Amino Acids and Peptides. LIII. Synthesis and Biological Activities of Some Pseudo-Peptide Analogs of PKSI-527, a Plasma Kallikrein Selective Inhibitor: The Importance of the Peptide Backbone[1,2)]

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Pseudo-peptide analogs of *trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanyl-4-aminophenyl acetic acid (PKSI-527, plasma kallikrein selective inhibitor), in which an amide bond (peptide bond) has been replaced by a CH_2–NH bond, *i.e. trans*-4-aminomethylcyclohexanecarbonyl-L-phenylalanyl-ψ(CH_2–NH)-4-aminophenyl acetic acid (I), *trans*-4-aminomethylcyclohexanecarbonyl-ψ(CH_2–NH)-L-phenylalanyl-4-aminophenyl acetic acid (II) and *trans*-4-aminomethylcyclohexanecarbonyl-D-phenylalanyl-ψ(CH_2–NH)-4-aminophenyl acetic acid (III) were synthesized. These pseudo-peptide analogs did not exhibit any detectable inhibitory activity against plasma kallikrein (PK), plasmin (PL), urokinase (UK), thrombine (TH) or trypsin (TRY). These results indicate that both carbonyl groups in the PKSI-527 are important for the manifestation of potent inhibitory activity against plasma kallikrein.

Key words plasma kallikrein inhibitor; pseudo-peptide bond; ψ(CH_2–NH); synthesis; biological activity

Plasma prekallikrein circulates in the blood as the zymogen of plasma kallikrein, and is activated by factor XIIa to form plasma kallikrein.[3)] Plasma kallikrein releases bradykinin from high molecular weight kininogen.[4)] Furthermore, it has been reported that plasma kallikrein activates factor XII,[5)] prourokinase[6)] and plasminogen.[7)] Plasma kallikrein is also known to cause neutrophile aggregation[8)] and elastase release.[9)] These observations suggest that plasma kallikrein has many functions, although its precise role remains to be determined. In order to study the precise role of plasma kallikrein and the relationship between plasma kallikrein and certain diseases, the development of selective plasma kallikrein inhibitors is required. The design of new drugs which will specifically inhibit plasma kallikrein is also a challenge for the future.

Previously, we succeeded in finding a novel highly selective plasma kallikrein inhibitor, *N*-(*trans*-4-aminomethylcyclohexanecarbonyl)-phenylalanyl-4-aminophenyl acetic acid, Tra-Phe-APAA (PKSI-527) (Fig. 1) with the following K_i values: 0.81 for plasma kallikrein, >500 for glandular kallikrein, 390 for plasmin, 200 for urokinase, >500 for factor Xa and >500 μM for thrombin.[10)] PKSI-527 is a phenylalanine derivative with a simple structure. Tra is located at the N-terminal of Phe and 4-aminophenyl acetic acid at the C-terminal position. The role of each moiety of PKSI-527 was analyzed for inhibitory activity.[11,12)] In this article, we present the synthesis and biological activities of pseudo-peptide analogs of PKSI-527 in which an amide bond was replaced by a CH_2–NH bond, an isosteric modification that was used in a series of renin inhibitors.[13)]

The pseudo-peptide, H-Tra-Phe-ψ(CH_2–NH)-APAA (I) was synthesized according to Chart 1. Boc-Phe-OH reacted with the hydrochloride salt of *N*,*O*-dimethylhydroxylamine in the presence of BOP reagent to yield Boc-Phe-N(OCH_3)CH_3. This compound was converted into the corresponding aldehyde in the presence of $LiAlH_4$,[14)] which reacted with 4-aminophenyl acetic acid benzyl ester in a methanol–acetic acid mixture with $NaBH_3CN$[15)] to yield Boc-Phe-ψ(CH_2–NH)-APAA-OBzl. After removal of the Boc group, Boc-Tra-OH was coupled with the corresponding amine to give Boc-Tra-Phe-ψ(CH_2–NH)-APAA-OBzl. After removal of the Boc group, the corresponding amine was hydrogenated over a Pd catalyst to give compound (I).

