Amino Acids and Peptides. XVII. Synthesis of Peptides Related to *N*-Terminal Portion of Fibrin α-Chain and Their Inhibitory Effect on Fibrinogen/Thrombin Clotting

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Various peptides related to *N*-terminal portion of fibrin α-chain were synthesized by the solution method and the solid-phase method, and their inhibitory effect on fibrinogen/thrombin clotting was examined. Extension of peptide chain from *N*-terminal tripeptide decreased the inhibitory effect. The most potent effect was shown by *N*-terminal decapeptide analog, H–Gly–Pro–Arg–Pro–Pro–Glu–Arg–His–Gln–Ser–NH_2.

Keywords fibrin-α *N*-terminal; anticoagulant; peptide synthesis; acid hydrolysis; nitroarginine; fibrinogen/thrombin clotting

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 prepared its analog, H–Gly–Pro–Arg–Pro–OH by the solid-phase method and reported it as a potent inhibitor of FTC. The inhibitory effect was due to its binding to fibrinogen, not to thrombin. The mechanism of the inhibitory effect suggested a feasible development of a new type of anticoagulant. To study structure–activity relationships of a fibrin *N*-terminal portion, various peptides were synthesized by the solution method and the solid-phase method.

Preparation of *N*-terminal tripeptide (H–Gly–Pro–Arg–OH) by the solution method was reported earlier.[3] *N*-Terminal tetrapeptide was prepared as shown in Fig. 1. The guanidino group of Arg was protected by nitro group.[4] N^{α}-*tert*-Butoxycarbonyl-N^{G}-nitroarginine (Boc–Arg(NO_2)–OH) was reacted with valine benzyl ester (H–Val–OBzl) by the mixed anhydride method,[5] followed by treatment with trifluoroacetic acid (TFA). The resulting H–Arg(NO_2)–Val–OBzl was reacted with Boc–Pro–OH by the mixed anhydride method, followed by treatment with TFA. The resulting H–Pro–Arg(NO_2)–Pro–OBzl was then reacted with benzyloxycarbonylglycine (Z–Gly–OH) by the mixed anhydride method, followed by hydrogenation to give H–Gly–Pro–Arg–Val–OH (I). The product was purified by carboxymethylcellulose (CM-cellulose) column chromatography using ammonium acetate buffer as an eluent.

For preparation of *N*-terminal heptapeptide, the γ-carboxyl group of Glu and the carboxyl group of *C*-terminal Arg were protected by benzyl group. Stepwise peptide-chain elongation from *C*-terminal H–Arg(NO_2)–OBzl was performed by the mixed anhydride method to give the pentapeptide, Z(OMe)–Arg(NO_2)–Val–Val–Glu(OBzl)–Arg(NO_2)–OBzl, followed by TFA treatment to remove Z(OMe) group. Z–Gly–Pro–OH was then introduced in the peptide chain by the diphenylphosphoryl azide (DPPA) method[6] to give Z–Gly–Pro–Arg(NO_2)–Val–Val–Glu(OBzl)–Arg(NO_2)–OBzl. The final deprotection was first performed by hydrogenation but the yield was low. The protected heptapeptide contained two nitro groups and their removal by hydrogenation was slow. The HF method[7] was then used for the final deprotection and the yield of the HF method was much higher than that of the hydrogenation method. The deblocked heptapeptide, H–Gly–Pro–Arg–Val–Val–Glu–Arg–OH (IV), was purified by CM-cellulose column chromatography using ammonium acetate buffer as an eluent. *N*-Terminal pentapeptide (H–Gly–Pro–Arg–Val–Val–OH, II) and hexapeptide (H–Gly–Pro–Arg–Val–Val–Glu–OH, III) were prepared in a similar manner as shown in Fig. 1.

During the synthesis, synthetic intermediates were hydrolyzed and their amino acid contents were examined. Arg contents in acid hydrolysates of Arg(NO_2)-containing peptides were always low compared with those of other amino acids in the same acid hydrolysate. Thus Arg content in an acid hydrolysate of Arg(NO_2) was compared with those of N^{G}-tosylarginine [Arg(Tos)] and N^{G}-2,4,6-triisopropylarginine [Arg(Tis)][8] derivatives. Acidolyses were performed for 24 and 48 h in 6 N HCl and the results are shown in Table I. Only Arg was detected in the acid hydrolysates of Arg, Boc–Arg(Tos)–OH and Boc–Arg(Tis)–OH, but about 30% of Orn was found in acid hydrolysates of H–Arg(NO_2)–OH and Boc–Arg(NO_2)–OH. Arg(NO_2) was detected in 24 h acid hydrolysate, but was not detected in 48 h acid hydrolysate. The retention time of Arg(NO_2) as determined by an amino acid analyzer was almost the same as that of Val. Considering these results, Arg content of Arg(NO_2)-containing peptides was determined as (Arg + Orn) content in 48 h acid hydrolysate.

