate was filtered off and dried over pellets of potassium hydroxide *in vacuo*; yield of hygroscopic powder was 3.96 g (98%); mp 65—67 °C; $[\alpha]_D^{20}$ −113.5° (*c* 1, methanol); R_f^1 0.65 and R_f^2 0.38.

Found: C, 57.38; H, 6.64; N, 10.43%. Calcd for $C_{19}H_{26}O_4N_3Cl$: C, 57.64; H, 6.62; N, 10.62%.

Z-Val-Tyr-Pro-Phe-Pro-Pro-Gly-OBzl (10): A solution of of DCC (2.06 g, 10 mmol) in THF was added to a chilled solution of **6** (6.59 g, 10 mmol), **9** (3.90 g, 10 mmol), NMM (1.1 ml, 10 mmol), and HOBt (1.48 g, 11 mmol) in THF (100 ml). The reaction mixture was held overnight at 0 °C. DCUrea was filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate. The solution was washed with 4% sodium hydrogencarbonate, 0.5 M hydrochloric acid and water, and dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo*, and the product was obtained by addition of ether; yield 8.68 g (76%); mp 115—117 °C; $[\alpha]_D^{20}$ −118.5° (*c* 1, methanol); R_f^1 0.91 and R_f^2 0.63.

Found: C, 65.74; H, 6.62; N, 9.72%. Calcd for $C_{55}H_{65}O_{11}N_7$: C, 66.05; H, 6.55; N, 9.80%.

Z-Val-Tyr-Pro-Phe-Pro-Pro-Gly-OH (11): To a solution of **10** (8.00 g, 8 mmol) in methanol (70 ml), 2 M sodium hydroxide (9 ml) was added. The reaction mixture was held for 2 h at room temperature, then diluted with 150 ml water and extracted with ethyl acetate. The aqueous layer was acidified with 2 M hydrochloric acid and extraced with ethyl acetate. The organic layer was washed with water and then dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo* and then was crystallized from ether; yield 5.79 g, (80%); mp 148—150 °C (decomp); $[\alpha]_D^{20}$ −128.0° (*c* 1, methanol); R_f^1 0.81 and R_f^2 0.36.

Found: C, 63.14; H, 6.46; N, 10.56%. Calcd for $C_{48}H_{59}O_{11}N_7$: C, 63.35; H, 6.54; N, 10.78%.

Boc-Ile-Asn-OH (12): Asparagine monohydrate (3.3 g, 22 mmol) and Et_3N (2.8 ml, 20 mmol) were dissolved in water (50 ml). Boc-Ile-ONSu[21] (6.65 g, 20 mmol) dissolved in dioxane (50 ml) was added at room temperature while the solution was being stirred. The mixture was stirred overnight, and then diluted with 5% sodium hydrogencarbonate (100 ml) and extracted with ether. The aqueous layer was acidified with citric acid and extracted with 1-butanol (300 ml). The butanol layer was washed with water and concentrated to 50 ml. The product was crystallized with ether and petroleum ether, then recrystallized from ethanol-ether; yield 4.69 g (68%); mp 159—161 °C; $[\alpha]_D^{20}$ −10.5° (*c* 1, methanol); R_f^1 0.86 and R_f^2 0.10.

Found: C, 52.34; H, 8.05; N, 11.88%. Calcd for $C_{15}H_{27}O_6N_3$: C, 52.16; H, 7.88; N, 12.17%.

Boc-Ile-Asn-His-OBzl (13): To a chilled solution of **12** (5.18 g, 15 mmol) and HOBt (2.44 g, 18 mmol) in THF (40 ml), DCC (3.09 g, 15 mmol) was added, then a solution of H-His-OBzl·2TsOH[22] (9.0 g, 15 mmol) and NMM (3.3 ml, 30 mmol) in DMF (20 ml) was added. The reaction mixture was held for 2 h at −5 °C and then refrigerated overnight at 0 °C. DCUrea was filtered off and washed with a small amount of DMF. The filtrate and the washing were combined and evaporated *in vacuo*, then dissolved in 1-butanol. The solution was washed successively with 4% sodium hydrogencarbonate and water, then dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo*, the product was crystallized by the addition of ether and recrystallized from methanol-ether; yield 5.38 g (63%); mp 181—182 °C (decomp); $[\alpha]_D^{20}$ −9.5° (*c* 1, DMF); R_f^1 0.77 and R_f^2 0.57.

