Studies of Bitter Peptides from Casein Hydrolyzate. II.[1] Syntheses of Bitter Peptide Fragments and Analogs of BPIa (Arg-Gly-Pro-Pro-Phe-Ile-Val) from Casein Hydrolyzate[2]

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In order to investigate the relationship between bitterness and chemical structure of BPIa, eleven kinds of fragments and analogs of BPIa were synthesized. des-Gly^2-BPIa and des-Pro^4-BPIa exhibited extremely bitter taste. However, the pentapeptide (Arg-Gly-Pro-Pro-Phe) of BPIa possessed weak bitterness. The bitterness exhibition of BPIa probably derived from the spatial structure of its molecule.

In the previous paper[1] and the preliminary reports,[3,4] the authors described the synthesis of the bitter peptide BPIa (Arg-Gly-Pro-Pro-Phe-Ile-Val) which was isolated by Minamiura *et al.*[5] and found that the peptide possesses extremely bitter taste, such as quinine and phenylthiourea. They also reported that the bitter taste exhibited by BPIa was associated with its characteristic conformation. In this paper, we describe the synthesis of eleven kinds of peptides as shown in Table 1 to investigate the participation of constituent amino acid residues of BPIa in producing the strong bitter taste.

Table 1. The threshold value for bitter taste of the synthetic peptides

	Compound	T.V.[b] / mM	Rcaf.[c]
(1)	Arg-Gly-Pro-Pro-Phe-Ile	0.025	40.00
(2)	Arg-Gly-Pro-Pro-Phe	2.30	0.43
(3)	Arg-Gly-Pro	13.00	0.08
(4)	Arg-Gly	10.00	0.10
(5)	Ile-Val	12.50	0.08
(6)	Phe-Ile-Val	1.50	0.67
(7)	Pro-Phe-Ile-Val	0.30	3.33
(8)	Pro-Pro-Phe-Ile-Val	1.20	0.83
(9)	Gly-Pro-Pro-Phe-Ile-Val	1.20	0.83
(10)	Arg-Pro-Pro-Phe-Ile-Val	0.08	12.50
(11)	Arg-Gly-Pro-Phe-Ile-Val	0.05	20.00
(12)	Arg-Gly-Pro-Pro-Phe-Ile-Val[a]	0.05	20.00

a) BPIa. b) Threshold value. c) The ratio of caffeine.

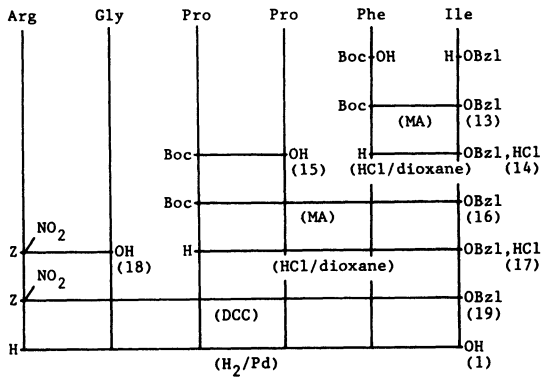

Fig. 1. The synthetic route of Arg-Gly-Pro-Pro-Phe-Ile.

In connection with the syntheses, the BPIa intermediates, as had been reported in the previous paper,[1] were efficiently employed. The synthetic route of the hexapeptide (Arg-Gly-Pro-Pro-Phe-Ile) is shown in Fig. 1. *N*-(*t*-Butoxycarbonyl)phenylalanine was coupled with isoleucine benzyl ester by the mixed anhydride method to yield *N*-(*t*-butoxycarbonyl)phenylalanylisoleucine benzyl ester (**13**). After treatment of the protected dipeptide with hydrogen chloride, the dipeptide benzyl ester hydrochloride (**14**) and *N*-(*t*-butoxycarbonyl)prolylproline (**15**) were coupled by the mixed anhydride method to yield *N*-(*t*-butoxycarbonyl)prolylprolylphenylalanylisoleucine benzyl ester (**16**). The *t*-butoxycarbonyl group was removed from the protected tetrapeptide with hydrogen chloride and the resulting tetrapeptide benzyl ester hydrochloride (**17**) was acylated with N^{α}-benzyloxycarbonyl-N^{G}-nitroarginylglycine (**18**) by the dicyclohexylcarbodiimide method to yield N^{α}-benzyloxycarbonyl-N^{G}-nitroarginylglycylprolylprolylphenylalanylisoleucine benzyl ester (**19**). The protected hexapeptide was hydrogenated in the presence of palladium black to give the desired hexapeptide (H-Arg-Gly-Pro-Pro-Phe-Ile-OH) (**1**).

