Amino Acids and Peptides. XVIII.[1] Synthetic Peptides Related to *N*-Terminal Portion of Fibrin α-Chain and Their Inhibitory Effect on Fibrinogen/Thrombin Clotting

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Peptide analogs of the *N*-terminal portion of fibrin α-chain were prepared and their inhibitory effects on fibrinogen/thrombin clotting were examined. Of the synthetic peptides, H–Gly–Pro–Arg–Pro–Pro–NH_2 exhibited the most potent inhibitory effect.

Keywords fibrin-related peptide; fibrin α-chain; anticoagulant; fibrinogen/thrombin clotting; peptide synthesis

N-Terminal tripeptide of fibrin α-chain, H–Gly–Pro–Arg–OH, was reported to be an inhibitor of fibrinogen/thrombin clotting (FTC) by Laudano and Doolittle.[2] They also reported that a synthetic analog of the tripeptide, H–Gly–Pro–Arg–Pro–OH, exhibited a potent inhibitory effect on FTC. These peptides bind to the *C*-terminal portion of the γ-chain of fibrin and prevent fibrin polymerization. On the basis of the inhibition mechanism, it should be possible to develop a new type of anticoagulant. In the preceding papers, we reported the structure–activity relationships of *N*-terminal tripeptide analogs[3] and tetrapeptide analogs.[4] Here we report the structure–activity relationship of *N*-terminal tetrapeptide analogs and pentapeptide analogs.

First of all, H–Gly–Pro–Arg–Leu–OH (I), corresponding to the *N*-terminal tetrapeptide of bovine[5] and lamprey[5] fibrin α-chains, and H–Gly–Pro–Arg–Ile–OH (II), corresponding to the *N*-terminal tetrapeptide of chicken[6] and dog[7] fibrin α-chains, were prepared by a solution method as shown in Fig. 1. Z(OMe)–Arg(NO_2)–OH and Leu–OBzl were reacted by the mixed anhydride method[8] to give a protected dipeptide, followed by TFA treatment to remove the Z(OMe) group. The resulting dipeptide was coupled with Z–Gly–Pro–OH[3,4] by the mixed anhydride method, followed by hydrogenation to give H–Gly–Pro–Arg–Leu–OH (I). H–Gly–Pro–Arg–Ile–OH (II) was prepared in the same way. Instead of Leu and Ile, tranexamic acid (Tra) was introduced into the tetrapeptide. H–Gly–Pro–Arg–Tra–NH_2 (III) was prepared by the solid-phase method using the N^{α}-Boc strategy. The mesitylenesulfonyl(Mts) group[9] was used for protection of the guanidino group of Arg. Final deprotection was performed by trifluoromethanesulfonic acid (TFMSA) treatment[10] and the product was purified by reverse-phase high performance liquid chromatography (RP-HPLC).

Next, various pentapeptide analogs of the *N*-terminal portion of the fibrin α-chain were prepared by the solid-phase method. In the *N*-terminal pentapeptide of human fibrin α-chain, H–Gly–Pro–Arg–Val–Val–, Val–Val portion was replaced by other amino acids. The following protecting groups were used for side chain protection: cyclohexyl ester (–OcHx)[11] for β- and γ-carboxyl groups of Asp and Glu, and the 2,4-dinitrophenyl group (DNP)[12] for imidazole of His. Synthetic peptides and their inhibitory effects on FTC are summarized in Table I. Inhibitory effects of the synthetic peptides on FTC were examined by the method described previously.[3] Since IC_{50} of each synthetic peptide varied when different lot of fibrinogen and thrombin were used, IC_{50} of H–Gly–Pro–Arg–Pro–OH was always measured for comparison when the inhibitory effect of a synthetic peptide was examined. Relative activities were calculated by dividing the IC_{50} of H–Gly–Pro–Arg–Pro–OH by that of each peptide. The IC_{50} of H–Gly–Pro–Arg–Pro–OH was between 28 and 65 μM. The inhibitory effects of I (bovine and lamprey fibrin α-chain *N*-terminal