N-Terminal decapeptide (H–Gly–Pro–Arg–Val–Val–Glu–Arg–His–Gln–Ser–NH_2), its analog (H–Gly–Pro–Arg–Pro–Pro–Glu–Arg–His–Gln–Ser–NH_2) and its *C*-terminal heptapeptide (H–Pro–Pro–Glu–Arg–His–Gln–Ser–NH_2) were prepared by the solid-phase method. Laudano and Doolittle reported that H–Gly–Pro–Arg–Pro–OH was a potent inhibitor of FTC.[2] The peptide is an analog of the *N*-terminal tetrapeptide of fibrin α-chain in which Val was replaced by Pro. Thus we prepared heptapeptide and decapeptide analogs in which Val–Val portions were replaced with Pro–Pro. The guanidino group of Arg was protected by 2,4,6-triisopropylbenzenesulfonyl (Tis) group,[8] the γ-carboxyl group of Glu was protected by benzyl group and α-amino groups were protected by Boc or Z(OMe) groups. The imidazole of His was protected by 2,4-dinitrophenyl (DNP) group.[9] A protected amino acid

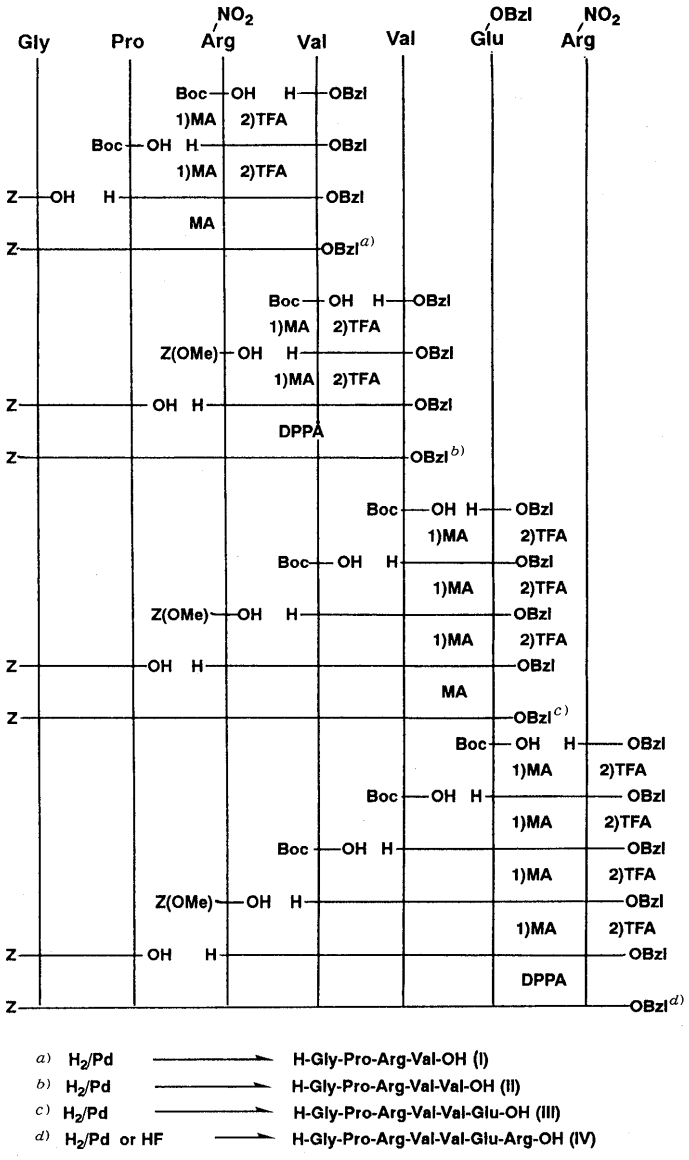

Fig. 1. Synthetic Scheme for *N*-Terminal Peptides of Fibrin α-Chain
MA: mixed anhydride method.