The synthetic route to compounds **2**, **3**, and **4**, compounds **6**, **7**, and **11**, and compounds **8**, **9**, and **10** are shown in Fig. 2, 3, and 4 respectively. However, the details of the synthetic route to those peptides is uneventful and is described in the experimental part. The purity of the synthetic peptides and their intermediates was confirmed by thin-layer examinations in two solvent systems and by elemental analyses.

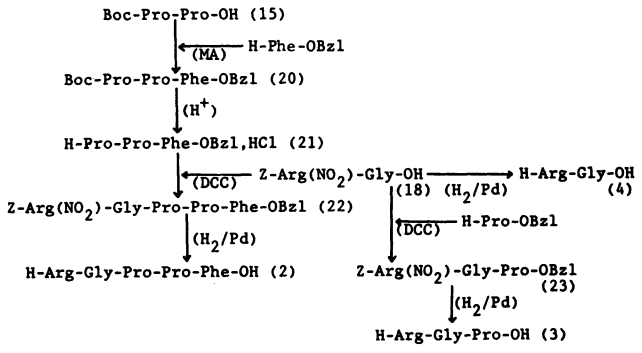

Fig. 2. The synthetic route of compound **2**, **3**, and **4**.

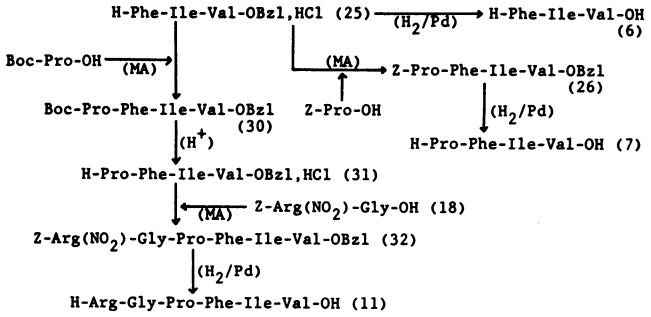

Fig. 3 The synthetic route of compounds **6**, **7**, and **11**.

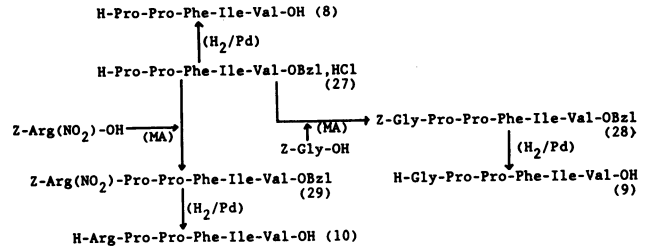

Fig. 4. The synthetic route of compounds **8**, **9**, and **10**.

The bitterness of the synthetic peptides were organoleptically determined by panel evaluation with five people (Table 1). As far as the N-terminal fragments of BPIa, compounds **2**—**4** exibited a weak bitter taste, but the hexapeptide (**1**) exhibited a strong bitter taste of the same level as BPIa. On the other hand, the C-terminal fragments of BPIa **5**—**9** that lack the arginine residue also exhibited a slightly bitter taste. These findings indicated that the L-arginine residue in the N-terminal and at least two hydrophobic amino acid residues in the C-terminal group are necessary for an intense bitter taste exhibition.

In order to confirm the participation of the strong bitter taste exhibition from the glycine residue at the 2-position and the L-proline residue at the 3-position, the bitterness of des-Gly^2-BPIa (**10**) and des-Pro^4-BPIa (**11**) were compared with that of BPIa. Both of these peptides exhibited a strong bitter taste of the same level as BPIa (Table 1). The results indicated that the glycine residue at the 2-position in BPIa is not always necessary, but the L-proline residue at the 3-position is necessary for an intense bitter taste exhibition.