TABLE I. Inhibitory Effects of Synthetic Peptides on FTC

Peptides	Relative activities
H–Gly–Pro–Arg–Pro–OH	1.00[2-4]
H–Gly–Pro–Arg–OH	0.33[2,3]
H–Gly–Pro–Arg–Leu–OH(I)	0.11
H–Gly–Pro–Arg–Ile–OH(II)	0.10
H–Gly–Pro–Arg–Tra–NH_2(III)	1.79
H–Gly–Pro–Arg–Pro–Tra–OH(IV)	0.04
H–Gly–Pro–Arg–Pro–Tra–NH_2(V)	1.68
H–Gly–Pro–Arg–Pro–Tyr–NH_2(VI)	0.04
H–Gly–Pro–Arg–Pro–Arg–NH_2(VII)	0.32
H–Gly–Pro–Arg–Pro–His–NH_2(VIII)	0.22
H–Gly–Pro–Arg–Pro–Asn–NH_2(IX)	0.39
H–Gly–Pro–Arg–Pro–Gln–NH_2(X)	0.34
H–Gly–Pro–Arg–Pro–Asp–OH(XI)	1.05
H–Gly–Pro–Arg–Pro–Asp–NH_2(XII)	0.03
H–Gly–Pro–Arg–Pro–Glu–OH(XIII)	0.37
H–Gly–Pro–Arg–Gly–Asp–NH_2(XIV)	0.03
H–Gly–Pro–Arg–Pro–Pro–NH_2(XV)	4.56

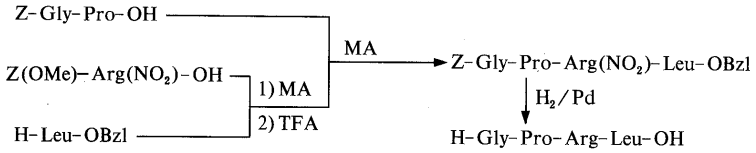

Fig. 1. Synthetic Scheme for H–Gly–Pro–Arg–Leu–OH
MA: mixed anhydride method.

tetrapeptide) and II (chicken and dog fibrin α-chain *N*-terminal tetrapeptide) were less potent than that of H–Gly–Pro–Arg–Pro–OH, which was reported to be a powerful inhibitor of FTC by Laudano and Doolittle.[2] Compound III contains Tra (an antifibrinolytic agent) and exhibited a potent inhibitory effect. The inhibitory effect of V was more potent than that of H–Gly–Pro–Arg–Pro–OH, but IV exhibited a sharp reduction of the inhibitory effect. Similar results were reported in the preceding papers.[3,4] In those papers, we found that the amide analogs of Gly–Pro–Arg and Gly–Pro–Arg–Pro exhibited more potent inhibitory effects than Gly–Pro–Arg and Gly–Pro–Arg–Pro with a free carboxyl group. The anionic charge of the carboxyl group in a synthetic peptide might prevent its binding to fibrin and fibrinogen. Introduction of an aromatic amino acid (Tyr) amide (VI), basic amino acid (Arg and His) amides (VII, VIII) and neutral amino acid (Asn and Gln) amides (IX, X) also resulted in decreases of the inhibitory effect. Introduction of Asp (XI) with a free carboxyl group did not change the potency of inhibitory effect, but Glu amide[5] and Asp amide[5] analogs (XII, XIII) were less active than the standard peptide, H–Gly–Pro–Arg–Pro–OH. It is interesting that the effect of the Asn–NH_2^5 analog (IX) was greater than that of Asp–NH_2^5 analog (XII) with a free β-carboxyl group and less active than that of the Asp [5] analog (XI) with both carboxyl groups (α and β) free. The [Gly[4], Asp–NH_2^5]-analog (XIV) exhibited sharply decreased inhibitory effect and the result implies an important role of Pro at the 4 position. Of the synthetic pentapeptides, H–Gly–Pro–Arg–Pro–Pro–NH_2 (XV) exhibited the most potent inhibitory effect on FTC. In the preceding paper,[1a] we found that the *N*-terminal fibrin α-chain decapeptide analog, H–Gly–Pro–Arg–Pro–Pro–Glu–Arg–His–Gln–Ser–NH_2, had a more potent inhibitory effect than the *N*-terminal fibrin α-chain decapeptide, H–Gly–Pro–Arg–Val–Val–Glu–Arg–His–Gln–Ser–NH_2. Furthermore, the *N*-terminal fibrin α-chain pentapeptide, H–Gly–Pro–Arg–Val–Val–OH, was less active than the *N*-terminal fibrin α-chain tripeptide, H–Gly–Pro–Arg–OH. The reason why such a structural change produced a remarkable change of the inhibitory effect is not clear. We speculate that the *N*-terminal portion of the fibrin α-chain binds to the γ-chain when fibrin polymerization occurs. Val has a bulky side chain but the Val–Val portion does not interfere with the binding in the natural molecule. Natural fibrin might have a conformation that allows the binding, but the synthetic peptide might not take the same conformation. Thus, the bulky side chain of Val in the synthetic peptide might interfere with the binding and the Pro–Pro portion might induce a conformation that allows easier binding.

