Structure–Inhibitory Activity Relationship of Plasmin and Plasma Kallikrein Inhibitors[1)]

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Based on the structure of Tra-Tyr(*O*-Pic)-octylamide, a portion of the octylamine was replaced with moieties bearing hydrophobic, basic or acidic groups. Replacement of the C-terminal residue with a moiety bearing a hydrophobic group gave the proper affinity of the inhibitor to both plasmin (PL) and plasma kallikrein (PK). While addition of a basic residue did not improve the affinity of the inhibitor, a carboxylic acid attached to the phenyl ring increased the PK selectivity of the inhibitor.

Key words enzyme-selective inhibitor; plasma kallikrein inhibitor; plasmin inhibitor; structure–inhibitory activity relationship

Plasmin (PL) (EC 3.4.21.7) is a serine protease, which plays a central role in the fibrinolytic process. Fibrinolysis is a physiological response to prevent excessive Fn accumulation in the circulating blood.[2)] The fibrinolytic system is a highly modulated enzyme system, in which a series of serine proteases are involved. The generation of PL is strongly regulated by two activators and three protease inhibitors. The two major activators, the tissue-type PA (t-PA)[3)] and the urinary-type PA (u-PA, urokinase),[4)] are serine proteases which cleave a peptide bond in plasminogen, the zymogen form, to generate PL on the biological surface. Two protease inhibitors include the PA inhibitors, which are called PAI,[5)] and the third is a PL inhibitor, namely α_2-antiplasmin.[6)] Additionally, the contribution of the contact system to the process of plasminogen activation should be considered. Plasma kallikrein (PK) (EC 3.4.21.34), which is one component of the contact system,[7)] probably mediates the activation of prourokinase and accelerates the PL formation,[8)] while PK was reported to activate the contact phase of coagulation.[9)]

Although the primary function of PL is to remove intravascularly formed thrombin by the degradation of Fn, PL has many other actions, such as the degradation of adhesive macromolecules,[10—13)] the activation of growth factors,[14,15)] coagulation factor modification,[16,17)] the activation of t-PA[18)] and u-PA,[19)] and so on. As a result, PL might function in pathologic phenomena, such as inflammation[20)] and tumor cells growth and metastasis.[21)] PK also has a broad activity spectrum. PK might be involved in the induction of several pathologic phases including allergic rhinitis, asthma, arthritis and so on, due to kinin generation.[9)] However, detailed studies of the role of these enzymes remain to be performed.

The regulation of PL and PK has been our interest in the regards of control of fibrinolytic actions as well as non-fibrinolytic actions. We have focused our attention on the synthesis of active center-directed PL and PK inhibitors. The first aim of our study was to obtain a powerful new tool to explore the role of PL and PK in coagulation–fibrinolytic system. An enzyme-selective inhibitor would be useful to discriminate the functions of PL and PK in the fibrinolytic system. The second aim was to develop novel type of PL or PK inhibitor available for clinical therapy. When massive activation of the fibrinolytic system occurs, as in thrombolytic therapy with streptokinase, t-PA or u-PA, ε-aminocaproic acid (EACA)[22)] and *trans*-4-aminomethylcyclohexanecarboxylic acid (*t*-AMCHA),[23)] which are employed clinically as a PL inhibitor, do not suffice to inhibit circulating PL, because they inhibit PL by blocking the lysine binding sites (LBS) but not the catalytic site. The active center-directed PL inhibitors might be beneficial in controlling excessive bleeding that frequently occurs in thrombolytic therapy and organ transplantation.

Among the active center-directed inhibitors we developed, Tra-Tyr(*O*-2-bromobenzyloxycarbonyl)-octylamide (compound I)[24)] specifically inhibited PL (IC_{50}=0.80 μM) and Tra-Phe-4-carboxymethylanilide (compound II)[25)] specifically inhibited PK (IC_{50}=1.3 μM) (Fig. 1). In order to improve solubility, a picolyl (*O*-Pic) residue was incorporated into the molecule as Tyr derivative to yield Tra-Tyr(*O*-Pic)-octylamide (compound III),[26)] which inhibited PL with an IC_{50} value of 0.53 μM. Thus, this replacement did not reduce the inhibitory effect of peptide and increased the solubility of the peptide. From these results, it was deduced that we could design enzyme-selective inhibitors by combination of the Tra-Tyr(*O*-Pic) sequence with various C-terminal residues. In this study, we present data on low molecular weight inhibitors based on the structure of compound III. The functional and structural roles of the C-terminal residue were investigated by replacement with moieties bearing hydrophobic, basic and acidic groups.

First of all, replacement of octylamine with aniline containing various methylene moieties provided peptides **1**—**6**. The IC_{50} values of those peptides are summarized with comparison with those of compound III in Table 1. In both PL and PK, systematic extension of the length by a methylene group (peptide **1**—**5**) increased the inhibitory activity. Peptide **5** (five methylene group) had an IC_{50} value of 0.58 and 0.88 μM, which were a 2.9-fold and a 1.9-fold increase in affinity, respectively, compared with those of peptide **1** (with one methylene group). It seems that the hydrophobic group enhanced the interaction between peptide and enzyme. However, an insertion of phenyl ring reduced the selectivity of PL/PK; peptide **5** inhibited PK as well as PL, although compound III inhibited PL a 60-fold more strongly than PK.