Then, the authors measured circular dichroism (CD) and optical rotatory dispersion (ORD) of the synthetic peptides above mentioned. The CD curves of compounds **1** and **11** afforded a similar shape of BPIa, but those of compound **2** afforded a different shape from that of BPIa as shown in Fig. 5. The ORD curve of compound **10** afforded a similar shape to that of BPIa.[3]

From the results described above, the strong bitter taste exhibition of BPIa is caused by its characteristic conformation. Furthermore, in this report, we will propose the following requirements for the strong bitter taste exhibition of BPIa; (a) at least six amino acid residues are necessary, (b) the proline residue at the 3-position must have the L-configuration and (c) the number of hydrophobic amino acid residues in the C-terminal is not important.

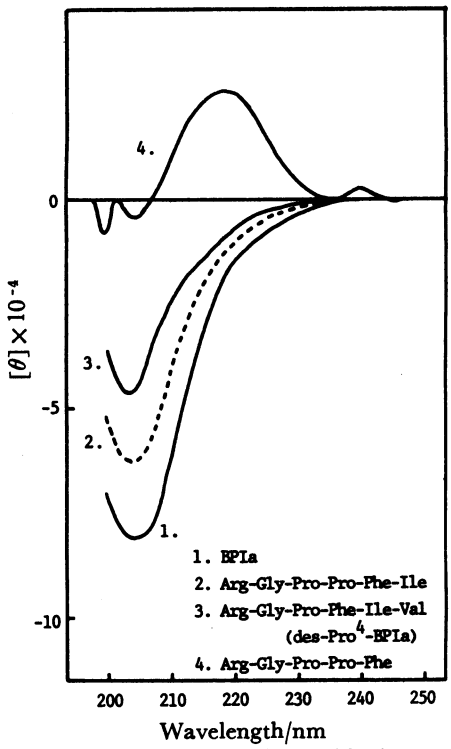

Fig. 5. CD curves of synthetic peptides in water.

Experimental

All the melting points are uncorrected. Thin-layer chromatography was carried out with Merck silica gel 60G. 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 a free amino group 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. The optical rotations were measured on an Union PM-101 polarimeter.

Boc–Phe–Ile–OBzl (13). To a solution of Boc–Phe–OH·DCHA[6] (9.83 g, 22 mmol) in THF (20 ml), ECF (2.0 ml, 20 mmol) and NMM (2.2 ml, 20 mmol) were added at −5 °C. After 10 min, a solution of H–Ile–OBzl·TsOH[7] (7.87 g, 20 mmol) and NMM(2.2 ml, 20 mmol) in chloroform (20 ml) was added. The reaction mixture was stirred in an ice bath for an hour, then at room temperature overnight. The mixture was evaporated *in vacuo*, and the oily residue was dissolved in ethyl acetate. The solution was washed with water, 4% citric acid, 4% sodium hydrogencarbonate, and water successively, then dried over anhydrous sodium sulfate. Sodium sulfate was removed by filtration, and the filtrate was evaporated *in vacuo*. The oily residue was crystallized with ether–petroleum ether: yield 9.04 g (96%); mp 87—88 °C $[\alpha]_D^{20}$ −18° (*c* 1, MeOH); R_f^1 0.99 and R_f^2 0.54. Found: C, 69.35; H, 7.69; N, 6.01%. Calcd for $C_{27}H_{36}O_5N_2$: C, 69.20; H, 7.74; N, 5.98%.

H–Phe–Ile–OBzl·HCl (14). To a solution of **13** (2.34 g, 5 mmol) in dioxane (10 ml), 4 M HCl(1 M=1 mol dm^{-3})/dioxane (20 ml) was added. The reaction mixture was allowed to stand at room temperature. After 2 h, the solution was evaporated *in vacuo*. The compound was obtained as an oily form: yield 1.86 g (92%); R_f^1 0.81 and R_f^2 0.68.

Boc–Pro–Pro–Phe–Ile–OBzl (16). Boc–Pro–Pro–OH[1] (**15**) (1.56 g, 5 mmol) and **14** (1.86 g, 4.8 mmol) were coupled

by the same method as has been described for the preparation of **13** This compound was obtained as an oily form: yield 2.89 g (92%); R_f^1 0.97 and R_f^2 0.67.

H-Pro-Pro-Phe-Ile-OBzl·HCl (17). Compound **16** (2.89 g, 4.5 mmol) was treated as has been described in the case of **14**. This compound was obtained as an oily form: yield 2.50 g (88%); R_f^1 0.81 and R_f^2 0.59.