Found: C, 58.50; H, 7.12; N, 14.69%. Calcd for $C_{29}H_{40}O_7N_6$: C, 58.72; H, 7.04; N, 14.68%.

H-Ile-Asn-His-OBzl·2HCl (14): Compound **13** (5.15 g, 9 mmol) was dissolved in 98% formic acid (50 ml) and the solution was cooled in an ice bath. A solution of 4 M hydrogen chloride in dioxane (50 ml) was added to it, and the mixture was held for 1 h at 10 °C. The solution was then evaporated *in vacuo* below 25 °C. A residue was obtained by the aid of ether as a hygroscopic solid and was recrystallized from methanol-ether; yield 4.79 g (94%); mp 156—157 °C; $[\alpha]_D^{20}$ +8.5° (*c* 1, pyridine); R_f^1 0.67 and R_f^2 0.11.

Found: C, 50.55; H, 6.56; N, 15.67%. Calcd for $C_{23}H_{34}O_5N_6Cl_2$: C, 50.64; H, 6.28; N, 15.41%.

Z-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His-OBzl (15): To a solution of **11** (3.64 g, 4 mmol), **14** (2.53 g, 4.5 mmol), HOBt (0.54 g, 4 mmol) and NMM (1.0 ml, 9 mmol) in DMF (40 ml), DCC (0.83 g, 4 mmol) was added. The reaction mixture was held overnight at 0 °C, then DCUrea was filtered off. The filtrate was dissolved in 1-butanol and washed with 4% sodium hydrogencarbonate and water. The solution was evaporated *in vacuo* and the crude product was precipitated with ether to give 4.43 g (81%, mp 135—140 °C). Two g of the crude product were dissolved in methanol (2 ml) and applied to a silica-gel column (Merck silica-gel G) (2×35 cm), then developed with a mixture of chloroform and methanol (5:1, v/v). The fractions (175—300 ml) which gave a single spot on thin-layer chromatography were collected and evaporated *in vacuo*. The residue was crystallized with ether; yield 1.15 g (57%); mp 144—145 °C; $[\alpha]_D^{20}$ −91.0° (*c* 1, methanol) R_f^1 0.79 and R_f^2 0.68.

Found: C, 62.77; H, 6.51; N, 13.06%. Calcd for $C_{71}H_{89}O_{15}N_{13}$: C, 62.49; H, 6.57; N, 13.35%.

H-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His-OH·2HCl (16): Compound **15** (0.45 g, 0.33 mmol) in 0.035 M methanolic hydrogen chloride (20 ml) was hydrogenated in the presence of palladium black. The filtrate was evaporated

in vacuo and the residue was crystallized from acetone. It was reacrystallized from methanol-acetone; yield of the air-dried product was 0.39 g (95%); $[\alpha]_D^{20}$ −96.4° (*c* 1.3, water); R_f^1 0.51. Amino acid ratios in acid hydrolyzate:[23] Val 1.0; Tyr 1.0; Pro 3.1; Phe 1.0; Gly 1.0; Ile 1.1; Asp 1.1; His 1.1.

Found: C, 50.13; H, 7.26; N, 13.31%. Calcd $C_{56}H_{79}O_{13}N_{13}Cl_2 \cdot 7H_2O$: C, 50.29; H, 7.01; N, 13.47%. The air-dried product lost 8.7% of its weight after drying for 2 h at 110 °C and 2 mmHg. Calcd for $7H_2O$: 8.8%.

Syntheses of BPIc Analogs and Fragments. Z-Gly-Gly-Gly-OEt (17): A solution of Z-Gly-OH[24] (1.88 g, 9 mmol) and NMM (0.99 ml, 9 mmol) in THF (20 ml) was chilled to −5 °C, and ECF (0.9 ml, 9 mmol) was added under stirring. After 10 min, the precooled solution of H-Gly-Gly-OEt·HCl[25] (1.77 g, 9 mmol) and NMM (0.99 ml, 9 mmol) in DMF (20 ml) was added to it. The reaction mixture was kept for 1 h in a refrigerator and then kept overnight at room temperature. The mixture was added to 4% sodium hydrogencarbonate (100 ml) and the precipitate was collected by filtration, then washed with 4% sodium hydrogencarbonate, 0.5 M hydrochloric acid and water. It was recrystallized ethanol-ether; yield 2.11 g (67%); mp 166 °C (lit,[26] 167 °C); R_f^1 0.91 and R_f^2 0.64.