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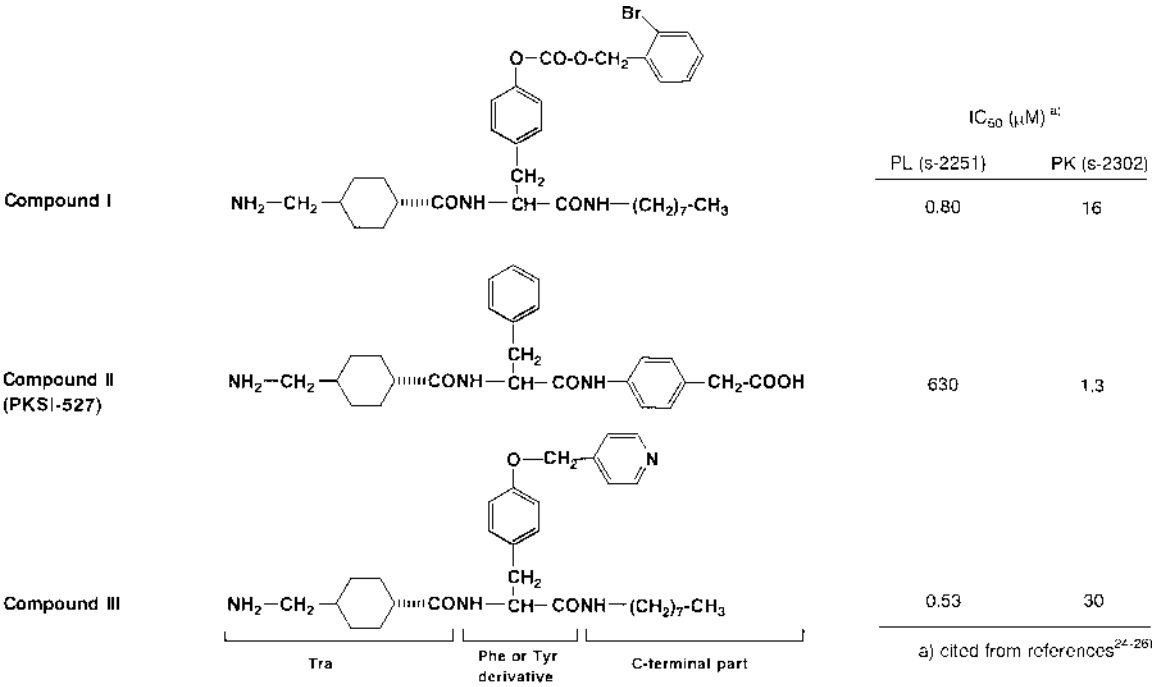

Fig. 1. Structure and Inhibitory Activity of Compounds I—III

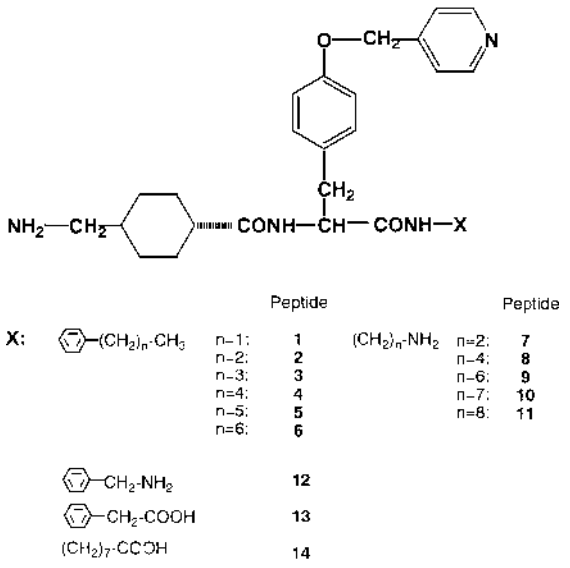

Fig. 2. Structure of Peptides **1**—**14**

Next, the addition of basic group to C-terminal part was examined. According to the previous structure–activity relationship (SAR) study, the presence of carboxyl group in C-terminal part is not favorable for the inhibition of PL. The introduction of a carboxyl group as in compound II contributed to an increase of PK/PL selectivity. Instead of carboxyl group, a series of peptides incorporated a basic group at the C-terminus. The resulting peptides **7**—**12** and their IC_{50} values are summarized in Table 2. Peptide **10** inhibited PL with an IC_{50} value of 3.8 μM; this affinity is one-seventh of that of compound III. Furthermore, addition of basic group also reduced the affinity of the peptide with PK, resulting in an IC_{50} value of 330 μM (peptide **10**), a loss of more than 10-fold relative to compound III. Thus, incorporation of a basic group showed no improvement in affinity; however, the PL/PK selectivity ratio was increased rather than being retained; the selectivity of PL/PK (reciprocal of PL-to-PK IC_{50} ratio) of peptide **10** was 86, while that of compound III was 56.