Z-Arg(NO$_2$)-Gly-Pro-Pro-Phe-Ile-OBzl (19). To a solution of Z-Arg(NO$_2$)-Gly-OH[1] (1.64 g, 4 mmol) in DMF (20 ml), DCC (0.81 g, 4 mmol) was added at 0 °C. After 10 min, to the mixture, a solution of **17** (2.50 g, 4 mmol) and NMM (0.44 ml, 4 mmol) in chloroform (20 ml) was added. The mixture was cooled at 0 °C for an hour and allowed to stand overnight at room temperature. The DCUrea was removed by filtration, and the filtrate was evaporated *in vacuo*. The oily residue was dissolved in ethyl acetate and the solution was washed with water, 2% hydrochloric acid, 4% sodium hydrogencarbonate, and water successively. The solution was dried over anhydrous sodium sulfate. Sodium sulfate was removed by filtration, and the filtrate was evaporated *in vacuo*. The residue was crystallized with ether-petroleum ether: yield 2.71 g (74%); mp 111—112 °C; $[\alpha]_D^{20}$ −87° (*c* 1, MeOH); R_f^1 0.91 and R_f^2 0.54. Found: C, 60.51; H, 6.53; N, 14.72%. Calcd for $C_{48}H_{62}O_{11}N_{10}$: C, 60.36; H, 6.56; N, 14.67%.

H-Arg-Gly-Pro-Pro-Phe-Ile-OH (1). A solution of **19** (1.24 g, 1.3 mmol) in methanol (2 ml) and acetic acid (2 ml) was hydrogenated in the presence of palladium black at room temperature for 72 h. The catalyst was removed by filtration, the filtrate was evaporated *in vauco*. The residue was crystallized with acetone: yield 0.72 g (74%) hygroscopic form; R_f^1 0.68 and R_f^2 0.00.

Boc-Pro-Pro-Phe-OBzl (20). Compound **15** (3.12 g, 10 mmol) and H-Phe-OBzl·TsOH[7] (4.28 g, 10 mmol) were coupled by the same method as has been described for the preparation of **13**: yield 5.22 g (95%) oily form; R_f^1 0.98 and R_f^2 0.78.

H-Pro-Pro-Phe-OBzl·HCl (21). Compound **20** (5.22 g, 9.5 mmol) was treated as has been described in the case of **14**: yield 4.25 g (92%); mp 84—86 °C; $[\alpha]_D^{20}$ −115.5° (*c* 1, H_2O); Found: C, 56.35; H, 6.01; N, 16.53%. Calcd for $C_{28}H_{35}O_8N_7$: C, 56.26; H, 5.91; N, 16.41%.

H-Arg-Gly-Pro-OH·HCl (3). Compound **23** (1.50 g, 2.50 mmol) was treated as has been described in the case of **1**: yield 0.43 g (66%) hygroscopic form; R_f^1 0.12.

H-Arg-Gly-OH·2AcOH (4). Z-Arg(NO$_2$)-Gly-OH (**19**) (1.00 g, 2 mmol) was treated as has been described in the case of **1**: yield 0.43 g (76%); $[\alpha]_D^{20}$ +36.7° (*c* 2, H_2O); R_f^1 0.21. Found: C, 42.35; H, 4.50; N, 20.36%. Calcd for $C_{16}H_{11}O_5N_5\cdot 2CH_3COOH$: C, 42.22; H, 4.44; N, 20.52%.

Z-Ile-Val-OH (24). To a solution of Z-Ile-OSu (3.60 g, 10 mmol) in dioxane (10 ml), a solution of H-Val-OH (1.32 g, 11 mmol), Et_3N (2.0 ml, 11 mmol) in water (15 ml) was added at room temperature. After 20 h, the mixture was evaporated *in vacuo*. The oily residue was dissolved in 8% sodium hydrogencarbonate and the solution washed with ether. The aqueous layer was acidified with 10% citric acid. The oily residue was extracted with ethyl acetate. The solution was dried over anhydrous sodium sulfate. Sodium sulfate was removed by filtration, and the filtrate was evaporated *in vacuo*. The oily residue was crystallized with ether: yield 2.86 g (81%); mp 133—134 °C; $[\alpha]_D^{20}$ +7.0° (*c* 1, DMF); R_f^1 0.87. Found: C, 62.36; H, 7.77; N, 7.62%. Calcd for $C_{19}H_{28}O_5N_2$: C, 62.62; H, 7.74; N, 7.69%.