H-Tra-ψ(CH_2–NH)-Phe-APAA (II) was prepared according to Chart 2. Boc-Tra-OH was converted to the corresponding aldehyde by the same method described above. Boc-Tra-H reacted with H-Phe-APAA-OBzl[16)] using $NaBH_3CN$ to yield Boc-Tra-ψ(CH_2–NH)-Phe-APAA-OBzl. After removal of the Boc group, the Bzl group was removed by hydrogenation over a Pd catalyst to give H-Tra-ψ(CH_2–NH)-Phe-APAA (II). Compounds I and II exhibited a single peak on analytical HPLC.

H-Tra-D-Phe-ψ(CH_2–NH)-APAA (III) was also prepared by the same route shown in Chart 1 in order to compare compound III with I.

As summarized in Table 1, compounds I, II and III did not exhibit potent inhibitory activities against various enzymes.

These results indicated that both amide bonds, between Phe and 4-aminophenyl acetic acid, and Tra and Phe in PKSI-527, are important for manifestation of potent inhibitory activity against plasma kallikrein.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Mass spectra were measured with a JEOL SX-102 mass spectrometer using the FAB tech-

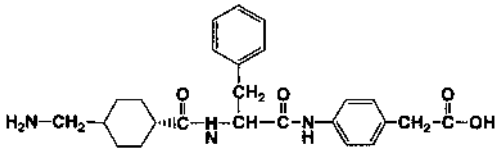

Fig. 1. Structure of Plasma Kallikrein Selective Inhibitor PKSI-527

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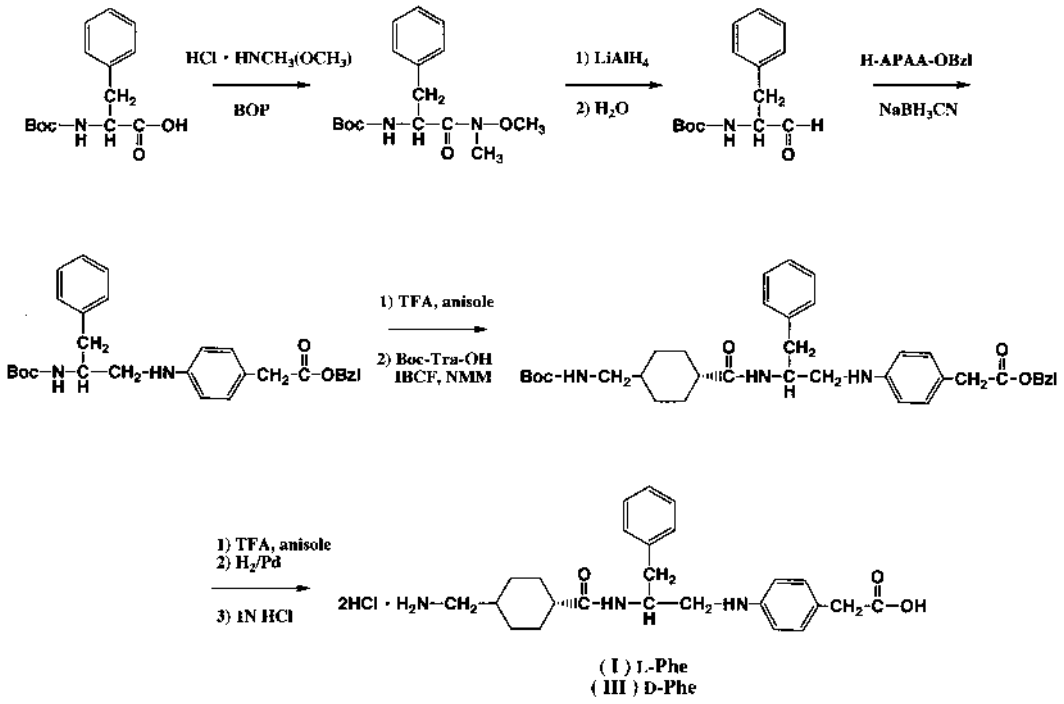

Chart 1. Synthetic Route to Tra-Phe-$\psi(CH_2NH)$-APAA (I and III)