Third, the effect of an acidic group was investigated. Peptide **13** and **14** were prepared and evaluated as shown in Table 2. Among peptides **1**, **12** and **13**, peptide **13** was the most potent PK inhibitor with an IC_{50} value of 1.3 μM, implying that incorporation of acidic group gave PK selectivity to inhibitor. In contrast, peptide **14** lost affinity for PK (IC_{50}=32 μM). Those findings indicated that the active-center of PL would have a rather wide hydrophobic region, while the active-center of PK would be a region consisting of both aromatic and basic side chains. The property of the C-terminal residue could regulate the enzyme selectivity ratio. On the other hand, the amino group of the Tra moiety in peptides **1**—**14** would be critical to bind the peptide to the enzyme. In fact, the study on X-ray crystal structures of trypsin–PKSI-527 (compound II) complex revealed that the amino group of the aminomethylcyclohexane moiety was hydrogen-bound to the carboxyl oxygens of Asp189.[27] We can expect that interactions between these enzymes and the amino group of Tra in peptides **1**—**14** are similar to that between trypsin and the amino group of Tra in PKSI-527, because the amino acid sequences of enzymes in coagulation–fibrinolysis system are highly homologous to that of trypsin. Indeed, peptides **1**—**14** had an inhibitory effect not only on fibrinolysis, but also on amidolysis by PL with micromole range (Tables 1, 2). *t*-AMCHA inhibited fibrinolytic and amidolytic actitivity of PL with IC_{50} of 60 μM and K*i* of 40 mM, respectively.[28] The inhibitory effect of peptides **1**—**14** on amidolysis was approximately 5000 times greater than that of *t*-AMCHA.

In conclusion, replacement of the C-terminal residue with moiety bearing an aromatic or hydrophobic group increased affinity of the inhibitor to both PL and PK. Incorporation of an acidic group was not favorable for inhibition of PL, but this modification yielded a PK specific inhibitor. In particu-

Table 1. The IC_{50} Values (μM) of Tra-Tyr(*O*-Pic)-NH-  -$(CH_2)_n$-CH_3 (**1**—**6**) for Various Enzymes

Peptide	*n*	PL		PK	Urokinase	Thrombin		Trypsin
		S-2251	Fn	S-2302	S-2444	S-2238	Fg	S-2238
1	1	1.7	0.51	1.7	60	480	>100	1.3
2	2	1.4	0.38	1.7	42	300	>100	1.7
3	3	0.99	0.39	1.2	40	130	>50	1.2
4	4	0.54	0.18	1.6	43	>500	>20	0.62
5	5	0.58	0.23	0.88	>200	>200	>25	0.85
6	6	0.97	0.35	0.82	>200	>200	>12.5	0.65
Compound III	$-NH(CH_2)_7-CH_3$	0.53	0.36	30	5.3	>400	>100	1.1

Table 2. The IC_{50} Values (μM) of Tra-Tyr(*O*-Pic)-NH-X (**7**—**14**) for Various Enzymes

Peptide	*X*	PL		PK	Urokinase	Thrombin		Trypsin
		S-2251	Fn	S-2302	S-2444	S-2238	Fg	S-2238
7	$-(CH_2)_2-NH_2$	84	71	>1000	51	>1000	>1000	28
8	$-(CH_2)_4-NH_2$	28	14	910	50	>1000	>1000	35
9	$-(CH_2)_6-NH_2$	5.4	3.8	510	30	>1000	>1000	4.0
10	$-(CH_2)_7-NH_2$[a]	3.8	2.4	330	31	>1000	>1000	2.0
11	$-(CH_2)_8-NH_2$	1.8	1.1	170	18	>1000	>1000	0.7
12	 -CH_2-NH_2	1.6	1.1	28	27	>1000	>1000	1.0
13	 -CH_2-COOH	9.6	7.4	1.3	52	>1000	>1000	3.8
14	$-(CH_2)_7-COOH$[a]	5.5	4.6	32	29	>500	>500	4.2
Compound III	$-(CH_2)_7-CH_3$	0.53	0.36	30	5.3	>400	>100	1.1

a) Cited from ref. 26).

lar, the addition of carboxylic acid to the phenyl ring produced a selective PK inhibitor. Replacement with basic residue did not improve the affinity of inhibitor. Thus, we described data involving the active site of PL and PK by the application of specific modifications in the peptide inhibitors. We can emphasize that there is possibility to develop more potent and selective PL or PK inhibitors by modification of Tyr/Phe part and/or C-terminal part (Fig. 1). More potent PL inhibitors than compound III are currently under study.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus without correction. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Time of flight-mass spectra (TOF-MS) were obtained on a KOMPACT MALDI IV mass spectra (Kratos Analytical). Waters model 600E was used for HPLC analysis and purification. Peptides were purified by reverse phase HPLC on a C18 semi-preparative column (20×250 mm, Nakarai). The column was eluted in 40 min using a linear gradient from 10 to 50% acetonitrile in water, both containing 0.05% TFA, at flow rate of 10 ml/min. The detection wavelength was 220 nm. On TLC (Kieselgel G. Merck) Rf^1, Rf^2, Rf^3, Rf^4, Rf^5, Rf^6 and Rf^7 values refer to the solvent systems consisting of $CHCl_3$–MeOH–AcOH (90 : 8 : 2); $CHCl_3$–MeOH–H_2O (89 : 10 : 1); $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase); *n*-BuOH–AcOH–H_2O (4 : 1 : 5, upper phase); *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2); *n*-BuOH–AcOH–pyridine–H_2O (1 : 1 : 1 : 1); and AcOEt–*n*-hexane (1 : 1), respectively.