TABLE I. Recoveries of Arg in Acid Hydrolysates of Arg Derivatives

		Arg (%)	Orn (%)	$Arg(NO_2)$ (%)
H–$Arg(NO_2)$–OH	24 h	59	27	14
	48 h	72	28	0
Boc–$Arg(NO_2)$–OH	24 h	64	24	12
	48 h	72	28	0
Boc–Arg(Tis)–OH	24 h	100	0	0
	48 h	100	0	0
Boc–Arg(Tos)–OH	24 h	100	0	0
	48 h	100	0	0
H–Arg–OH	24 h	100	0	0
	48 h	100	0	0

Times indicated are those of acid hydrolysis.

was converted to its anhydride by the DCC method and then subjected to a coupling reaction. Prior to the final deprotection, the peptide-resin was treated with thiophenol to remove the DNP group on His. The final deprotection was performed by the HF method and by the trifluoromethanesulfonic acid (TFMSA) method,[10] and the product was purified by reverse-phase high performance liquid chromatography (RP-HPLC).

TABLE II. Inhibitory Effects of Synthetic Peptides on FTC

Synthetic peptides	Relative activities
H–Gly–Pro–Arg–Pro–OH[2]	1.00
H–Gly–Pro–Arg–OH[2,3]	0.20
H–Gly–Prp–Arg–Val–OH (I)	0.06
H–Gly–Pro–Arg–Val–Val–OH (II)	0.06
H–Gly–Pro–Arg–Val–Val–Glu–OH (III)	<0.02
H–Gly–Pro–Arg–Val–Val–Glu–Arg–OH (IV)	<0.02
H–Gly–Pro–Arg–Val–Val–Glu–Arg–His–Gln–Ser–NH_2 (V)	0.13
H–Gly–Pro–Arg–Pro–Pro–Glu–Arg–His–Gln–Ser–NH_2 (VI)	2.33
H–Pro–Pro–Glu–Arg–His–Gln–Ser–NH_2 (VII)	<0.03

Inhibitory effects of the synthetic peptides on FTC were examined by the method described previously[3] and the results are summarized in Table II. Since IC_{50} of each synthetic peptide varied when different lots 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 the synthetic peptides was examined. Relative activities were calculated by dividing the IC_{50} of H–Gly–Pro–Arg–Pro–OH by that of each peptide. As shown in Table II, extension of the peptide chain from *N*-terminal tripeptide resulted in a sharp reduction in the inhibitory effect. The reason is not clear, but it is interesting that the *N*-terminal decapeptide analog (VI) exhibited a potent inhibitory effect. The inhibitory effect of VI was 2.33 times as high as that of H–Gly–Pro–Arg–Pro–OH which was reported as a potent inhibitor of FTC.[2] Since H–Pro–Pro–Glu–Arg–His–Gln–Ser–NH_2 (VII) did not exhibit the inhibitory effect, the inhibitory effect of VI derived from Gly–Pro–Arg portion. The structural difference between *N*-terminal decapeptide (V) and its analog (VI) is Val–Val and Pro–Pro bonds. The reason such a structural change produced remarkable potentiation of the inhibitory effect is not clear but the following might be speculated: *N*-terminal portion of fibrin α-chain binds to γ-chain when fibrin polymerization occurs. Val has a bulky side chain but Val–Val portion does not interfere with the binding between *N*-terminal portion of α-chain and *C*-terminal portion of γ-chain in a natural molecule. The conformation of natural fibrin might make the binding easy but the synthetic *N*-terminal decapeptide might not take the same conformation as natural fibrin. Pro–Pro portion might make the binding between VI and natural fibrin γ-chain easy.

Various *N*-terminal pentapeptide analogs of fibrin α-chain including H–Gly–Pro–Arg–Pro–Pro–NH_2 are being prepared and their inhibitory effects on FTC will be published.

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^4 = AcOEt–benzene (1:1), Rf^5 = $CHCl_3$–MeOH–AcOH (90:8:2), Rf^6 = $CHCl_3$–MeOH–H_2O (16:3:1, lower phase). Rotations were measured with a JASCO DIP-360 polarimeter. The synthetic peptides were hydrolyzed in 6 N HCl at 100 °C for 20 h. As described in this paper, $Arg(NO_2)$-containing peptides were hydrolyzed for 48 h. During the acid hydrolysis, nitroarginine was mainly converted to Arg, but partially converted to Orn. Thus Arg content of $Arg(NO_2)$-containing peptide in an acid hydrolysate was calculated as (Arg+Orn) content. Peptides

containing Val–Val bond were hydrolyzed for 48 h. Amino acid compositions of acid hydrolysates were determined with a Hitachi 835 amino acid analyzer. Inhibitory effect of the synthetic peptides on FTC was examined as reported.[3]

RP-HPLC was conducted with Waters 600 on YMC Pack AQ-ODS-5 using a mixture of 0.1% TFA-containing CH_3CN/H_2O as an eluent. CM-cellulose column chromatography was conducted using ammonium acetate buffer in stepwise elution from 0.01 to 0.05 M concentration. Conversion of AcOH salt (or TFA salt) of synthetic peptide to its hydrochloride was achieved by lyophilization from H_2O containing an equivalent amount of HCl. Fast atom bombardment mass spectra (FAB-MS) were measured on a VG Analytical ZAV-SE spectrometer.