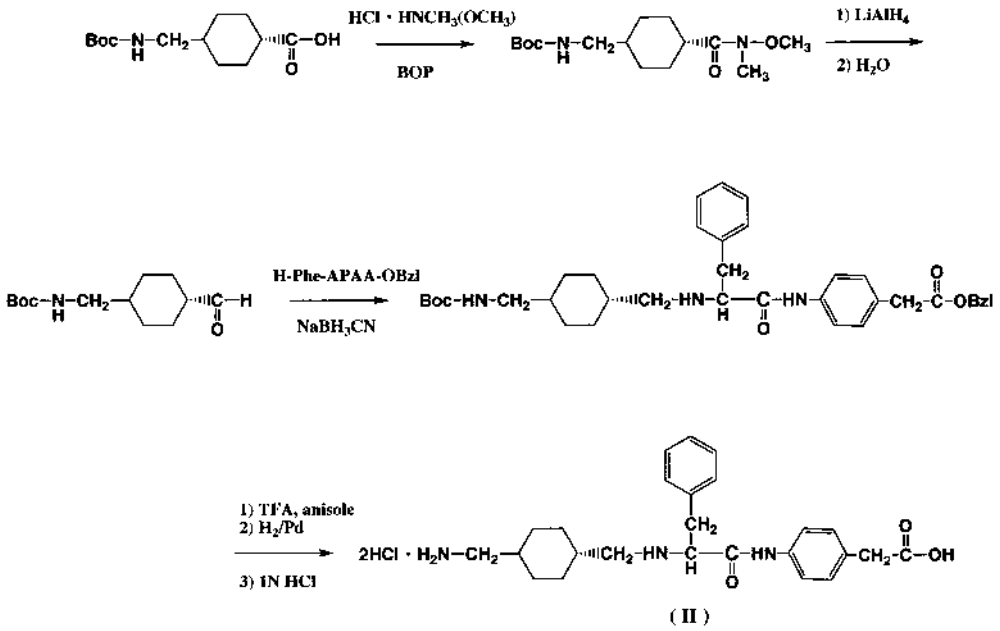

Chart 2. Synthetic Route to Tra-$\psi(CH_2NH)$-Phe-APAA (II)

nique. Waters model 600E was used for HPLC analysis. The solvents were as follows: A, 0.05% TFA in water; B, 0.05% TFA in CH_3CN. The retention time was reported as t_R. On TLC (Kieselgel G 60, Merck), Rf^{-1}, Rf^{-2} and Rf^{3} values refer to the systems of AcOEt and hexane (1 : 1), AcOEt and hexane (1 : 2) and *n*-BuOH, AcOH and water (4 : 1 : 5, upper phase), respectively.

Boc-D-Phe-N(OCH$_3$)CH$_3$ Triethylamine (1.40 ml, 10.0 mmol) was added to a stirred solution of a Boc-D-Phe-OH (2.65 g, 10.0 mmol) in dichloromethane. Then, BOP (3.48 g, 11.0 mmol) was added, followed after a few minutes by *N*,*O*-dimethylhydroxylamine hydrochloride (1.11 g, 11.0 mmol) and triethylamine (1.54 ml, 11.0 mmol). The reaction mixture was stirred at 0 °C for 30 min. The mixture was extracted with dichloromethane (120 ml). The extract was washed with 3 N HCl, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography using $CHCl_3$. Removal of the solvent gave the desired compound as an oil: yield 2.65 g (85.9 %), $[\alpha]_D^{25}$ −3.97° (*c*=1.0, MeOH), Rf^{-1} 0.65.

Boc-D-Phe-H Lithium aluminum hydride (377 mg, 9.93 mmol) was added to a stirred solution of Boc-D-Phe-N(OCH$_3$)CH$_3$ (2.45 g, 7.94 mmol) in THF (60 ml). The reaction mixture was stirred at 0 °C for 1 h. The mixture was hydrolyzed with a solution of $KHSO_4$ (1.85 g, 13.8 mmol) in water (40 ml). The reaction mixture was stirred at 0 °C for 30 min. Then, ether (100 ml) was added; the aqueous phase was separated and extracted with ether. The combined organic layer was washed with 3 N HCl, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. Hexane was added to the residue to give crystals, which were collected by filtration: yield 1.39 g (70.2%), mp 69—71 °C, $[\alpha]_D^{25}$ −37.9° (*c*=1.0, CH_2Cl_2) [Ref. 14: Boc-L-Phe-H, mp 86 °C, $[\alpha]_D^{25}$ +40.4° (*c*=1.0, CH_2Cl_2)], Rf^{-1} 0.81. *Anal*. Calcd for $C_{14}H_{19}NO_3 \cdot 0.2H_2O$: C, 66.5; H, 7.68; N, 5.54. Found: C, 66.7; H, 7.64; N, 5.67.