Experimental

Melting points are uncorrected. Solvent systems for ascending thin-layer chromatography on Silica gel G (type 60, E. Merck) are indicated as follows: Rf^1 = BuOH–AcOH–H_2O (4 : 1 : 5, upper phase), Rf^2 = BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf^3 = $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase), Rf^5 = $CHCl_3$–MeOH–AcOH (90 : 8 : 2). Synthetic peptides were hydrolyzed in 6 N HCl at 110 °C for 20 h. Arg(NO_2)-containing peptides were hydrolyzed for 48 h. During the hydrolysis, Arg (NO_2) was mainly converted to Arg, but partially converted to Orn.[1a] Thus Arg content of Arg (NO_2)-containing peptides in an acid hydrolysate was calculated as (Arg + Orn) content.

Solid-Phase Peptide Synthesis *p*-Methylbenzhydrylamine resin and chloromethylated resin were purchased from Peptide Institute, Inc. The following amino acid derivatives were used; Z(OMe)–Gly–OH, Boc–Pro–OH, Boc–Arg(Mts)–OH, Boc–Leu–OH, Boc–Ile–OH, Boc–Glu(OBzl)–OH, Boc–Asp(OcHx)–OH, Z(OMe)–His(DNP)–OH, Z(OMe)–Gln–OH, Z(OMe)–Asn–OH. The synthetic protocol for solid-phase peptide synthesis is shown below.

step	reagents	reaction time	
1	NMM/DCM	10 min	×2
2	DCM	3 min	×3
3	Boc- or Z(OMe)-amino acid (2 eq) in DMF (or DCM) 1 M DCC/DCM (2 eq)	120 min	
4	50% MeOH/DCM	5 min	×3
5	DCM	2 min	1
6	50% TFA/DCM, anisole	2 min	1
		45 min	1
7	DCM	3 min	×3

1 M HOBt/DMF (2 eq) was added when Boc–Arg(Mts)–OH, Z(OMe)–Gln–OH and Z(OMe)–Asn–OH were activated. Prior to the final deprotection, the DNP group was removed by using thiophenol (20-fold excess over peptide in molar ratio) in DMF for 1 h at room temperature. Final deprotection was performed by TFMSA–thioanisole treatment[10] in TFA for 0.5 h at 0 °C and 2 h at room temperature. The product was treated with Amberlite 400 (acetate) and purified by RP-HPLC on a YMC-Pack AQ ODS5 column using a 0.1% TFA-containing mixture of CH_3CN and water, or by carboxymethyl(CM)-cellulose column chromatography using 0.005—0.04 M ammonium acetate. Yields were calculated from crude deblocked material. The peptides purified by HPLC were converted to their hydrochlorides by lyophilization from HCl-containing water.