General Procedure for Preparation of Boc-Tyr(*O*-Pic)-NH-  -$(CH_2)_n$-CH_3 (*n*=1—6) Boc-Tyr(*O*-Pic)-OH (1.9 g, 5.0 mmol) was added to the corresponding amine component (5.0 mmol) in DMF (30 ml) containing Et_3N (1.5 ml, 11 mmol) and BOP reagent (2.7 g, 6.0 mmol). The reaction mixture was stirred at room temperature for 15 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give a white precipitate, which was collected by filtration and recrystallized from EtOH. Yield, mp, $[\alpha]_D^{25}$, elemental analysis and *Rf* value are summarized in Table 3.

General Procedure for Preparation of Boc-Tra-Tyr(*O*-Pic)-NH-  -$(CH_2)_n$-CH_3 (*n*=1—6) Boc-Tra-OH (0.26 g, 1.0 mmol) was added to the corresponding amine component [prepared from the corresponding Boc-Tyr(*O*-Pic)-NH-$(CH_2)_n$-CH_3 derivative (1.0 mmol) and TFA (1.53 ml, 20 mmol) in the presence of anisole (0.15 ml, 1.4 mmol) as usual] in DMF (30 ml) containing Et_3N (0.34 ml, 2.4 mmol) and BOP reagent (0.53 g, 1.2 mmol). The reaction mixture was stirred at room temperature for 15 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Diethyl ether was added to the residue to give a white precipitate, which was collected by filtration and recrystallized from EtOH. Yield, mp, $[\alpha]_D^{25}$, elemental analysis and *Rf* values are summarized in Table 4.

General Procedure for Preparation of H-Tra-Tyr(*O*-Pic)-NH-  -$(CH_2)_n$-CH_3·TFA (*n*=1—6) Boc-Tra-Tyr(*O*-Pic)-NH-  -$(CH_2)_n$-CH_3 (0.30 mmol) was dissolved in TFA (1.28 ml, 17 mmol) containing anisole (0.04 ml, 0.34 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 60 min. Diethyl ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. Each product was lyophilized from water afford an amorphous powder. Yield, mp, $[\alpha]_D^{25}$, elemental analysis and *Rf* values are summarized in Table 5.

General Procedure for Preparation of Fmoc-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH-Boc (*n*=2, 4, 6, 8) A solution of Boc-NH-$(CH_2)_n$-NH_2 (5.5 mmol)[29] in DMF (50 ml) was added to Fmoc-Tyr(*O*-Pic)-OH (6.0 mmol) in DMF (50 ml) containing BOP (2.6 g, 6.0 mmol), HOBt (0.81 g, 6.0 mmol) and DIPEA (1.0 ml, 6.0 mmol) at 0 °C and the reaction mixture was stirred at the same temperature for 5 min and was further stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Diethyl ether was added to the residue to give a white precipitate, which was collected by filtration and recrystallized from EtOH. Yield, mp, $[\alpha]_D^{25}$, elemental analysis and *Rf* value

Table 3. Yield, Melting Point, Optical Rotation, *Rf* Value and Elemental Analysis of Boc-Tyr(*O*-Pic)-NH-  -$(CH_2)_n$-CH_3

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^7
$n=1$	50.9	165—169	+44.4 ($c=1.0$)	$C_{28}H_{33}N_3O_4$	70.7 (70.8)	6.99 6.96	8.83 8.69)		0.47
=2	84.0	114—116	+39.3 ($c=1.0$)	$C_{29}H_{35}N_3O_4$	71.1 (71.4)	7.21 7.47	8.58 8.07)		0.46
=3	90.0	130—142	+37.7 ($c=1.0$)	$C_{30}H_{37}N_3O_4 \cdot 1.2H_2O$	68.6 (68.5)	7.50 7.02	8.00 7.79)	0.56	
=4	58.0	153—156	+36.6[a] ($c=1.0$)	$C_{31}H_{39}N_3O_4$	69.5 (69.2)	7.71 7.32	7.84 7.63)	0.45	
=5	95.0	162—165	+41.4 ($c=1.0$)	$C_{32}H_{41}N_3O_4$	72.3 (72.4)	7.77 7.89	7.90 7.94)		0.27
=6	33.0	164—167	+39.3[a] ($c=1.0$)	$C_{33}H_{43}N_3O_4 \cdot 0.2H_2O$	72.2 (72.5)	7.90 7.98	7.66 7.61)		0.58

a) DMF.

