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Amino Acids and Peptides. VI.[1] Synthesis of the N-Terminal Pentapeptide of α_2-Plasmin Inhibitor and Its Analogue

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The N-terminal pentapeptide of α_2-plasmin inhibitor, H–Asn–Gln–Glu–Gln–Val–OH, and its analogue, H–Asp–Gln–Glu–Gln–Val–OH, were synthesized and their inhibitory effects on the cross-linking reaction of α_2-plasmin inhibitor to fibrin mediated by factor $XIII_a$ were examined. The synthetic peptides were inhibitory at high concentration.

Keywords——α_2-plasmin inhibitor; synthetic pentapeptide; cross-linking; fibrin

Aoki and Moroi isolated and characterized human α_2-plasmin inhibitor (α_2-PI), which cross-links to fibrin at Gln^2 in the presence of factor $XIII_a$ when blood coagulation takes place.[2] They demonstrated that the N-terminal dodecapeptide of α_2-PI, H–Asn–Gln–Glu–

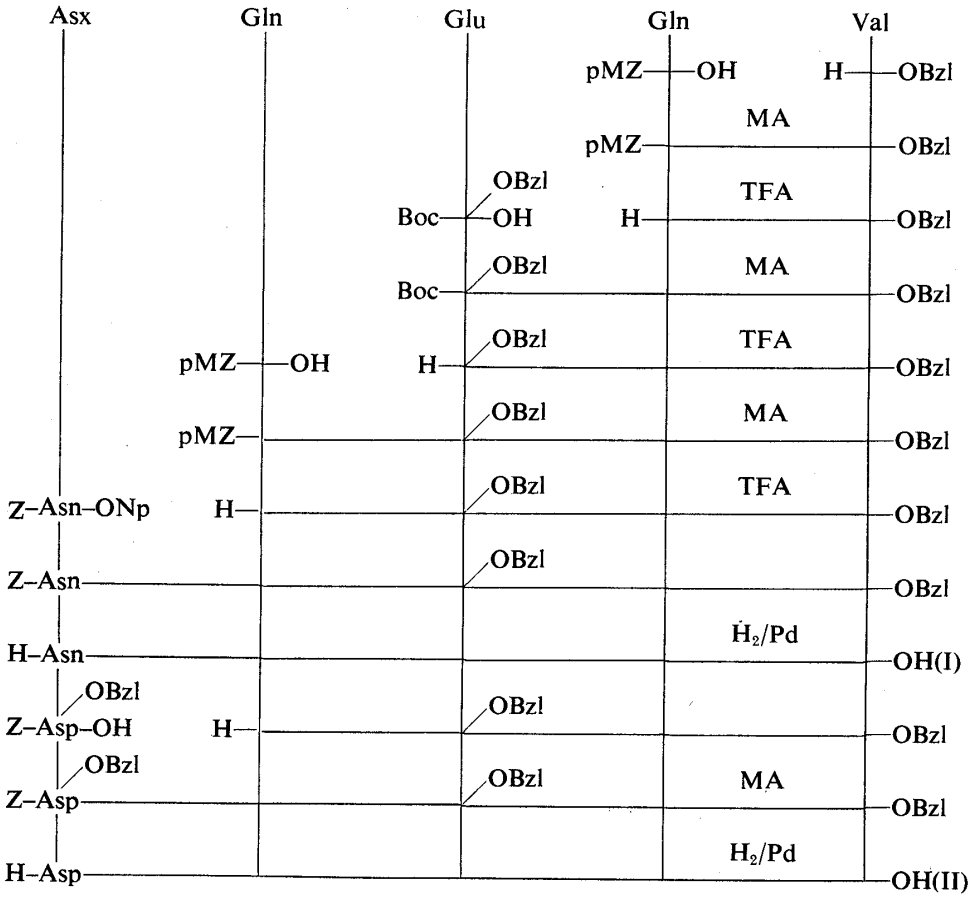

Fig. 1. Synthetic Scheme for I and II

MA: Mixed anhydride method.