Found: C, 54.76; H, 6.04; N, 11.92%. Calcd for $C_{16}H_{21}O_6N_3$: C, 54.69; H, 6.02; N, 11.96%.

H-Gly-Gly-Gly-OEt·HCl (18): A solution of **17** (2.0 g, 5.7 mmol) dissolved in 0.3 M methanolic hydrogen chloride (30 ml) was hydrogenated in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo* and the residue was crystallized by addition of ether; yield 1.25 g (87%); mp 219—220 °C (decomp) (lit, [27] 214—217 °C (decomp)); R_f^1 0.64 and R_f^2 0.08.

Found: C, 37.98; H, 6.43; N, 16.79%. Calcd for $C_8H_{16}O_4N_3Cl$: C, 37.87; H, 6.36; N, 16.56%.

Z-Val-Tyr-Pro-Phe-Gly-Gly-Gly-OEt (19): To a chilled solution of **6** (2.64 g, 4 mmol) and HOBt (0.57 g, 4.2 mmol) in THF (20 ml), DCC (0.83 g, 4 mmol) was added. After 20 min, the precooled solution of **18** (1.01 g, 4 mmol) and NMM (0.44 ml, 4 mmol) in DMF (20 ml) was added and the mixture was kept for 5 h at 0 °C, then stood overnight at room temperature. DCUrea was filtered off and the filtrate was evaporated *in vacuo* and then dissolved in ethyl acetate. The solution was washed successively with 4% sodium hydrogencarbonate, 0.5 M hydrochloric acid and water, then dried over anhydrous sodium sulfate. The filtrate was evaporated *in vacuo* and the residue was solidified by addition of petroleum ether. It was recrystallized from ethanol and petroleum ether; yield 2.78 g (81%); mp 96 °C; $[\alpha]_D^{20}$ −59.0° (*c* 1, methanol); R_f^1 0.92 and R_f^2 0.63.

Found: C, 61.07; H, 6.73; N, 11.11%. Calcd for $C_{34}H_{55}O_{11}N_7 \cdot 1/2\,H_2O$: C, 60.95; H, 6.51; N, 11.31%.

Z-Val-Tyr-Pro-Phe-Gly-Gly-Gly-OH (20): Compound **19** (2.53 g, 3 mmol) was subjected to the saponification in the mixture of methanol (10 ml) and 2 M sodium hydroxide (3.3 ml) for 1 h. The following procedure was employed for the preparation of **11**; yield 2.07 g (83%); mp 114—116 °C; $[\alpha]_D^{20}$ −65.0° (*c* 1, methanol); R_f^1 0.73 and R_f^1 0.29.

Found: C, 59.76; H, 6.22; N, 11.43%. Calcd for $C_{32}H_{51}O_{11}N_7 \cdot H_2O$: C, 59.45; H, 6.30; N, 11.55%.

Z-Val-Tyr-Pro-Phe-Gly-Gly-Gly-Ile-Asn-His-OBzl (21): This compound was prepared from **20** (1.25 g, 1.5 mmol) and **14** (0.98 g, 1.8 mmol) as described for the preparation of **15**; yield 1.36 g (71%); mp 153—155 °C; $[\alpha]_D^{20}$ −58.5° (*c* 1, methanol); R_f^1 0.84 and R_f^2 0.48.

Found: C, 60.92; H, 6.29; N, 14.13%. Calcd for $C_{65}H_{81}O_{15}N_{13}$: C, 60.78; H, 6.36; N, 14.18%.

H-Val-Tyr-Pro-Phe-Gly-Gly-Gly-Ile-Asn-His-OH·HCl (22): A solution of **20** (1.0 g, 0.78 mmol) in 0.3 M methanolic hydrogen chloride (4 ml) was subjected to the hydrogenation as described for **16**; yield 0.75 g (79%); mp 184—187 °C; $[\alpha]_D^{20}$ −39.5° (*c* 1, methanol); R_f^1 0.60. Amino acid ratios in acid hydrolyzate:[23] Val 1.1; Tyr 1.0; Pro 1.1; Phe 1.0; Gly 3.1; Ile 1.0; Asp 1.0; His 1.0.