Table 4. Yield, Melting Point, Optical Rotation, *Rf* Value and Elemental Analysis of Boc-Tra-Tyr(*O*-Pic)-NH-  -$(CH_2)_n$-CH_3

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (DMF)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^7
$n=1$	61.9	175.185	+23.7 ($c=1.0$)	$C_{36}H_{46}N_4O_5 \cdot 0.2H_2O$	69.9 (69.9)	7.56 7.66	9.06 8.93)	0.63	
=2	81.2	181—183	+13.5 ($c=1.0$)	$C_{37}H_{48}N_4O_5 \cdot 1.2H_2O$	68.3 (68.0)	7.80 7.60	8.60 8.55)	0.77	
=3	61.1	167—170	+18.6 ($c=1.1$)	$C_{38}H_{50}N_4O_5 \cdot 0.8H_2O$	69.5 (69.4)	7.86 7.69	8.53 8.17)	0.58	
=4	64.1	161—170	+22.3 ($c=1.0$)	$C_{39}H_{52}N_4O_5 \cdot 0.3H_2O$	70.7 (70.9)	8.03 7.93	8.45 8.42)	0.50	
=5	41.2	155—157	+23.9 ($c=1.0$)	$C_{40}H_{54}N_4O_5$	71.6 (71.3)	8.11 8.05	8.35 8.27)		0.23
=6	63.4	172—176	+16.9 ($c=1.0$)	$C_{41}H_{56}N_4O_5 \cdot 0.5H_2O$	70.9 (70.6)	8.30 8.06	8.19 7.97)		0.63

Table 5. Yield, Melting Point, Optical Rotation, *Rf* Values and Elemental Analysis of H-Tra-Tyr(*O*-Pic)-NH-  -$(CH_2)_n$-CH_3

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (10% AcOH)	Formula	Elemental analysis Calcd (Found)			MS *m/z*: [Calcd]	TLC		
					C	H	N		Rf^3	Rf^4	Rf^5
$n=1$	95.1	Amorphous	+36.4 ($c=1.0$)	$C_{31}H_{38}N_4O_3 \cdot 2TFA \cdot 0.2H_2O$	69.9 (69.9)	7.56 7.66	9.06 8.93)	515 $[(M+H)^+]$ [515]		0.38	
=2	86.0	Amorphous	+26.4 ($c=1.0$)	$C_{32}H_{40}N_4O_3 \cdot 2.5TFA \cdot 1.2H_2O$	68.3 (68.0)	7.80 7.60	8.60 8.55)	529 $[(M+H)^+]$ [529]	0.43	0.17	0.44
=3	87.7	Amorphous	+19.0 ($c=1.0$)	$C_{33}H_{42}N_4O_3 \cdot 2TFA \cdot 0.8H_2O$	69.5 (69.4)	7.86 7.69	8.53 8.17)	543 $[(M+H)^+]$ [543]	0.50		
=4	95.0	Amorphous	+29.8 ($c=1.0$)	$C_{34}H_{44}N_4O_3 \cdot 2.5TFA$	70.7 (70.9)	8.03 7.93	7.45 7.42)	557 $[(M+H)^+]$ [557]		0.48	0.66
=5	74.0	Amorphous	+14.4 ($c=1.0$)	$C_{35}H_{46}N_4O_3 \cdot 2TFA$	71.6 (71.3)	8.11 8.05	8.35 8.27)	571 $[(M+H)^+]$ [571]			
=6	95.0	Amorphous	+18.2 ($c=1.0$)	$C_{36}H_{48}N_4O_3 \cdot 2.8TFA$	70.9 (70.6)	8.30 8.06	8.19 7.97)	585 $[(M+H)^+]$ [585]			0.65

are summarized in Table 6.

General Procedure for Preparation of Boc-Tra-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH-Boc ($n=2, 4, 6, 8$) Piperidine (8 ml) was added to the solution of Fmoc-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH-Boc (1.5 mmol) in DMF (32 ml) and stirred at room temperature for 90 min. After removal of the solvent, ether was added to the residue to yield crystals [H-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH-Boc], which were collected by filtration. A solution of H-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH-Boc in DMF (20 ml) was added to Boc-Tra-OH (0.36 g, 1.4 mmol) in DMF (20 ml) containing BOP (0.75 g, 1.7 mmol), HOBt (0.23 g, 1.7 mmol) and DIPEA (0.30 ml, 1.7 mmol) at 0 °C. The reaction mixture was stirred at room temperature overnight. After removal of the solvents, the residue was extracted with AcOEt. The extract was washed with 10% citrtic acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Diethyl ether was added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH. Yield, mp, $[\alpha]_D^{25}$, elemental analysis and Rf value are summarized in Table 7.