Solid-Phase Peptide Synthesis *p*-Methylbenzhydrylamine resine was purchased from Peptide Institute, Inc. The following amino acid derivatives were used: Z(OMe)–Gly–OH, Boc–Pro–OH, Boc–Arg(Tis)–OH, Boc–Val–OH, Boc–Glu(OBzl)–OH, Z(OMe)–His(DNP)–OH, Z(OMe)–Gln–OH, Boc–Ser(Bzl)–OH. Synthetic protocol for solid-phase peptide synthesis is shown below;

step	reagent	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)[a]	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

a) 1 M HOBt/DMF (2 eq) was added when Boc–Arg(Tis)–OH was activated.

Final deprotection was performed by HF treatment or TFMSA treatment.

General Procedure for the Mixed Anhydride Method Equimolar reagents were used. Isobutyl chloroformate and Et_3N (or NMM) were added to a N^{α}-protected amino acid (or peptide) solution in DMF at −10 °C and stirred for 15 min. The mixture was combined with an amino-component and stirred overnight. The solvent was removed and the product was extracted with AcOEt, followed by washing with 5% Na_2CO_3, 5% citric acid and H_2O. The AcOEt was dried with Na_2SO_4 and evaporated off. The product was purified by a suitable method.

General Procedure for TFA Treatment Boc- or Z(OMe)-containing peptide (1 g) was dissolved in 5% anisole/TFA (8—10 ml) and the solution was stirred for 20 min at 0 °C and 20 min at room temperature. Chilled ether was added and the resulting precipitate was collected by filtration or centrifugation. The precipitate was neutralized in DMF with Et_3N and the mixture was subjected to the next reaction.

Boc–Arg(NO_2)–Val–OBzl Prepared from Boc–Arg(NO_2)–OH (7 g, 21.9 mmol) and H–Val–OBzl [prepared from its tosylate (8.32 g, 21.9 mmol) by neutralization with Et_3N] in DMF (140 ml) by the mixed anhydride method in the usual manner. The product was precipitated from petroleum ether/AcOEt. Yield 9.18 g (82%), mp 67—70 °C, Rf^3 0.67, $[\alpha]_D^{22}$ −59.0° (c=1.0, MeOH). *Anal.* Calcd for $C_{23}H_{36}N_6O_6$: C, 54.32; H, 7.14; N, 16.53. Found: C, 53.99; H, 7.32; N, 16.30.

Boc–Pro–Arg(NO_3)–Val–OBzl Synthesized from Boc–Pro–OH (0.85 g, 3.95 mmol) and H–Arg(NO_2)–Val–OBzl [prepared from its Boc derivative (2 g, 3.93 mmol) by TFA treatment, followed by neutralization with Et_3N] by the mixed anhydride method in the usual manner. The product was purified by silicagel column chromatography using MeOH-containing $CHCl_3$ as an eluent. Yield 2.00 g (90%), mp 97—105 °C, Rf^6 0.55, $[\alpha]_D^{22}$ −60.0° (c=1.0, MeOH). *Anal.* Calcd for $C_{28}H_{43}N_7O_8$: C, 55.52; H, 7.17; N, 16.19. Found: C, 55.22; H, 7.22; N, 16.04.

Z–Gly–Pro–Arg(NO_2)–Val–OBzl The above protected tripeptide (2 g, 3.3 mmol) was treated with TFA in the usual manner and reacted with Z–Gly–OH (0.83 g, 3.97 mmol) by the mixed anhydride method in the usual manner. The product was purified by silica gel column chromatography using MeOH-containing $CHCl_3$ as an eluent. Yield 1.84 g (80%), mp 89—95 °C, Rf^6 0.72, $[\alpha]_D^{22}$ −63.9° (c=1.0, MeOH). *Anal.* Calcd for $C_{33}H_{44}N_8O_9$: C, 56.87; H, 6.38; N, 16.08. Found: C, 56.73; H, 6.52; N, 15.99.

H–Gly–Pro–Arg–Val–OH (I) The above protected tetrapeptide (264 mg, 0.4 mmol) was hydrogenated with Pd catalyst in MeOH for 20 h. The product was purified by CM-cellulose column chromatography using 0.005—0.05 M $AcONH_4$. Yield 87 mg (54%), hygroscopic powder, Rf^2 0.15, $[\alpha]_D^{22}$ −84.3° (c=0.5, H_2O). FAB-MS m/z: 428 (M^++1). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.12, Arg 0.89, Val 1.07 (average recovery 87%).