Gln–Val–Ser–Pro–Leu–Thr–Gly–Leu–Lys–NH_2, inhibited the cross-linking reaction of α_2-PI to fibrin.[3]

To examine the effect of the N-terminal portion of α_2-PI on the cross-linking reaction, a smaller N-terminal peptide, H–Asn–Gln–Glu–Gln–Val–OH[4] (I) and its analogue, H–Asp–Gln–Glu–Gln–Val–OH (II), were synthesized. The synthetic scheme is shown in Fig. 2. The carboxyl group of C-terminal valine was protected as the benzyl ester and the C-terminal tetrapeptide was synthesized stepwise by the mixed anhydride method.[5] N-Protecting groups were removed by TFA-treatment at each step, and N-terminal asparagine was introduced onto the tetrapeptide benzyl ester by the *p*-nitrophenyl ester method.[6] Introduction of the N-terminal asparagine by the mixed anhydride method afforded a by-product which was difficult to remove. All coupling reactions for preparation of the Asp^1-analogue (II) were done by the mixed anhydride method. The protecting groups on I and II were removed by catalytic hydrogenation to give the free pentapeptides. The purities I and II were confirmed by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC).

The inhibitory effects of the synthetic peptides on the cross-linking reaction between α_2-PI and fibrin mediated by factor $XIII_a$ were determined by measuring the amount of α_2-PI incorporated into a fibrin clot. A mixture of the synthetic peptide (I or II), human citrated plasma (containing α_2-PI, factor XIII and fibrinogen), calcium chloride and thrombin was incubated in Tris buffer and the resulting clot was squeezed and removed with a spstula. The resulting clot was insoluble in 1% monochloroacetic acid but a clot formed in a mixture containing ethylenediaminetetraacetic acid (EDTA) instead of calcium chloride was soluble. These results indicated that factor XIII was activated by thrombin in the presence of calcium and catalyzed the cross-linking reaction to form a covalent bond between α_2-PI and fibrin. The amount of α_2-PI in the supernatant was determined by the single radial immunodiffusion method.[7] An aliquot of the supernatant was added to a well in agarose gel containing anti-α_2-PI IgG and the amount of α_2-PI was determined from the resulting precipitation ring. The results are summarized in Table I.

Both synthetic peptides inhibited the factor XIII-mediated cross-linking reaction between α_2-PI and fibrin, and the effects were dependent on the concentration of the synthetic peptide. The inhibitory potency of I was higher than that of II. The carboxyl group of Asp^1 in II might decrease the affinity between II and fibrin or between II and factor $XIII_a$.

When the inhibitory effect of I was compared with that of the N-terminal dodecapeptide of α_2-PI reported by Aoki *et al.*,[3] I showed 55% inhibition at 10 mmol/l, while the dodecapeptide exhibited 50% inhibition at 350 to 1 μmol α_2-PI. Even though the molecular weight of I is lower than that of the dodecapeptide, the potency of I is lower than that of the

TABLE I. Inhibitory Effects of the Synthetic Peptides on the Cross-Linking Reaction of α_2-PI to Fibrin

	Concentration (mM)	Amount of α_2-PI (%)	Inhibition[a] (%)
EDTA plasma		100	
H–Asn–Gln–Glu–Gln–Val–OH	0	72.5	0
	1	77.5	18.2
	10	87.5	54.6
H–Asp–Gln–Glu–Gln–Val–OH	0	72.5	0
	1	73.8	4.7
	10	81.3	32.0

a) $\text{Inhibition} = \dfrac{(\text{amount of } \alpha_2\text{-PI}) - 72.5}{100 - 72.5} \times 100.$

dodecapeptide on a weight per liter basis. Aoki *et al.*[3] reported that 50% inhibition of the cross-linking reaction was achieved by addition of 1000-fold molar excess of the dodecapeptide to α_2-PI. Shortening the peptide chain of α_2-PI might cause a conformational change leading to a decrease of the binding affinity to factor $XIII_a$ or to fibrin.

Experimental

Melting points are uncorrected. Solvent systems for ascending TLC on silica gel G (type 60, E. Merck) are indicated as follows: Rf^1 = *n*-BuOH–AcOH–H_2O (4:1:5, upper phase), Rf^2 = *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf^3 = $CHCl_3$–MeOH–H_2O (8:3:1, lower phase). Acid hydrolyses were performed in constant-boiling HCl at 110 °C for 24 h in evacuated tubes.

pMZ–Gln–Val–OBzl——Triethylamine (1.93 ml) and isobutylchloroformate[5] (1.84 ml) were added to a tetrahydrofuran (THF) solution of pMZ–Gln–OH[8] (4.38 g) at −10 °C and the reaction mixture was stirred for 10 min. The mixture was then combined with a solution of H–Val–OBzl tosylate[9] (5.3 g) and triethylamine (1.93 ml), and the whole was stirred for 2 h. The solvent was evaporated off and the residue was washed successively with H_2O, 10% Na_2CO_3, H_2O, 5% citric acid and H_2O in a mortar. The material was recrystallized from MeOH. Yield 5.72 g (82%), mp 171—174 °C, $[\alpha]_D^{33}$ −9.5° (c=0.9, DMF), Rf^3 0.69. *Anal.* Calcd for $C_{26}H_{33}N_3O_7$: C, 62.5; H, 6.7; N, 8.4. Found: C, 62.3; H, 6.7; N, 8.4. Amino acid ratios in an acid hydrolysate: $Glu_{0.91}Val_{1.00}$ (average recovery 89%).