Table 6. Yield, Melting Point, Optical Rotation, *Rf* Value and Elemental Analysis of Fmoc-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH-Boc

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ ($CHCl_3$)	Formula	Elemental analysis Calcd (Found)			TLC
					C	H	N	Rf^1
n=2	57.8	148—150	−19.5[a] (c=1.0)	$C_{37}H_{40}N_4O_6\cdot H_2O$	69.8 (69.5)	6.33 6.32	8.80 8.67)	0.55
=4	38.6	124—126	−9.7 (c=1.0)	$C_{39}H_{44}N_4O_6$	66.6 (66.6)	6.79 6.60	8.21 8.07)	0.50
=6	76.7	117—119	−9.0 (c=1.0)	$C_{41}H_{48}N_4O_6\cdot 1.2H_2O$	70.5 (70.7)	7.18 6.91	7.83 7.96)	0.52
=8	61.4	123—125	−9.4 (c=1.0)	$C_{43}H_{52}N_4O_6$	71.6 (71.5)	7.27 7.04	7.77 7.82)	0.58

a) DMF.

Table 7. Yield, Melting Point, Optical Rotation, *Rf* Value and Elemental Analysis of Boc-Tra-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH-Boc

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ ($CHCl_3$ c=1.0)	Formula	Elemental analysis Calcd (Found)			TLC
					C	H	N	Rf^1
n=2	66.4	161—163	−9.6[a]	$C_{35}H_{51}N_5O_7\cdot H_2O$	62.6 (62.4)	7.95 7.72	10.4 10.5)	0.25
=4	20.7	141—142	−4.8	$C_{37}H_{55}N_5O_7$	65.2 (65.3)	8.13 7.90	8.21 8.07)	0.56
=6	77.0	132—134	−3.1	$C_{39}H_{59}N_5O_7$	66.0 (65.7)	8.38 8.62	7.83 7.96)	0.53
=8	77.0	144—146	−2.6	$C_{41}H_{63}N_5O_7\cdot 0.5H_2O$	65.9 (65.7)	8.64 8.73	7.77 7.82)	0.60

a) DMF.

Table 8. Yield, Melting Point, Optical Rotation, *Rf* Value and Elemental Analysis of H-Tra-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH_2

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (10% AcOH)	Formula	Elemental analysis Calcd (Found)			MS *m*/*z*: [Calcd]	TLC	
					C	H	N		Rf^4	Rf^7
n=2	39.7	Amorphous	+6.26 (c=1.0)	$C_{25}H_{35}N_5O_3\cdot$ 3TFA$\cdot H_2O$	45.8 (45.4	4.95 4.92	8.60 8.73)	454 [(M+H)$^+$] [454]	0.15	
=4	88.0	Amorphous	−8.7 (c=1.0)	$C_{27}H_{39}N_5O_3\cdot$ 3TFA$\cdot 0.2H_2O$	47.4 (47.3	5.18 5.18	8.38 8.53)	482 [(M+H)$^+$] [482]		0.60
=6	93.2	Amorphous	−9.0 (c=1.0)	$C_{29}H_{43}N_5O_3\cdot$ 3TFA	48.3 (48.7	5.55 5.76	8.05 8.29)	510 [(M+H)$^+$] [510]		0.69
=8	85.3	Amorphous	−8.6 (c=1.0)	$C_{31}H_{47}N_5O_3\cdot$ 3TFA$\cdot 2.5H_2O$	48.1 (47.8	7.57 7.48	7.57 7.67)	538 [(M+H)$^+$] [538]		0.79

General Procedure for Preparation of H-Tra-Tyr(*O*-Pic)-NH-$(CH_2)_n$-NH_2·3TFA (n=2, 4, 6, 8) Boc-Tra-Tyr(*O*-Pic)-NH$(CH_2)_n$-NH-Boc (0.76 mmol) was dissolved in TFA (1.1 ml, 15 mmol) containing anisole (0.21 ml, 1.9 mmol) at 0 °C and the reaction mixture was stirred at 0 °C for 10 min and was further stirred at room temperature for 60 min. After removal of the solvent, dry diethyl ether was added to the residue to afford a precipitate, which was collected by filtration. Yield, mp, $[\alpha]_D^{25}$, elemental analysis and *Rf* values are summarized in Table 8.

4-(Boc-Aminomethyl)aniline A solution of di-*tert*-butyldicarbonate (5.0 g, 0.0225 mol) in dioxane (20 ml) was added over a period of 2.5 h to a solution of 4-aminomethylaniline (6.1 g, 0.05 mol) in water (20 ml) containing Et_3N (3.5 ml, 0.025 mol). The mixture was allowed to stir for 22 h and the solvent was removed. AcOEt (40 ml) was added to the residue and the insoluble product was removed by filtration. The filtrate was washed with water (5×40 ml) dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to afford a precipitate. The crude product in solvent (AcOEt–*n*-hexane, 1 : 1 v/v, 5 ml) was applied to a silica gel column (3×26 cm), equilibrated and eluted with solvent (AcOEt–*n*-hexane, 1 : 2 v/v). Individual fractions (200 ml) were collected. The solvent of the effluent (tubes 1 and 2) was removed by evaporation. Petroleum ether was added to the residue to give a powder: yield 4.7 g (42%), mp 75—76 °C, Rf^1 0.50. *Anal.* Calcd for $C_{12}H_{18}N_2O_2$: C, 64.8; H, 8.16; N, 12.6. Found; C, 64.7; H, 8.27; N, 12.8.