Boc–Val–Val–OBzl Prepared from Boc–Val–OH (6.29 g, 27 mmol) and H–Val–OBzl·Tos–OH (10.24 g, 27 mmol) by the mixed anhydride method in the usual manner. Recrystallized from AcOEt/petroleum ether. Yield 7.2 g (67%), mp 53—55 °C, Rf^4 0.90, $[\alpha]_D^{30}$ −48.1° (c=1.0, MeOH). *Anal.* Calcd for $C_{22}H_{34}N_2O_5$: C, 65.00; H, 8.45; N, 6.89. Found: C, 65.02; H, 8.47; N, 6.83.

Z(OMe)–Arg(NO_2)–Val–Val–OBzl The above protected dipeptide (2.7 g, 6.6 mmol) was treated with TFA in the usual manner and reacted with Z(OMe)–Arg(NO_2)–OH (3.6 g, 9.4 mmol) by the mixed anhydride method in the usual manner. Recrystallized from MeOH/ether. Yield 3.96 g (88%), mp 138—140 °C, Rf^4 0.64, $[\alpha]_D^{29}$ −40.9 (c=0.9, MeOH). *Anal.* Calcd for $C_{32}H_{45}N_7O_9$: C, 57.21; H, 6.78; N, 14.60. Found: C, 57.20; H, 6.89; N, 14.70.

Z–Gly–Pro–Arg(NO_2)–Val–Val–OBzl The above protected tripeptide (700 mg, 0.88 mmol) was treated with TFA in the usual manner and reacted with Z–Gly–Pro–OH (780 mg, 2.54 mmol) by the mixed anhydride method in the usual manner. The product was recrystallized from MeOH. Yield 590 mg (70%), mp 115—118 °C, Rf^5 0.37, $[\alpha]_D^{26}$ −75.1° (c=1.0, MeOH). *Anal.* Calcd for $C_{38}H_{53}N_9O_{10}\cdot H_2O$: C, 56.07; H, 6.83; N, 15.49. Found: C, 55.88; H, 6.50; N, 15.11. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.09, Arg+Orn 0.94, Val 2.00 (average recovery 84%).

H–Gly–Pro–Arg–Val–Val–OH (II) The above protected pentapeptide (270 mg, 0.34 mmol) was hydrogenated with Pd catalyst in 10% AcOH/MeOH for 12 h. The product was purified by CM-cellulose column chromatography using 0.005—0.05 M $AcONH_4$ as an eluent. Yield 138 mg (69%), hygroscopic powder, Rf^2 0.37, $[\alpha]_D^{26}$ −127.0° (c=0.5, H_2O). *Anal.* Calcd for $C_{23}H_{42}N_8O_6\cdot AcOH\cdot 3H_2O$: C, 46.86; H, 8.20; N, 17.49. Found: C, 47.04; H, 7.88; N, 17.82. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.04, Val 1.99, Arg 0.92 (average recovery 84%).

Boc–Val–Glu(OBzl)–OBzl Prepared from Boc–Val–OH (6.95 g, 32 mmol) and H–Glu(OBzl)–OBzl·Tos–OH (15.83 g, 31.7 mmol) by the mixed anhydride method in the usual manner. Recrystallized from ether/petroleum ether. Yield 14.3 g (87%), mp 98—101 °C, Rf^5 0.88, $[\alpha]_D^{25}$ −35.6° (c=1.0, MeOH). *Anal.* Calcd for $C_{29}H_{38}N_2O_7$: C, 66.14; H, 7.29; N, 5.32. Found: C, 66.24; H, 7.29; N, 5.43. Amino acid ratio in an acid hydrolysate: Glu 1.04, Val 1.00 (average recovery 84%).

Boc–Val–Val–Glu(OBzl)–OBzl The Boc group of the above protected dipeptide (1.54 g, 2.92 mmol) was removed by TFA treatment in the usual manner and reacted with Boc–Val–OH (1.22 g, 5.61 mmol) by the mixed anhydride method in the usual manner. The product was recrystallized from ether/petroleum ether. Yield 1.41 g (77%), mp 133—135 °C, Rf^4 0.76, $[\alpha]_D^{26}$ −14.9° (c=1.0, MeOH). *Anal.* Calcd for $C_{34}H_{47}N_3O_8$: C, 65.26; H, 7.59; N, 6.72. Found: C, 65.47; H, 7.61; N, 6.48. Amino acid ratios in an acid hydrolysate: Val 2.00, Glu 1.03 (average recovery 85%).