Found: C, 51.43; H, 6.53; N, 15.45%. Calcd for $C_{50}H_{70}O_{13}N_{13}Cl \cdot 4H_2O$: C, 51.38; H, 6.73; N, 15.58%.

Boc-Ile-Gly-Gly-OBzl (23): This compound was prepared from Boc-Ile-OH·DCHA[28] (4.14 g, 10 mmol) and H-Gly-Gly-OBzl·HCl[29] (2.60 g, 10 mmol) as described for the preparation of **1**; yield 3.70 g (85%); mp 93—94 °C; $[\alpha]_D^{20}$ +3.0° (*c* 1, methanol); R_f^1 0.95 and R_f^2 0.67.

Found: C, 60.68; H, 7.73; N, 9.67%. Calcd for $C_{22}H_{33}O_6N_3$: C, 60.67; H, 7.64; N, 9.65%.

H-Ile-Gly-Gly-OBzl·HCl (24): This was prepared from **23** (1.74 g, 4 mmol) as described for the preparation of **9**; yield 1.40 g (94%); mp 186—187 °C; $[\alpha]_D^{20}$ +29.0° (*c* 1, methanol); R_f^1 0.80 and R_f^2 0.50.

Found: C, 54.58; H, 7.23; N, 11.49%. Calcd for $C_{17}H_{26}O_4N_3Cl$: C, 54.90; H, 7.31; N, 11.30%.

Z-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Gly-Gly-OBzl (25): This was prepared from **11** (1.86 g, 2 mmol) and **24** (0.78 g, 2.2 mmol) as described for the preparation of **15**. Purification of the product was done by the recrystallization from ethanol-ether; yield 2.06 g (84%); mp 115—118 °C; $[\alpha]_D^{20}$ −91.0° (*c* 1, methanol); R_f^1 0.92 and R_f^2 0.57.

Found: C, 62.43; H, 6.90; N, 11.15%. Calcd for $C_{65}H_{82}O_{14}N_{10} \cdot H_2O$: C, 62.53; H, 7.02; N, 11.22%.

H-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Gly-Gly-OH (26): Compound **25** (1.23 g, 1 mmol) was hydrogenated as described for the preparation of **16**; yield 0.95 g (85%); mp 168—171 °C; $[\alpha]_D^{20}$ −89.0° (*c* 1, methanol); R_f^1 0.71 and R_f^2 0.31. Amino acid ratios in acid hydrolyzate:[23] Val 1.0; Tyr 1.0; Pro 2.9; Phe 1.0; Gly 3.2; Ile 1.0.

Found: C, 56.77; H, 6.95; N, 13.05%. Calcd for $C_{50}H_{70}O_{12}N_{10} \cdot 3H_2O$: C, 56.80; H, 7.25; N, 13.25%.

Boc-Gly-Ile-OBzl (27): This was prepared from Boc-Gly-OH[30] (3.50 g, 20 mmol) and H-Ile-OBzl·TsOH[31] (9.00 g, 22 mmol) as described for the preparation of **1**. The product was obtained as an oily form; yield 7.50 g (100%); R_f^1 0.97 and R_f^2 0.81.

H-Gly-Ile-OBzl·HCl (28): Compound **27** (7.50 g, 20 mmol (oily form)) was treated with hydrogen chloride as described for the preparation of **9**; yield 5.10 g (81%); mp 108—110 °C; $[\alpha]_D^{20}$ −19.5° (*c* 1, methanol); R_f^1 0.81 and R_f^2 0.47.

Found: C, 55.52; H, 7.66; N, 8.95%. Calcd for $C_{15}H_{23}O_3N_2Cl \cdot 1/2\,H_2O$: C, 55.64; H, 7.47; N, 8.65%.

Boc-Pro-Pro-Gly-Ile-OBzl (29): This was prepared from **7** (1.87 g, 6 mmol) and **28** (1.88 g, 6 mmol) as described for the preparation of **1**. The product was obtained as an oily form; yield 3.43 g (100%) R_f^1 0.91 and R_f^2 0.63.

H-Pro-Pro-Gly-Ile-OBzl·HCl (30): Compound **29** (3.43 g, 6 mmol (oily form)) was treated with hydrogen chloride as described for the preparation of **9**; yield 2.54 g (83%); mp 68—70 °C; $[\alpha]_D^{20}$ −99.0° (*c* 1, methanol); R_f^1 0.77 and R_f^2 0.35.