Z(OMe)–Arg(NO_2)–Leu–OBzl Isobutyl chloroformate (1.03 ml, 7.94 mmol) was added to a solution of Z(OMe)–Arg(NO_2)–OH (3 g, 7.83 mmol) and triethylamine (1.08 ml, 7.83 mmol) in DMF (20 ml) at −10 °C and the mixture was stirred for 10 min. DMF solution (20 ml) of H–Leu–OBzl (prepared from 3.08 g of its tosylate by neutralization with triethylamine) was then combined with the mixed anhydride solution and the whole was stirred overnight. The solvent was removed *in vacuo* and the residue was dissolved in AcOEt, followed by washing with 5% Na_2CO_3, 5% citric acid and water. The AcOEt layer was dried with sodium sulfate and evaporated. The residue was purified by silica gel column chromatography using 3% $CHCl_3$ as an eluent. Yield 3.57 g (78%), mp 62—64 °C, Rf^5 0.56, $[\alpha]_D^{19}$ −26.9° (c = 1.0, MeOH). *Anal.* Calcd for $C_{28}H_{38}N_6O_6$: C, 57.33; H, 6.54; N, 14.33. Found: C, 57.12; H, 6.57; N, 14.46.

Z–Gly–Pro–Arg(NO_2)–Leu–OBzl Isobutyl chloroformate (0.22 ml, 1.7 mmol) was added to a DMF (15 ml) solution of Z–Gly–Pro–OH (0.52 g, 1.7 mmol) and triethylamine (0.23 ml, 1.7 mmol) at −10 °C and the mixture was stirred for 10 min. A DMF (20 ml) solution of H–Arg(NO_2)–Leu–OBzl [prepared from 1 g of Z(OMe)–Arg(NO_2)–Leu–OBzl by TFA treatment followed by triethylamine treatment] was then added to the mixture and the whole was stirred overnight. The solvent was removed and the residue was extracted with AcOEt, followed by washing of the AcOEt layer with 5% Na_2CO_3, 5% citric acid and water. The AcOEt was removed and the residue was purified by silica gel column chromatography using 3% MeOH/CHC_3 as an eluent. Yield 0.61 g (51%), mp 78—81 °C, Rf^3 0.75, $[\alpha]_D^{22}$ −64.8° (c = 1.0, MeOH). *Anal.* Calcd for $C_{34}H_{46}N_8O_9$: C, 57.45; H, 6.54; N, 15.77. Found: C, 57.16; H, 6.54; N, 15.81. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.15, Arg + Orn 0.94, Leu 1.06 (average recovery 91%).

H–Gly–Pro–Arg–Leu–OH (I) Z–Gly–Pro–Arg(NO_2)–Leu–OBzl (305 mg, 0.43 mmol) was hydrogenated in a mixture of MeOH (17 ml) and AcOH (3 ml) in the presence of Pd catalyst for 18 h at 40 °C. The product was purified by CM-cellulose column chromatography using 0.01—0.05 M ammonium acetate. Yield 129 mg (60%), hygroscopic powder, Rf^2 0.17, $[\alpha]_D^{20}$ −101.6° (c = 0.5, H_2O), *Anal.* Calcd for $C_{19}H_{35}N_7O_5 \cdot AcOH \cdot H_2O$: C, 48.54; H, 7.97; N, 18.87. Found: C, 48.44; H, 7.98; N, 18.95. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.15, Arg 0.92, Leu 1.04 (average recovery 88%).