Boc–Glu(OBzl)–Gln–Val–OBzl——Boc–Glu(OBzl)–OH[10] (2.95 g) dissolved in THF (30 ml) and H–Gln–Val–OBzl·TFA (prepared from 4.37 g of pMZ–Gln–Val–OBzl by TFA treatment) dissolved in DMF (30 ml) were coupled by the mixed anhydride method[5] in the usual manner. The solvents were evaporated off and the residue was extracted with AcOEt. The AcOEt layer was washed successively with 10% Na_2CO_3, H_2O, 5% citric acid and H_2O, then dried over Na_2SO_4 and evaporated down. The residue was recrystallized from AcOEt–petroleum ether. Yield 3.92 g (69%), mp 123—125 °C, $[\alpha]_D^{33}$ −12.8° (c=1.1, DMF), Rf^1 0.85, Rf^3 0.82. *Anal.* Calcd for $C_{34}H_{46}N_4O_9$: C, 62.4; H, 7.1; N, 8.6. Found: C, 62.1; H, 7.2; N, 8.7. Amino acid ratio in an acid hydrolysate: $Glu_{2.01}Val_{1.00}$ (average recovery 90%).

pMZ–Gln–Glu(OBzl)–Glu–Val–OBzl——pMZ–Gln–OH (1.85 g) and H–Glu(OBzl)–Gln–Val–OBzl·TFA (prepared from 3.9 g of Boc–Glu(OBzl)–Gln–Val–OBzl by TFA treatment) were coupled by the mixed anhydride method[5] in DMF in the usual manner. The solvent was evaporated off and the resulting residue was washed successively with H_2O, 5% Na_2CO_3, 10% citric acid, H_2O and AcOEt in a mortar. Yield 3.77 g (75%), mp 239—245 °C, $[\alpha]_D^{22}$ −14.6° (c=1.0, DMF), Rf^3 0.71. *Anal.* Calcd for $C_{43}H_{54}N_6O_{12}$: C, 61.0; H, 6.4; N, 9.9. Found: C, 60.9; H, 6.5; N, 9.9. Amino acid ratio in an acid hydrolysate: $Glu_{2.89}Val_{1.00}$ (average recovery 89%).

Z–Asn–Gln–Glu(OBzl)–Gln–Val–OBzl——Z–Asn–ONp[11] (358 mg) was added to a solution of H–Gln–Glu(OBzl)–Gln–Val–OBzl·TFA (prepared from 627 mg of pMZ–Gln–Glu(OBzl)–Gln–Val–OBzl by TFA treatment) in DMF (10 ml) and the mixture was adjusted to pH 8 with triethylamine. The reaction mixture was stirred in a cold room overnight, then the solvent was evaporated off. The residue was washed successively with 10% Na_2CO_3, H_2O, 5% citric acid, H_2O and MeOH in a mortar. Yield 427 mg (62%) mp 263—267 °C, $[\alpha]_D^{23}$ −20.8° (c=1.0, DMF), Rf^2 0.85, Rf^3 0.84. *Anal.* Calcd for $C_{46}H_{58}N_8O_{13}\cdot 1/2H_2O$: C, 58.8; H, 6.3; N, 11.9. Found: C, 58.6; H, 6.2; N, 11.9. Amino acid ratios in an acid hydrolysate: $Asp_{1.00}Glu_{2.99}Val_{1.02}$ (average recovery 94%).

H–Asn–Gln–Glu–Gln–Val–OH (I)——Z–Asn–Gln–Glu(OBzl)–Gln–Val–OBzl (198 mg) was hydrogenated over Pd catalyst in 90% AcOH (30 ml) for 5 h. The reaction mixture was concentrated and lyophilized to afford a hygroscopic fluffy powder. Yield 125 mg (100%), $[\alpha]_D^{21}$ −18.3° (c=1.0, AcOH), Rf^2 0.28. *Anal.* Calcd for $C_{24}H_{40}N_8O_{11}\cdot 2.5H_2O$: C, 43.6; H, 6.9; N, 16.9. Found: C, 43.8; H, 7.1; N, 17.0. Amino acid ratios in an acid hydrolysate: $Asp_{0.92}Glu_{3.00}Val_{1.09}$ (average recovery 78%).