Z(OMe)–Arg(NO_2)–Val–Val–Glu(OBzl)–OBzl The Boc group of the above protected tripeptide (700 mg, 1.12 mmol) was removed by TFA treatment and reacted with Z(OMe)–Arg(NO_2)–OH (980 mg, 2.56 mmol) by the mixed anhydride method in the usual manner. The reaction mixture was condensed and the product was precipitated by addition of MeOH, followed by washing with H_2O and hot EtOH. Yield 650 mg (65%), mp 190—193 °C, Rf^5 0.68, $[\alpha]_D^{26}$ −28.1° (c=1.0, DMF). *Anal.* Calcd for $C_{44}H_{58}N_8O_{12}$: C, 59.24; H, 6.66; N, 12.56. Found: C, 59.18; H, 6.59; N, 12.46.

Z–Gly–Pro–Arg(NO_2)–Val–Val–Glu(OBzl)–OBzl The Z(OMe) group of the above protected tetrapeptide (530 mg, 0.59 mmol) was removed by TFA treatment in the usual manner. Z–Gly–Pro–OH (490 mg, 1.6 mmol), Et_3N (0.08 ml, 0.59 mmol) and DPPA (0.36 ml, 0.59 mmol) were dissolved in DMF (15 ml) and stirred for 30 min at 0 °C. The deblocked material was dissolved in DMF (10 ml) and neutralized with Et_3N (0.08 ml, 0.59 mmol). Both DMF solutions were then combined and stirred overnight in a cold room. The solvent was removed and the residue was washed with H_2O and dried. Recrystallized from MeOH. Yield 460 mg (81%), mp 196—198 °C, Rf^5 0.47, $[\alpha]_D^{26}$ −28.1° (c=1.0, DMF). *Anal.* Calcd for $C_{50}H_{66}N_{10}O_{13}\cdot 1/2H_2O$: C, 58.64; H, 6.61; N, 13.68. Found: C, 58.56; H, 6.54; N, 13.38. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.11, Arg+Orn 0.91, Val 2.08, Glu 1.03 (average recovery 98%).

H–Gly–Pro–Arg–Val–Val–Glu–OH (III) The above protected hexapeptide (257 mg, 0.25 mmol) was hydrogenated with Pd catalyst in 5% AcOH/MeOH in the usual manner. The material was purified by CM-cellulose column chromatography using 0.005—0.03 M $AcONH_4$ as an eluent. Yield 127 mg (71%), hygroscopic powder, Rf^2 0.39, $[\alpha]_D^{26}$ −107.1° (c=0.5, H_2O). *Anal.* Calcd for $C_{28}H_{49}N_9O_9\cdot 5/2H_2O$: C, 47.99; H, 7.78; N, 17.99. Found: C, 47.98; H, 7.68; N, 17.67. Amino acid ratios

in an acid hydrolysate: Gly 1.00, Pro 1.08, Arg 0.90, Val 1.94, Glu 1.11 (average recovery 79%).

Boc–Glu(OBzl)–Arg(NO_2)–OBzl Prepared from Boc–Glu(OBzl)–OH (2 g, 5.9 mmol) and H–Arg(NO_2)–OBzl·2Tos–OH (3.88 g, 5.9 mmol) by the mixed anhydride method in the usual manner. The product was purified by silica gel column chromatography using MeOH-containing $CHCl_3$. Yield 2.68 g (72%), amorphous powder, Rf^3 0.87, $[\alpha]_D^{20}$ −21.9° ($c=1.0$, MeOH). *Anal.* Calcd for $C_{35}H_{49}N_7O_{10}$: C, 57.75; H, 6.80; N, 13.47. Found: C, 57.49; H, 6.86; N, 13.74.

Boc–Val–Glu(OBzl)–Arg(NO_2)–OBzl The above protected dipeptide (4.96 g, 7.89 mmol) was treated with TFA to remove its Boc group in the usual manner. The material was reacted with Boc–Val–OH (2.15 g, 9.9 mmol) by the mixed anhydride method in the usual manner. The product was recrystallized from AcOEt/ether. Yield 4.4 g (76%), mp 117—119 °C, Rf^4 0.88, $[\alpha]_D^{26}$ −21.9° ($c=1.0$, MeOH). *Anal.* Calcd for $C_{35}H_{49}N_7O_{10}$: C, 57.75; H, 6.80; N, 13.47. Found: C, 57.49; H, 6.86; N, 13.74. Amino acid ratios in an acid hydrolysate: Val 1.00, Glu 1.03, Arg + Orn 0.89 (average recovery 82%).