Table 1. Inhibitory Activity of Compounds (I, II and III) against Various Enzymes[a)]

Compound (Peptide)	Inhibition (%)						
	PK	PL		UK	TH		TRY
	S-2302	S-2251	Fibrin	S-2444	S-2238	Fibrinogen	S-2238
H-Tra-L-Phe-APAA[b)] (PKSI-527)	0.81	390	—	200	500	—	—
H-Tra-L-Phe-ψ(CH_2–NH)-APAA (I)	7% (1000)	5% (1000)	24% (1000)	0% (1000)	0% (1000)	0% (1000)	0% (300)
H-Tra-ψ(CH_2–NH)-L-Phe-APAA (II)	20% (1000)	0% (1000)	26% (1000)	39% (1000)	0% (1000)	0% (1000)	0% (300)
H-Tra-D-Phe-ψ(CH_2–NH)-APAA (III)	5% (1000)	0% (1000)	23% (1000)	0% (1000)	0% (1000)	0% (1000)	0% (300)

a) Inhibition % at the concentration (μM) described in parentheses. *b*) K_i value (μM). PK, plasma kallikrein; PL, plasmin; UK, urokinase; TH, thrombine; TRY, trypsin.

Boc-Tra-N(OCH_3)CH_3 Triethylamine (1.40 ml, 10.0 mmol) was added to a stirred solution of Boc-Tra-OH (2.57 g, 10.0 mmol) in dichloromethane. Then, BOP (3.48 g, 11.0 mmol) was added, followed after a few minutes by *O*,*N*-dimethylhydroxylamine hydrochloride (1.11 g, 11.0 mmol) and triethylamine (1.54 ml, 11.0 mmol). The reaction mixture was stirred at 0 °C for 30 min. The mixture was extracted with dichloromethane (120 ml). The extract was washed with 3 N HCl, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. Petroleum ether was added to the residue to give crystals, which were collected by filtration: yield 2.65 g (88.2%), mp 58—60 °C, Rf^1 0.55. *Anal*. Calcd for $C_{15}H_{28}N_2O_4$: C, 60.0; H, 9.33; N, 9.33. Found: C, 59.7; H, 9.33; N, 9.42.

Boc-Tra-H Lithium aluminum hydride (237 mg, 6.25 mmol) was added to a stirred solution of a Boc-Tra-N(OCH_3)CH_3 (1.50 g, 5.00 mmol) in THF (40 ml). The reaction mixture was stirred at 0 °C for 40 min. The mixture was hydrolyzed with a solution of $KHSO_4$ (1.19 g, 8.75 mmol) in water (30 ml). The reaction mixture was stirred at 0 °C for 30 min. Ether (70 ml) was added; the aqueous phase was separated and extracted with ether. The combined organic layer was washed with 3 N HCl, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. Hexane was added to the residue to give crystals, which were collected by filtration: yield 838 mg (69.4%), mp 43—45 °C, Rf^1 0.71. *Anal*. Calcd for $C_{13}H_{23}NO_3$: C, 64.7; H, 9.54; N, 5.81. Found: C, 64.5; H, 9.51; N, 5.81.

Boc-L-Phe-ψ(CH_2–NH)-APAA-OBzl Boc-L-Phe-H (402 mg, 1.61 mmol) was dissolved in a mixture of MeOH–AcOH (99 : 1, 30 ml) containing H-APAA-OBzl·Tos (512 mg, 1.24 mmol). $NaBH_3CN$ (101 mg, 1.61 mmol) was added portionwise for 45 min. The reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was cooled in an ice water bath, and a saturated sodium bicarbonate solution (30 ml) was added with stirring, followed by AcOEt (70 ml). The organic layer was collected, washed with 3 N HCl and water, dried over Na_2SO_4 and evaporated. Ether was added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH: yield 353 mg (60.0%), mp 117—119 °C, $[\alpha]_D^{25}$ +4.6° (c=1.0, $CHCl_3$), Rf^2 0.67. *Anal*. Calcd for $C_{29}H_{34}N_2O_4$: C, 73.4; H, 7.17; N, 5.91. Found: C, 73.1; H, 7.31; N, 5.92.