Found: C, 58.08; H, 7.42; N, 10.59%. Calcd for $C_{25}H_{37}O_5N_4Cl \cdot 1/2\,H_2O$: C, 57.96; H, 7.39; N, 10.82%.

Z-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-OBzl (31): This compound was prepared from **6** (3.29 g, 5 mmol) and **30** (2.40 g, 5 mmol) as described for the preparation of **10**; yield 4.21 g (76%); mp 114—116 °C; $[\alpha]_D^{20}$ −104.0° (*c* 1, methanol); R_f^1 0.86 and R_f^2 0.71.

Found: C, 65.29; H, 7.01; N, 9.98%. Calcd for $C_{61}H_{76}O_{12}N_8 \cdot 1/2\,H_2O$: C, 65.28; H, 6.92; N, 9.98%.

H-Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-OH (32): A solution of **31** (3.34 g, 3 mmol) in methanol (8 ml) and acetic acid (8 ml) was hydrogenated in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo* and

the residue was crystallized from ether; yield 2.54 g (92%); mp 164—168 °C (decomp); $[\alpha]_D^{20}$ −124.5° (*c* 0.92, water); R_f^1 0.76 and R_f^2 0.40. Amino acid ratios in acid hydrolyzate:[23] Val 0.9; Tyr 1.0; Pro 3.1; Phe 1.0; Gly 1.0; Ile 1.0.

Found: C, 60.06; H, 7.10; N, 12.21%. Calcd for $C_{46}H_{64}O_{10}N_8 \cdot 3/2\ H_2O$: C,60.31; H, 7.37; N, 12.23%.

H-Val-Tyr-Pro-Phe-Pro-Pro-Gly-OH (33): Compound **11** (1.36 g, 1.5 mmol) was hydrogenated as described for the preparation of **32**; yield 1.15 g (91%); mp 172—174 °C (decomp); $[\alpha]_D^{20}$ −125.9° (*c* 0.84, water); R_f^1 0.67 and R_f^2 0.28. Amino acid ratios in acid hydrolyzate:[23] Val 1.0; Tyr 1.0; Pro 2.9; Phe 1.0; Gly 0.9.

Found: C, 58.63; H, 6.65; N, 11.93%. Calcd for $C_{40}H_{53}O_9N_7 \cdot 1/2\ CH_3COOH \cdot 2H_2O$: C, 58.49; H, 7.06; N, 11.65%.

A Comparison of Synthetic and Natural BPIc.[32] *Thin Layer Chromatography*: Both compounds gave a single spot and identical R_f values. R_f^1 values of both are 0.51.

Paper Electrophoresis: This was carried out under the following conditions. Paper, Toyo Roshi No. 51A chromatography paper; solvent, pyridine–acetic acid–water (10:0.4:90, v/v) (pH 6.4); voltage gradient, 15 V/cm; charge period, 2 h. Electrophoretic mobilities were recorded as R_{His}, the ratio of the distance the compounds moved to that which a standard histidine spot moved on the same electrophoreogram. BPIc migrated toward the cathode and gave a single spot. The R_{His} value of both natural and synthetic BPIc was 0.82. The R_{His} values of **22**, **26**, **32**, and **33** were 0.83, 0.70, 0.70, and 0.69 respectively.

Carboxymethylcellulose Column Chromatography: A portion (*ca.* 1 mg) of synthetic or natural BPIc was dissolved in 0.3 ml of 0.2 M pyridinium acetate (pH 5.0). The solution was applied to a column (1.0×60 cm) of carboxymethylcellulose. Elution was carried out with the same solvent and 2 ml fractions were collected at a flow rate of 8 ml h^{-1}. The peptide content was determined by the method described by Yemm and Cocking.[33] The results shown in Fig. 2 indicate that the pattern of the chromatogram of synthetic BPIc is indistinguishable from that of the natural material. The cochromatography of synthetic and natural BPIc was carried out under the same conditions and a single peak was obtained. The chromatographic patterns of other synthetic peptides are also presented in Fig. 2.

Sensory Test. Bitterness of the synthetic peptides was organoleptically determined by panel evaluation employing four people. A series of solutions of decreasing concentration, each half as strong as the proceeding 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.