Boc–Val–Val–Glu(OBzl)–Arg(NO_2)–OBzl The Boc group of the above protected tripeptide (4.36 g, 5.99 mmol) was removed by TFA treatment in the usual manner. The deblocked material was reacted with Boc–Val–OH (4.01 g, 18.4 mmol) by the mixed anhydride method in the usual manner. The product was recrystallized from EtOH/ether. Yield 4.24 g (86%), mp 175—178 °C, Rf^6 0.88, $[\alpha]_D^{26}$ −42.3° ($c=1.0$, DMF). *Anal.* Calcd for $C_{40}H_{58}N_8O_{11}$: C, 58.10; H, 7.08; N, 13.55. Found: C, 57.95; H, 7.02; N, 13.47. Amino acid ratios in an acid hydrolysate: Val 1.87, Glu 1.00, Arg + Orn 0.89 (average recovery 83%).

Z(OMe)–Arg(NO_2)–Val–Val–Glu(OBzl)–Arg(NO_2)–OBzl The Boc group of the above protected tetrapeptide (507 mg, 0.46 mmol) was removed by TFA treatment in the usual manner. The deblocked material was reacted with Z(OMe)–Arg(NO_2)–OH (375 mg, 0.98 mmol) by the mixed anhydride method in the usual manner. The solvent of the reaction mixture (DMF) was removed and the residue was washed with H_2O and hot MeOH. Yield 479 mg (72%), mp 203—209 °C, Rf^7 0.33, $[\alpha]_D^{24}$ −13.4° ($c=1.0$, DMF). *Anal.* Calcd for $C_{50}H_{69}N_{13}O_{15}\cdot 1/2H_2O$: C, 54.53; H, 6.42; N, 16.54. Found: C, 54.59; H, 6.38; N, 16.19.

Z–Gly–Pro–Arg(NO_2)–Val–Val–Glu(OBzl)–Arg(NO_2)–OBzl The Z(OMe) group of the above protected pentapeptide (1 g, 0.9 mmol) was removed by TFA treatment in the usual manner. The material was dissolved in DMF and neutralized with Et_3N.

DPPA (2.17 ml, 9 mmol) was added to a solution of Z–Gly–Pro–OH (2.81 g, 9 mmol) and Et_3N (1.39 ml, 10 mmol) in DMF (15 ml) at −10 °C and the solution was stirred for 30 min. This solution was combined with the deblocked-pentapeptide solution and the whole was stirred for 36 h in a cold room. The solvent was removed *in vacuo* and the residue was washed with H_2O and chilled MeOH. Recrystallized from a mixture of MeOH and $CHCl_3$. Yield 900 mg (80%), mp 242—246 °C, Rf^3 0.77, $[\alpha]_D^{23}$ −36.5° ($c=1.0$, DMSO). *Anal.* Calcd for $C_{56}H_{77}N_{15}O_{16}$: C, 55.30; H, 6.39; N, 17.28. Found: C, 55.59; H, 6.40; N, 16.90.

H–Gly–Pro–Arg–Val–Val–Glu–Arg–OH (IV) Deblocking by Hydrogenation: The protected heptapeptide (200 mg, 0.16 mmol) was hydrogenated with Pd catalyst in 80% AcOH (20 ml) for 5 h and the solvent was diluted to 50% AcOH. The hydrogenation was continued further 6 h and the solvent was evaporated off. The product was purified by CM-cellulose column chromatography using 0.01—0.05 M $AcONH_4$ as an eluent. Yield 73 mg (51%), hygroscopic powder, Rf^2 0.09, $[\alpha]_D^{23}$ −90.4 ($c=0.4$, H_2O). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.08, Arg 1.87, Val 1.93, Glu 1.04 (average recovery 85%).

Deblocking by HF Treatment: The protected heptapeptide (206 mg, 0.17 mmol) was dissolved in 5% anisole/HF (20 ml) and the solution was stirred for 1 h at 0°C. The HF was removed and the residue was dissolved in H_2O, followed by washing with AcOEt. The aqueous layer was treated with Amberlite IRA-45 (acetate) and lyophilized. The material was purified by CM-cellulose column chromatography using 0.01—0.05 M $AcONH_4$ as an eluent. Yield 118 mg (75%), hygroscopic powder, Rf^2 0.09, $[\alpha]_D^{20}$ −94.0° ($c=0.5$, H_2O). *Anal.* Calcd for $C_{34}H_{61}N_{13}O_{10}\cdot AcOH\cdot 3H_2O$: C, 46.68; H, 7.74; N, 19.66. Found: C, 46.97; H, 7.69; N, 19.66. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.13, Arg 1.87, Val 1.92, Glu 1.02 (average recovery 86%).