Boc-D-Phe-ψ(CH_2–NH)-APAA-OBzl The title compound was prepared from Boc-D-Phe-H (1.20 g, 4.81 mmol) and H-APAA-OBzl·TosOH (1.53 g, 3.70 mmol): yield 859 mg (46.2%), mp 119—120 °C, $[\alpha]_D^{25}$ −4.5° (c=1.0, $CHCl_3$), Rf^2 0.67. *Anal.* Calcd for $C_{29}H_{34}N_2O_4$: C, 73.4; H, 7.17; N, 5.91. Found: C, 73.2; H, 7.27; N, 5.91.

Boc-Tra-L-Phe-ψ(CH_2–NH)-APAA-OBzl A mixed anhydride [prepared from Boc-Tra-OH (540 mg, 2.10 mmol), isobutyl chloroformate (0.275 ml, 2.10 mmol) and NMM (0.277 ml, 2.52 mmol) as usual] in THF (40 ml) was added to an ice-cold solution of H-L-Phe-ψ(CH_2–NH)-APAA-OBzl·TFA [prepared from the corresponding Boc-L-Phe-ψ(CH_2–NH)-APAA-OBzl (997 mg, 2.10 mmol), TFA (4.82 ml, 63.0 mmol) and anisole (0.45 ml, 4.20 mmol)] in THF (40 ml) containing NMM (0.23 ml, 2.10 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$, and water, dried over Na_2SO_4 and evaporated. Ether was added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH: yield 504 mg (39.1%), mp 141—143 °C, $[\alpha]_D^{25}$ −1.7° (c=1.0, $CHCl_3$), Rf^1 0.59. *Anal*. Calcd for $C_{37}H_{47}N_3O_5 \cdot 0.25H_2O$: C, 71.9; H, 7.74; N, 6.82. Found: C, 72.0; H, 7.55; N, 6.62.

Boc-Tra-D-Phe-ψ(CH_2–NH)-APAA-OBzl The title compound was prepared from a mixed anhydride [prepared from Boc-Tra-OH (407 mg, 1.58 mmol), isobutyl chloroformate (0.207 ml, 1.58 mmol) and NMM (0.208 ml, 1.90 mmol) as usual] and H-D-Phe-ψ(CH_2–NH)-APAA-OBzl·TFA [prepared from the corresponding Boc-D-Phe-ψ(CH_2–NH)-APAA-OBzl (750 mg, 1.58 mmol), TFA (3.63 ml, 47.4 mmol) and anisole (0.342 ml, 3.16 mmol)]: yield 315 mg (32.5%), mp 141—143 °C, $[\alpha]_D^{25}$ +1.6° (c=1.0, $CHCl_3$), Rf^1 0.59. *Anal*. Calcd for $C_{37}H_{47}N_3O_5$: C, 72.4; H, 7.72; N, 6.85. Found: C, 72.3; H, 7.74; N, 6.91.

Boc-Tra-ψ(CH_2–NH)-Phe-APAA-OBzl Boc-Tra-H (483 mg, 2.00 mmol) was dissolved in a mixture of MeOH–AcOH (99 : 1, 30 ml) containing the H-Phe-APAA-OBzl·TFA [prepared from the corresponding Boc-Phe-APAA-OBzl[16)] (733 mg, 1.50 mmol), TFA (3.45 ml, 45.0 mmol) and anisole (0.33 ml, 3.00 mmol)]. $NaBH_3CN$ (126 mg, 2.00 mmol) was added to the above solution portionwise during 45 min. The reaction mixture was stirred at 0 °C for 1 h and then cooled in an ice-water bath. A saturated sodium bicarbonate solution (50 ml) was added to the above solution with stirring, followed by AcOEt (70 ml). The organic layer was collected, washed with 3 N HCl and water, dried over Na_2SO_4 and evaporated. Ether was added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH: yield 219 mg (23.8%), mp 95—97 °C, $[\alpha]_D^{25}$ −2.6° (c=1.0, MeOH), Rf^1 0.43. *Anal*. Calcd for $C_{37}H_{47}N_3O_5 \cdot H_2O$: C, 70.3; H, 7.82; N, 6.65. Found: C, 70.4; H, 7.76; N, 6.70.