The author wishes to express his thanks to Professor Nobuo Izumiya of Kyushu University and Associate Professor Hideo Okai of Hiroshima University for their helpful discussions. The author would like to thank Dr. Noshi Minamiura of Osaka City University for his kind offer of the natural sample of BPIc. Thanks are also due to Dr. Shinji Okumura of Ajinomoto Co. Inc., for supplying some amino acids.

References

1) Part V. H. Kanehisa and H. Okai, *Bull. Chem. Soc. Jpn.*, **57**, 301 (1984).

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 unless otherwise noted. Additional abbreviations: DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DCUrea, *N,N'*-dicyclohexylurea; DMF, *N,N*-dimethylformamide; ECF, ethyl chloroformate; Et_3N, triethylamine; HOBt, 1-hydroxybenzotriazole; HONSu, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; THF, tetrahydrofuran; TsOH, *p*-toluenesulfonic acid.

3) Part I. H. Fukui, H. Kanehisa, N. Ishibashi, I. Miyake, and H. Okai, *Bull. Chem. Soc. Jpn.*, **56**, 766 (1983).

4) N. Minamiura, Y. Matsumura, J. Fukumoto, and T. Yamamoto, *Agric. Biol. Chem.*, **36**, 588 (1972).

5) Part II. K. Otagiri, I. Miyake, N. Ishibashi, H. Fukui, H. Kanehisa, and H. Okai, *Bull. Chem. Soc. Jpn.*, **56**, 1116 (1983).

6) Part. III. I. Miyake, K. Kouge, H. Kanehisa, and H. Okai, *Bull. Chem. Soc. Jpn.*, **56**, 1678 (1983).

7) Part IV. K. Otagiri, T. Shigenaga, H. Kanehisa, and H. Okai, *Bull. Chem. Soc. Jpn.*, **57**, 90 (1984).

8) These peptides were synthesized in the usual manners. Details of di-, tri-, and tetrapeptide fragments of BPIc will be described in another investigation.

9) E.Shröder, *Ann. Chem.*, **692**, 241 (1966).

10) E. Wünsch, G. Friss, and A. Zwick, *Chem. Ber.*, **91**, 543 (1958).

11) H. Ramachandran and C. H. Li, *J. Org. Chem.*, **28**, 173 (1963).

12) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *Helv. Chim. Acta*, **41**, 1273 (1958).

13) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 462 (1958).

14) T. Kato, S. Makisumi, M. Ohno, and N. Izumiya, *Nippon Kagaku Zasshi*, **83**, 1151 (1962).

15) H. Aoyagi, K. Arakawa, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **41**, 433 (1968).

16) N. Izumiya and J. S. Fruton, *J. Biol. Chem.*, **218**, 59 (1956).

17) E. Boyer, G. Jung, and H. Hagenmaier, *Tetrahedron*, **24**, 4853 (1968).

18) R. E. Newman and E. L. Smith, *J. Biol. Chem.*, **193**, 97 (1951).

19) G. R. Pettit and S. K. Das Gupta, *Can. J. Chem.*, **45**, 1600 (1967).

20) C. R. Harington and T. H. Mead, *Biochem. J.*, **30**, 1598 (1936).

21) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

22) S. Akabori, S. Sakakibara, and S. Shiina, *Bull. Chem. Soc. Jpn.*, **31**, 784 (1958).

23) The synthetic peptides were hydrolyzed with 6 M hydrochloric acid in a sealed tube at 110 °C for 65 h. Molar ratios of amino acids are based on isoleucine or valine value.

24) M. Bergmann and L. Zervas, *Chem. Ber.*, **65**, 1192 (1932).

25) A. H. Cook and A. L. Levy, *J. Chem. Soc.*, **1950**, 646.

26) P. M. Hardy, B. Ridge, H. N. Rydon, and F. O. Dos. S. P. Seräo, *J. Chem. Soc.*, **1971**, 1722.

27) J. C. Sheehan and W. L. Richardson, *J. Am. Chem. Soc.*, **76**, 6329 (1954).

28) H. C. Beyerman, C. A. M. Boers-Boonekamp, and H. Maassen van den Brink-Zimmermannova, *Recl. Trav. Chim. Bays-Bas*, **87**, 257 (1968).

29) L. Zervas and D. M. Theodoropoulos, *J. Am. Chem. Soc.*, **78**, 1359 (1959).

30) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957).

31) L. Zervas, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).

32) Natural BPIc was received from Dr. N. Minamiura.

33) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).