H-Ile-Val-OH (5). Compound **24** (1.77 g, 5 mmol) was treated as has been described in the case of **1**: yield 1.02 g (89%); $[\alpha]_D^{20}$ +12.5° (*c* 1, H_2O); R_f^1 0.81. Found: C, 57.06; H, 9.76; N, 12.08%. Calcd for $C_{11}H_{22}O_3N_2$: C, 57.36; H, 9.63; N, 12.17%.

H-Phe-Ile-Val-OH (6). H-Phe-Ile-Val-OBzl·HCl[1] (**25**) (1.51 g, 3 mmol) was treated as has been described in the case of **1**; yield 0.91 g (80%); $[\alpha]_D^{20}$ +10.5° (*c* 0.5, H_2O); R_f^1 0.77. Found: C, 61.39; H, 8.19; N, 10.74%. Calcd for $C_{20}H_{31}O_4N_3\cdot 2/3\ H_2O$: C, 61.67; H, 8.37; N, 10.79%.

Z-Pro-Phe-Ile-Val-OBzl (26). Z-Pro-OH[10-12] (0.59 g, 2 mmol) and **25** (1.01 g, 2 mmol) were coupled by the same method as has been described for the preparation of **13**: yield 1.15 g (85%); mp 196 °C; $[\alpha]_D^{20}$ −57.1° (*c* 1, $CHCl_3$); R_f^1 0.99. Found: C, 67.97; H, 7.32; N, 7.98%. Calcd for $C_{40}H_{50}O_7N_4\cdot 1/2\ H_2O$: C, 67.87; H, 7.12; N, 7.92%.

H-Pro-Phe-Ile-Val-OH (7). Compound **26** (0.91 g, 1.3 mmol) was treated as has been described in the case of **1**: yield 0.54 g (88%); $[\alpha]_D^{20}$ −51.7° (*c* 0.5, H_2O); R_f^1 0.70. Found: C, 57.41; H, 7.84; N, 10.48%. Calcd for $C_{25}H_{38}O_5N_4\cdot 1/2\ CH_3COOH\cdot 2H_2O$: C, 57.76; H, 8.20; N, 10.36%.

H-Pro-Pro-Phe-Ile-Val-OH (8). H-Pro-Pro-Phe-Ile-Val-OBzl·HCl[1] (0.69 g, 1 mmol) was treated as has been described in the case of **1**: yield 0.47 g (82%); mp 182 °C; $[\alpha]_D^{20}$ −122.0° (*c* 0.7, H_2O); R_f^1 0.61. Found: C, 60.54; H, 7.90; N, 11.14%. Calcd for $C_{30}H_{45}O_6N_5\cdot CH_3COOH$: C, 60.82; H, 7.83; N, 11.09%.

Z-Gly-Pro-Pro-Phe-Ile-Val-OBzl (28). Z-Gly-OH[13-15] (0.32 g, 1.5 mmol) and H-Pro-Pro-Phe-Ile-Val-OBzl·HCl (**27**) (0.90 g, 1.3 mmol) were coupled by the same method as has been described for the preparation of **13**: yield 1.02 g (93%); mp 78—82 °C; $[\alpha]_D^{20}$ −54.0° (*c* 1, DMF); R_f^1 0.93 and R_f^2 0.66. Found: C, 65.55: H, 7.13: N, 9.62%. Calcd for $C_{47}H_{60}O_9N_6\cdot 1/2H_2O$: C, 65.80; H, 7.17; N, 9.80%.

H-Gly-Pro-Pro-Phe-Ile-Val-OH (9). Compound **28** (0.80 g, 0.94 mmol) was treated as has been described in the case of **1**: yield 0.46 g (77%): $[\alpha]_D^{20}$ −133.5° (*c* 1, H_2O); R_f^1 0.75. Found: C, 57.53; H, 7.68; N, 12.31%. Calcd for $C_{32}H_{48}O_7N_6\cdot 2H_2O$: C, 57.81; H, 7.88; N, 12.64%.