H-Tra-L-Phe-ψ(CH_2–NH)-APAA-OH (I) Boc-Tra-L-Phe-ψ(CH_2–NH)-APAA-OBzl (200 mg, 0.326 mmol) was dissolved in TFA (0.75 ml, 9.78 mmol) containing anisole (70.5 μl, 0.652 mmol). The reaction mixture was stored at 0 °C for 5 min and at room temperature for 2 h. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. The resulting TFA salt was dissolved in MeOH (30 ml) and hydrogenated in the presence of a Pd catalyst. After the removal of Pd and solvent, the resulting product was dissolved in water (10 ml) containing 1 N HCl (0.65 ml), which was lyophilized to give an amorphous powder: yield 126 mg (84.0%), $[\alpha]_D^{25}$ −13.9° (c=0.2, H_2O), Rf^3 0.38, t_R 22.78 (min). FAB-MS *m*/*z*: 424 $(M+H)^+$. HPLC conditions: column, COSMOSIL C18 (4.6×250 mm); solvents, A : B (90 : 10) for 5 min, to A : B (10 : 90) in 40 min; flow rate, 1 ml/min; detection, 220 nm.

H-Tra-D-Phe-ψ(CH_2–NH)-APAA-OH (III) The title compound was prepared from Boc-Tra-D-Phe-ψ(CH_2–NH)-APAA-OBzl (200 mg, 0.326 mmol): yield 115 mg (76.7%), $[\alpha]_D^{25}$ +13.5° (c=0.2, H_2O), Rf^3 0.38, t_R 22.78 (min). FAB-MS *m*/*z*: 424 $(M+H)^+$. HPLC conditions: column, COSMOSIL C18 (4.6×250 mm); solvents, A : B (90 : 10) for 5 min, to A : B (10 : 90) in 40 min; flow rate, 1 ml/min; detection, 220 nm.

H-Tra-ψ(CH_2–NH)-Phe-APAA-OH (II) The title compound was prepared from Boc-Tra-ψ(CH_2–NH)-Phe-APAA-OBzl (100 mg, 0.163 mmol) by the same procedure described above: yield 78.5 mg (96.7%), $[\alpha]_D^{25}$ +74.3° (c=0.2, H_2O), Rf^3 0.21, t_R 25.34 (min). FAB-MS *m*/*z*: 424 $(M+H)^+$. HPLC conditions: column, COSMOSIL C18 (4.6×250 mm); solvents, A : B (90 : 10) for 5 min, to A : B (10 : 90) in 40 min; flow rate, 1 ml/min; detection, 220 nm.

Assay Procedure The enzymes used were as follows: human plasmin and plasma kallikrein (KABI Co.), bovine thrombin (Mochida Seiyaku Co.), porcine glandular kallikrein (Sigma Chemical Co.), human urokinase (Green Cross) and trypsin (Sigma Chemical Co.). Enzymatic activities of plasmin, plasma kallikrein, thrombin, glandular kallikrein, urokinase and trypsin were

determined by the method described previously,[17] using D-Val-Leu-Lys-*p*NA (S-2251), D-Pro-Phe-Arg-*p*NA (S-2302), D-Phe-Pip-Arg-*p*NA (S-2238), D-Val-Leu-Arg-*p*NA (S-2266), <Glu-Gly-Arg-*p*NA (S-2444) and D-Phe-Pip-Arg-*p*NA (S-2238), respectively. Fibrin and fibrinogen were used as substrates for plasmin and thrombin, respectively. IC_{50} values were determined as follows: 1) Antiamidolytic assay:[18] the IC_{50} value was taken as the concentration of inhibitor which decreased the absorbancy at 405 nm by 50% compared with the absorbancy measured under the same conditions without the inhibitor. 2) Antifibrinolytic assay:[18] the IC_{50} value was taken as the concentration of inhibitor which prolonged the complete lysis time two-fold in comparison to that without the inhibitor. 3) Antifibrinogenolytic assay: to a borate saline buffer (pH 7.4), a solution of various concentrations of an inhibitor to be tested (0.5 ml), and 0.2% bovine fibrinogen in the above buffer (0.4 ml), bovine thrombin 4 U/ml (0.1 ml) was added. The assay was carried out at 37 °C and the clotting time was measured. The IC_{50} value was taken as the concentration of inhibitor which prolonged the clotting time two-fold in comparison to that without the inhibitor.

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