Z–Asp(OBzl)–Gln–Glu(OBzl)–Gln–Val–OBzl——Z–Asp(OBzl)–OH[12] (597 mg) dissolved in 10 ml of THF was coupled with H–Gln–Glu(OBzl)–Gln–Val–OBzl (prepared from 1.41 g of pMZ–Gln–Glu(OBzl)–Gln–Val–OBzl by TFA treatment) in DMF (10 ml) by the mixed anhydride method[5] in the usual manner. The solvents were evaporated off and the residue was washed successively with H_2O, 10% Na_2CO_3, H_2O, 5% citric acid, H_2O and AcOEt in a mortar. Yield 1.3 g (76%), mp 230—239 °C, $[\alpha]_D^{32}$ −17.5° (c=1.0, DMF), Rf^3 0.75. *Anal.* Calcd for $C_{53}H_{63}N_7O_{14}\cdot 1/2H_2O$: C, 61.7; H, 6.3; N, 9.5. Found: C, 61.7; H, 6.2; N, 9.6. Amino acid ratios in an acid hydrolysate: $Asp_{1.00}Glu_{3.06}Val_{1.09}$ (average recovery 99%).

H–Asp–Gln–Glu–Gln–Val–OH (II)——Z–Asp(OBzl)–Gln–Glu(OBzl)–Gln–Val–OBzl (200 mg) was hydrogenated over Pd catalyst in 90% AcOH (50 ml) for 5 h. The reaction mixture was evaporated and lyophilized to afford a hygroscopic fluffy powder. Yield 123 mg (96%), $[\alpha]_D^{32}$ −20.0° (c=0.9, DMF), Rf^2 0.22. *Anal.* Calcd for $C_{24}H_{39}N_7O_{12}\cdot 2H_2O$: C, 44.1; H, 6.6; N, 15.0. Found: C, 43.8; H, 6.5; N, 14.9. Amino acid ratios in an acid hydrolysate: $Asp_{1.05}Glu_{3.00}Val_{1.06}$ (average recovery 89%).

Inhibitory Effects of the Synthetic Peptides on the Cross-Linking Reaction of α_2-PI to Fibrin——Human blood was collected from the antecubital vein of normal subjects into a syringe containing 0.1 volume of 3.8% sodium citrate, and centrifuged at 1800 *g* for 20 min to prepare platelet-poor plasma. This plasma (0.41 ml) was mixed with a synthetic peptide (I or II) dissolved in 20 mM Tris buffer (0.05 ml, pH 7.4) containing 0.8% NaCl and with 0.5 M $CaCl_2$

(0.02 ml) or 0.05 M EDTA (0.02 ml) instead of $CaCl_2$. The mixture was incubated with thrombin (0.02 ml of 40 U/ml, Parke Davis Co.) at 37 °C for 1 h. After incubation, the plasma clot was squeezed and removed with a spatula and the concentration of α_2-PI in the supernatant was measured by the single radial immunodiffusion method[7] as follows. A 1% solution of agarose in barbitone buffer (16 ml, pH 8.6, ionic strength 0.05, Daiichi Chemical Co., Ltd.) was mixed with rabbit immunoglobuling G (IgG 0.11 ml) purified from rabbit anti-human α_2-PI serum by using protein A-Sepharose (Sigma Chemical Co., Ltd.). The agarose solution containing IgG was layered onto a slide (11 × 7.5 cm) and 3 mm diameter wells were cut out with a gel punch. The supernatant (0.02 ml) of the plasma clot was added to a well, and diluted human plasma was used as a standard. The gel slide was incubated for 30 h at 37 °C under a humid atmosphere and the diameter of each precipitation ring was measured. The concentration of α_2-PI in the supernatant was determined from a standard curve. The results are shown in Table I.

HPLC——The purities of the synthetic peptides were checked by chromatography on a Cosmosil $5C_{18}$ column (4.6 × 150 mm, Nakarai Chem. Co.) with the following eluents at a flow rate of 0.1 ml/min. The eluents: MeOH–H_2O (6:4); CH_3CN–H_2O (7:3); 0.05% H_3PO_4–CH_3CN (4:6); 0.1% TFA.

References and Notes

1) Amino acids and peptides and their derivatives mentioned in this paper are of L-configuration. Abbreviations used in this paper are: Z = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, pMZ = *p*-methoxybenzyloxycarbonyl, OBzl = benzyl ester, ONp = *p*-nitrophenyl ester, TFA = trifluoroacetic acid, DMF = dimethylformamide.
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