H–Gly–Pro–Arg–Val–Val–Glu–Arg–His–Gln–Ser–NH_2 (V) Prepared by the solid-phase method. Final deprotection was performed by HF treatment and by TFMSA treatment.

Deblocking by HF Treatment: The protected decapeptide resin (1 g) was suspended in a mixture of DMF (10 ml) and thiophenol (0.4 ml) and the mixture was stirred for 30 min to remove the DNP group on His. The resin was collected by filtration, washed with DMF and dried. It was suspended in 5% anisole/HF (20 ml) and the mixture was stirred at 0 °C for 1 h. The HF was removed *in vacuo* and the resin was dried. The product was extracted with 5% AcOH and the aqueous solution was washed with ether, followed by Amberlite IRA-400 (acetate) treatment. After lyophilization, the material was purified by RP-HPLC on YMC Pack AQ-5 column using 0.1% TFA-containing H_2O/CH_3CN. The purified material was lyophilized from HCl-containing H_2O. Yield 31 mg, hygroscopic powder, Rf^2 0.08, $[\alpha]_D^{26}$ −85.9° ($c=0.5$, H_2O). FAB-MS *m/z*: 1164 ($M^+ + 1$). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 1.03, Arg 2.11, Val 1.87, Glu 2.15, His 1.00, Ser 0.90 (average recovery 80%).

Deblocking by TFMSA Treatment: The protected decapeptide resin (1 g) was treated with thiophenol in DMF to remove the DNP group on His as described above. The dried resin was suspended in a mixture of TFA (15 ml), thioanisole (1 ml, 8.5 mmol) and ethanedithiol (0.5 ml, 6 mmol), and then TFMSA (1.4 ml, 15.8 mmol) was added at 0 °C. The mixture was stirred at 0 °C for 30 min and at room temperature for 1.5 h. Chilled ether was added and the resulting precipitate was collected by centrifugation, followed by washing with ether. The deblocked peptide was extracted by 5% AcOH and the extract was treated with Amberlite IRA-400 (acetate). The final purification was performed by RP-HPLC on YMC Pack AQ-5 column using 0.1% TFA-containing H_2O/CH_3CN as an eluent. The purified material was lyophilized from HCl-containing H_2O. Yield 8.5 mg, hygroscopic powder, Rf^2 0.08, $[\alpha]_D^{25}$ −87.8° ($c=0.5$, H_2O). Identified with above sample by RP-HPLC. Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 0.93, Arg 2.13, Val 1.87, Glu 2.17, His 0.90, Ser 0.89 (average recovery 73%).

H–Gly–Pro–Arg–Pro–Pro–Glu–Arg–His–Gln–Ser–NH_2 (VI) Prepared by the solid-phase method and the protected peptide resin (1 g) was deblocked by TFMSA treatment in the same manner described above. The final purification was performed by RP-HPLC on YMC Pack AQ-5 column using 0.1% TFA-containing H_2O/CH_3CN as an eluent. The purified material was lyophilized from HCl-containing H_2O. Yield 48 mg, hygroscopic powder, Rf^2 0.08, $[\alpha]_D^{22}$ −127.3° ($c=1.0$, H_2O). FAB-MS *m/z*: 1160 ($M^+ + 1$). Amino acid ratios in an acid hydrolysate: Gly 1.00, Pro 3.10, Arg 2.10, Glu 2.17, His 0.92, Ser 0.79 (average recovery 92%).

H–Pro–Pro–Glu–Arg–His–Gln–Ser–HN_2 (VII) Prepared by the solid-phase method. The protected heptapeptide resin (1.0 g) was treated with thiophenol to remove the DNP group and was treated with TFMSA as described above. The deblocked material was treated with Amberlite IRA-400 (acetate) and purified by CM-cellulose column chromatography using 0.01—0.05 M $AcONH_4$. The purified material was lyophilized from HCl-containing H_2O. Yield 26 mg, hygroscopic powder, Rf^2 0.10, $[\alpha]_D^{23}$ −90.1° ($c=0.3$, H_2O). FAB-MS *m/z*: 850 ($M^+ + 1$). Amino acid ratios in an acid hydrolysate: Pro 2.23, Arg 1.19, Glu 2.13, His 1.00, Ser 0.86 (average recovery 86%).

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References and Notes

1) Standard abbreviations for amino acids, peptides and protecting groups are used [*Eur. J. Biochem.*, **138**, 9 (1984)]. Other abbreviations include: DMF = dimethylformamide, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, DPPA = diphenylphosphoryl azide, NMM = *N*-methylmorpholine, DCM = dichloromethane, HOBt = *N*-hydroxybenzotriazole.
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