[Fmoc-Tyr(*O*-Pic)]-4-(Boc-aminomethyl)anilide A mixed anhydride [prepared from Fmoc-Tyr(*O*-Pic)-OH (500 mg, 1.0 mmol), NMM (0.11 ml, 1.0 mmool) and isobutyl chloroformate (0.13 ml, 1.0 mmol) as usual] in DMF (20 ml) was added to an ice-cooled solution of 4-(Boc-aminomethyl)-aniline (0.45 g, 1.0 mmol) in THF–DMF (1 : 1, v/v, 10 ml each). The reaction mixture was stirred at 0 °C for 1 h and at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Diethyl ether was added to the residue to give a white precipitate, which was collected by filtration. The crude product in $CHCl_3$ (5 ml) was applied to a silica gel column (3×18 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (200 ml) were collected. The solvent of the effluent (fractions 3—6) was removed by evaporation. Petroleum ether was added to the residue to give a powder: yield 250 mg (36%), mp 140—142 °C, $[\alpha]_D^{25}$ +11.2° (c=0.67, DMF), Rf^1 0.58, Rf^2 0.54. *Anal.* Calcd for

$C_{42}H_{42}N_4O_6$: C, 72.2; H, 6.06; N, 8.20. Found; C, 71.9; H, 6.18; N, 7.91.

[Boc-Tra-Tyr(*O*-Pic)]-4-(aminometyl)anilide A mixed anhydride [prepared from Boc-Tra-OH (74 mg, 0.29 mmol), NMM (0.032 ml, 0.29 mmool) and isobutyl chloroformate (0.037 ml, 0.29 mmol) as usual] in THF (10 ml) was added to an ice-cooled solution of [H-Tyr(*O*-Pic)]-4-(aminomethyl)-anilide {prepared from [Fmoc-Tyr(*O*-Pic)]-4-Boc-aminomethyl) anilide (0.20 g, 0.29 mmol) and 20% piperidine/DMF} in DMF (10 ml). The reaction mixture was stirred at 0 °C for 1 h and at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Diethyl ether was added to the residue to give a white precipitate, which was collected by filtration. The crude product was recrystallized from AcOEt: yield 130 mg (63%), mp 186—188 °C, Rf^1 0.56, Rf^2 0.37. *Anal.* Calcd for $C_{40}H_{53}N_5O_7 \cdot 0.25H_2O$: C, 66.7; H, 7.48; N, 9.72. Found; C, 66.6; H, 7.36; N, 9.71.

[H-Tra-Tyr(*O*-Pic)]-4-(aminomethyl)anilide·3TFA [Boc-Tra-Tyr(*O*-Pic)]-4-(Boc-aminomethyl)anilide (100 mg, 0.14 mmol) was dissolved in TFA (0.50 ml, 6.5 mmol) containing anisole (0.050 ml, 0.46 mmol) at 0 °C and the reaction mixture was stirred at 0 °C for 10 min, and then further stirred at room temperature for 1 h. Anhydrous ether was added to the reaction mixture to afford a precipitate, which was collected by filtration. The crude peptide was applied to a column of Sephadex G-15 (2.2×0.49 cm), equilibrated and eluted with 3% AcOH. Individual fractions (13 g each) were collected and the solvent of effluent (tubes 4—10) was removed by lyophilization to give a white amorphous powder: yield 100 mg (83%), $[\alpha]_D^{25}$ +22.9° (c=0.86, 10% AcOH), Rf^5 0.10, Rf^6 0.62. TOF-MS *m/z*: 516 $(M+H)^+$. *Anal.* Calcd for $C_{30}H_{37}N_5O_3 \cdot 3TFA \cdot 0.75H_2O$: C, 49.6; H, 4.80; N, 8.04. Found: C, 49.5; H, 4.60; N, 8.18.

Boc-Tyr(*O*-Pic)-APAA-OBzl A mixed anhydride of Boc-Tyr(*O*-Pic)-OH [prepared from Boc-Tyr(O-Pic)-OH (1.5 g, 4.0 mmol), isobutyl chloroformate (0.55 ml, 4.0 mmol) and NMM (0.88 ml, 8.0 mmol) as usual] in THF (10 ml) was added to a solution of H-APAA-OBzl. TosOH (1.52 g, 4.0 mmol), in DMF (10 ml) containing NMM (0.44 ml, 4.0 mmol) at 0 °C and the reaction mixture was stirred at 6 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give a white precipitate, which was collected by filtration and recrystallized from EtOH: yield 940 mg (42%), mp 162—165 °C, $[\alpha]_D^{25}$ +5.7° (c=1.0, $CHCl_3$), Rf^1 0.53. *Anal.* Calcd for $C_{35}H_{37}N_3O_6$: C, 70.5; H, 6.26; N, 7.05. Found; C, 70.3; H, 6.14; N, 7.01.