Z-Arg(NO$_2$)-Pro-Pro-Phe-Ile-Val-OBzl (29). Z-Arg(NO$_2$)-OH[16,17] (0.54 g, 1.5 mmol) and **27** (1.04 g, 1.5 mmol) were coupled by the same method as has been described for the preparation of **13**: yield 1.06 g (73%); mp 104—107 °C; $[\alpha]_D^{20}$ −60.2° (*c* 0.6, EtOH); R_f^1 0.96. Found: C, 61.00; H, 6.89; N, 13.44%. Calcd for $C_{50}H_{68}O_{10}N_{10}\cdot H_2O$: C, 60.83; H, 7.15; N, 13.19%.

H-Arg-Pro-Pro-Phe-Ile-Val-OH·2AcOH (10). Compound **29** (0.76 g, 0.8 mmol) was treated as has been described in the case of **1**: yield 0.60 g (95%); mp 139—141 °C; $[\alpha]_D^{20}$ −114.0° (*c* 1, H_2O); R_f^1 0.66. Found: C, 54.34; H, 7.48; N, 14.43%. Calcd for $C_{36}H_{57}O_7N_9\cdot 2CH_3COOH\cdot 2H_2O$: C, 54.34; H, 7.87; N, 14.25%.

Boc-Pro-Phe-Ile-Val-OBzl (30). Boc-Pro-OH[19,20] (2.15 g, 10 mmol) and **25** (5.04 g, 10 mmol) were coupled by the same method as has been described for the preparation of **13**: yield 6.06 g (91%); mp 111—112 °C; $[\alpha]_D^{20}$ −83° (*c* 1, MeOH); R_f^1 0.98 and R_f^2 0.88. Found: C, 67.01; H, 7.92; N, 8.39%. Calcd for $C_{37}H_{52}O_7N_4$: C, 66.84; H, 7.88; N, 8.43%.

H-Pro-Phe-Ile-Val-OBzl·HCl (31). Compound **30** (1.99 g, 3 mmol) was treated as has been described in the case of **14**: yield 1.71 g (95%); mp 90 °C (decomp); $[\alpha]_D^{20}$ −66° (*c* 1, MeOH); R_f^1 0.88 and R_f^2 0.61. Found: C, 63.99; H, 7.29; N, 9.36%. Calcd for $C_{32}H_{44}O_5N_4\cdot HCl$: C, 63.93; H, 7.38; N, 9.32%.

Z-Arg(NO$_2$)-Gly-Pro-Phe-Ile-Val-OBzl (32). Z-Arg-(NO$_2$)-Gly-OH (**21**) (0.41 g, 1 mmol) and **31** (0.60 g, 1 mmol) were coupled by the same method as has been described for the preparation of **13**: yield 0.72 g (75%); mp 175—180 °C; $[\alpha]_D^{20}$ −24° (*c* 1, DMF); R_f^1 0.88 and R_f^2 0.74. Found: C,

60.12; H, 6.74; N, 14.52%. Calcd for $C_{48}H_{64}O_{11}N_{10}$: C, 60.23; H, 6.74; N, 14.64%.

H–Arg–Gly–Pro–Phe–Ile–Val–OH (11). Compound **32** (0.50 g, 0.52 mmol) was treated as has been described in the case of 1: yield 0.27 g (69%); $[\alpha]_D^{20}$ −114° (*c* 1, H_2O); R_f^1 0.74 and R_f^2 0.00. Found: C, 55.21; H, 7.95; N, 16.81%. Calcd for $C_{33}H_{53}O_7N_9 \cdot CH_3COOH \cdot 1/2\ H_2O$: C, 55.53; H, 7.72; N, 16.66%.

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References

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2) The abbreviations recommended by the IUPAC-IUB commission of Biochemical Nomenclature (*J. Biol. Chem.*, **247**, 977 (1972)) have been used. Amino acid symbols except glycine denote the L-configuration. Additional abbreviations: AcOH, acetic acid; DCC, dicyclohexylcarbodiimide; DCUrea, *N,N'*-dicyclohexylurea; DMF, *N,N*-dimethylformamide; ECF, ethyl chloroformate; EtOH, ethanol; Et_3N, triethylamine; MA, mixed anhydride; MeOH, methanol; NMM, *N*-methylmorpholine; TsOH, *p*-toluenesulfonic acid; THF, tetrahydrofuran.

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