Z(OMe)–Arg(NO_2)–Ile–OBzl Z(OMe)–Arg(NO_2)–OH (3 g, 7.8 mmol) and H–Ile–OBzl·Tos–OH (3.21 g, 7.8 mmol) were reacted by the mixed anhydride method in the same way as described for preparation of Z(OMe)–Arg(NO_2)–Leu–OBzl. The product was purified by silica gel column chromatography using 3% MeOH/$CHCl_3$ as an eluent. Yield 2.1 g (46%), mp 77—79 °C, Rf^5 0.62, $[\alpha]_D^{21}$ −23.9° (c = 1.0, MeOH). *Anal.* Calcd for $C_{28}H_{38}N_6O_6$: C, 57.33; H, 6.54; N, 14.33. Found: C, 57.55; H,

6.68; N, 14.38.

Z–Gly–Pro–Arg(NO_2)–Ile–OBzl Prepared from Z–Gly–Pro–OH (250 mg, 0.8 mmol) and H–Arg(NO_2)–Ile–OBzl (prepared from 470 mg of above protected dipeptide by TFA treatment) by the mixed anhydride method in the same way as described for preparation of Z–Gly–Pro–Arg(NO_2)–Leu–OBzl. The product was purified by silica gel column chromatography using 3% MeOH/$CHCl_3$ as an eluent. Yield 380 mg (67%), mp 85—90 °C, Rf^1 0.73, $[\alpha]_D^{21}$ −61.0° (c=1.0, MeOH). *Anal.* Calcd for $C_{34}H_{46}N_8O_9$: C, 57.45; H, 6.54; N, 15.77. Found: C, 57.28; H, 6.58; N, 15.89. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.11, Arg+Orn 0.90, Ile 1.03 (average recovery 87%).

H–Gly–Pro–Arg–Ile–OH (II) Z–Gly–Pro–Arg(NO_2)–Ile–OBzl (240 mg, 0.34 mmol) was hydrogenated in a mixture of MeOH (16 ml) and AcOH (4 ml) in the presence of Pd catalyst for 16 h at 40 °C. The product was purified by CM-cellulose column chromatography using 0.01—0.04 M ammonium acetate. Yield 105 mg (62%), hygroscopic powder, Rf^2 0.18, $[\alpha]_D^{19}$ −81.9° (c=0.5, H_2O). *Anal.* Calcd for $C_{19}H_{35}N_7O_5 \cdot AcOH \cdot 7/4H_2O$: C, 47.31; H, 8.05; N, 18.40. Found: C, 47.60; H, 7.97; N, 18.07. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.09, Arg 0.93, Ile 1.07 (average recovery 84%).

H–Gly–Pro–Arg–Tra–NH_2 (III) Prepared by the solid-phase method and purified by CM-cellulose column chromatography using 0.01—0.05 M ammonium acetate buffer as an eluent. Yield 33%, hygroscopic powder, Rf^2 0.46, $[\alpha]_D^{26}$ −63.0° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 0.98, Arg 1.01, Tra 0.76 (average recovery 75%).

H–Gly–Pro–Arg–Pro–Tra–OH (IV) Prepared by the solid-phase method and purified by RP-HPLC. Yield 9%, hygroscopic powder, Rf^2 0.38, $[\alpha]_D^{25}$ −79.2° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 2.15, Arg 0.94, Tra 0.87 (average recovery 82%).

H–Gly–Pro–Arg–Pro–Tra–NH_2 (V) Prepared by the solid-phase method and purified by CM-cellulose column chromatography. Yield 40%, hygroscopic powder, Rf^2 0.42, $[\alpha]_D^{26}$ −111.0° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 2.17, Arg 0.99, Tra 0.83 (average recovery 88%).

H–Gly–Pro–Arg–Pro–Tyr–NH_2 (VI) Prepared by the solid-phase method and purified by RP-HPLC. Yield 13%, hygroscopic powder, Rf^2 0.40, $[\alpha]_D^{26}$ −128.6° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.98, Arg 1.05, Tyr 0.89 (average recovery 74%).