Boc-Tra-Tyr(*O*-Pic)-APAA-OBzl A mixed anhydride of Boc-Tra-OH [prepared from Boc-Tra-OH (0.33 g, 1.3 mmol), isobutyl chloroformate (0.18 ml, 1.3 mmol) and Et_3N (0.18 ml, 1.3 mmol) as usual] in THF (10 ml) was added to a solution of H-Tyr(*O*-Pic)-APAA-OBzl [prepared from Boc-Tyr(*O*-Pic)-APAA-OBzl (0.80 g, 1.3 mmol) and TFA (2.0 ml, 26 mmol) in the presence of anisole (0.19 ml, 1.8 mmol) as usual] in DMF (30 ml) containing Et_3N (0.36 ml, 2.6 mmol) at 0 °C and the reaction mixture was stirred at 6 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give a white precipitate, which was collected by filtration and recrystallized from DMF and AcOEt: yield 0.59 mg (57%), mp 162—165 °C, $[\alpha]_D^{25}$ +23.7° (c=1.0, DMF), Rf^1 0.53. *Anal.* Calcd for $C_{43}H_{50}N_4O_7$: C, 70.3; H, 6.85; N, 7.62. Found; C, 70.1; H, 6.82; N, 7.84.

Tra-Tyr(*O*-Pic)-APAA·2TFA Boc-Tra-Tyr(*O*-Pic)-APAA-OBzl (230 mg, 0.31 mmol) in DMF (10 ml) and MeOH (10 ml) was hydrogenated for 2 h over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to yield crystals, which were collected by filtration. The crude material in $CHCl_3$ (3 ml) was applied to a silica gel column (3×8 cm), equilibrated and eluted with $CHCl_3$ (2.2 l), followed by $CHCl_3$, MeOH and H_2O (16 : 3 : 1, lower phase, 0.80 l). The solvent of the latter effluent (200—800 ml) was removed by evaporation to yield a powder, which was collected by filtration, yield 180 mg (80%), mp 198—200 °C, Rf^1 0.25. Boc-Tra-Tyr(*O*-Pic)-APAA (120 mg, 1.9 ml) was dissolved in TFA (0.30 ml, 3.8 mmol) containing anisole (0.050 ml, 0.46 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min and was further stirred at room temperature for 1 h. Anhydrous ether was added to the reaction mixture to afford a precipitate, which was collected by filtration. The crude peptide was applied to a column of Sephadex G-15 (2.5×53 cm), equilibrated and eluted with 3% AcOH. Individual fractions (13 g each) were collected and the solvent of effluent (tubes 8—15) was removed by lyophilization to afford a white amorphous powder: 150 mg (90%), $[\alpha]_D^{25}$ +36.6° (c=0.34, 10% AcOH), Rf^1 0.25. *Anal.* Calcd for $C_{30}H_{37}N_5O_3 \cdot 2TFA \cdot 0.5H_2O$: C, 52.9; H, 5.12; N, 7.08. Found: C, 52.6; H, 4.83; N, 7.04.

Assay Procedure The enzymes used were as follows: human PL and PK (Chromogenix AB, Molndal), human urokinase (Green Cross Co., Osaka), bovine thronbin (Mochida Pharmaceutical, Tokyo) and trypsin (Sigma Chemical Co., St. Louis). Enzymatic activities of PL, PK, urokinase, thrombin and trypsin were determined by the method described previously, using D-Vla–Leu–Lys–*p*NA (s-2251), D-Pro–Phe–Arg–*p*NA (s-2302), <Glu–Gly–Arg–*p*NA (s-2444) and D-Phe–Pip–Arg–*p*NA (s-2238), respectively. Fn and Fg were used as substrates for PL and thrombin, respectively. IC_{50} values were determined as follows: (1) Antiamidolytic assay[30]: the IC_{50} value was taken as the concentration of inhibitor which reduced the absorbance at 405 nm by 50% compared with the absorbance measured under the same conditions without inhibitor. (2) Antifibrinolytic assay[30]: the IC_{50} value was taken as the concentration of inhibitor, which doubled the complete lysis time compared to control samples without inhibitor. (3) Antifibrinolytic assay: to a borate saline buffer (pH 7.4) was added solutions containing various concentrations of the inhibitor to be tested (0.5 ml), 0.2% bovine Fg in the above buffer (0.4 ml) and bovine thrombin 4 U/ml (0.1 ml). 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 doubled the clotting time compared to the controls without inhibitor.

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References and Notes

1) The customary L-configuration for amino acid residues is omitted. Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485—2489 (1966); **6**, 362—364 (1967); **11**, 1726—1732 (1972). The following additional abbreviations are used: AcOEt, ethyl acetate; APAA, 4-carboxymethyanilide; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazole-1-yl-oxy-tris(dimethylamino)phosphoniumhexafluorophosphate; Bzl, benzyl; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fg, fibrinogen; Fn, fibrin; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methylmorpholine; *p*NA, *p*-nitroanilide; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Tos, *p*-toluensulfonyl; Tra, 4-aminomethylcyclohexanecarbonyl.
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