H–Gly–Pro–Arg–Pro–Arg–NH_2 (VII) Prepared by the solid-phase method and purified by CM-cellulose column chromatography. Yield 11%, hygroscopic powder, Rf^2 0.38, $[\alpha]_D^{24}$ −48.3° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 2.23, Arg 2.05 (average recovery 81%).

H–Gly–Pro–Arg–Pro–His–NH_2 (VIII) Prepared by the solid-phase method and purified by CM-cellulose column chromatography. Yield 50%, hygroscopic powder, Rf^2 0.28, $[\alpha]_D^{26}$ −83.4° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 2.19, Arg 1.05, His 0.95 (average recovery 86%).

H–Gly–Pro–Arg–Pro–Asn–NH_2 (IX) Prepared by the solid-phase method and purified by RP-HPLC. Yield 16%, hygroscopic powder, Rf^2 0.34, $[\alpha]_D^{26}$ −90.4° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 2.13, Arg 1.05, Asp 0.92 (average recovery 84%).

H–Gly–Pro–Arg–Pro–Gln–NH_2 (X) Prepared by the solid-phase method and purified by CM-cellulose column chromatography. Yield 30%, Rf^2 0.39, $[\alpha]_D^{26}$ −109.8° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.91, Arg 0.90, Glu 1.03 (average recovery 86%).

H–Gly–Pro–Arg–Pro–Asp–OH (XI) Prepared by the solid-phase method and purified by CM-cellulose column chromatography. Yield 8%, Rf^2 0.32, $[\alpha]_D^{26}$ −103.6° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 2.15, Arg 1.05, Asp 0.93 (average recovery 84%).

H–Gly–Pro–Arg–Pro–Asp–NH_2 (XII) Prepared by the solid phase method and purified by CM-cellulose column chromatography. Yield 54%, hygroscopic powder, Rf^2 0.45, $[\alpha]_D^{26}$ −88.6° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 2.12, Arg 0.94, Asp 1.01 (average recovery 76%).

H–Gly–Pro–Arg–Pro–Glu–OH (XIII) Prepared by the solid-phase method and purified by RP-HPLC. Yield 10%, hygroscopic powder, Rf^2 0.35, $[\alpha]_D^{26}$ −122.4° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 2.20, Arg 1.05, Glu 1.09 (average recovery 79%).

H–Gly–Pro–Arg–Gly–Asp–NH_2 (XIV) Prepared by the solid-phase method and purified by CM-cellulose column chromatography. Yield 63%, hygroscopic powder, Rf^2 0.40, $[\alpha]_D^{26}$ −53.6° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate; Gly 2.00, Pro 1.01, Arg 0.98, Asp 1.00 (average recovery 86%).

H–Gly–Pro–Arg–Pro–Pro–NH_2 (XV) Prepared by the solid-phase method and purified by CM-cellulose column chromatography. Yield 45%, hygroscopic powder, Rf^2 0.32, $[\alpha]_D^{27}$ −152.3° (c=1.0, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 3.24, Arg 0.99 (average recovery 84%).

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References and Notes

1) *a*) Part XVII: K. Kawasaki, T. Tsuji, K. Hirase, M. Miyano, S. Inouye, and M. Iwamoto, *Chem. Pharm. Bull.*, **41**, 525 (1993); *b*) Standard abbreviations for amino acids and peptides and protecting groups are used [*Eur. J. Biochem.*, **138**, 9 (1984)]; Other abbreviations include DMF=dimethylformamide, TFA=trifluoroacetic acid, TFMSA=trifluoromethanesulfonic acid, DCC=dicyclohexylcarbodiimide, HoBt=1-hydroxybenzotriazole, N^G=nitrogen of a guanidino group, FTC=fibrinogen/thrombin clotting, Tos=tosyl, cHx=cyclohexyl, Tra=tranexamic acid, NMM=*N*-methylmorpholine, DCM=dichloromethane.
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