Design of Plasma Kallikrein Inhibitors: Functional and Structural Requirements of Plasma Kallikrein Inhibitors[1)]

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The synthetic plasma kallikrein (PK) inhibitor *trans*-4-aminomethylcyclohexanecarbonylphenylalanine-4-carboxymethylanilide (PKSI-527) consists of three parts. Each part was replaced by analogues in an attempt to improve the potency and the selectivity of PKSI-527. Among the peptides examined, *trans*-4-aminomethylcyclohexanecarbonylphenylalanine-4-carboxyanilide (peptide 16) inhibited PK with a high selectivity and an IC_{50} value of 2.7 μM, being as potent as PKSI-527.

Key words plasma kallikrein; potent-selective inhibitor; substituted peptide; *trans*-4-aminomethylcyclohexanecarbonylphenylalanine-4-carboxymethylanilide

Plasma kallikrein (PK) (EC. 3. 4. 21. 34) is a serine proteinase required for the activation and inhibition of the surface-mediated pathway.[2)] Once PK is generated from its precursor (prekallikrein) by factor XIIa, PK initiates surface-mediated activation of coagulation, fibrinolysis, and kinin generation.[3)] Generally, PK formed is rapidly inactivated by C1 inhibitor, and it is inhibited by α_2-macroglobulin and antithrombin III.[2,3)] An imbalance between PK and its plasma inhibitors causes several diseases (allergic rhinitis, asthma, arthritis). In those pathological states, a highly specific PK inhibitor is an important therapeutic candidate.[4)] Furthermore, a highly specific PK inhibitor would be a useful tool to examine the physiological role of PK.

Recently, a specific inhibitor for PK (PKSI-527) was reported[5)] and used in elucidating several PK functions.[6,7)] PKSI-527 inhibits the amidolytic activity of PK with a K_i value of 0.81 μM, while it only slightly inhibits the amidolytic activity of plasmin or urokinase, and hardly inhibits that of glandular kallikrein, thrombin or factor Xa.[5)] PKSI-527 consists of *trans*-4-aminomethylcyclohexanecarboxylic acid (*t*-AMCHA), phenylalanine (Phe), and 4-aminophenylacetic acid (APAA) moieties, as shown in Fig. 1. It might be possible to improve the potency and selectivity of PKSI-527 by investigating the structure–activity relationships of the *t*-AMCHA, Phe and APAA residues. In this study, the functional and structural roles of the *t*-AMCHA, Phe and APAA residues were investigated by replacement of each moiety with analogues.

First, replacement of *t*-AMCHA with EACA, Ac-Lys, Lys and Arg afforded peptides **1**—**4**. The IC_{50} values of these peptides are summarized in Table 1. Plasmin-to-PK IC_{50} ratio (described as PL/PK in Table 1) was used as an indication of enzyme selectivity, together with trypsin-to-PK IC_{50} ratio (described as Trypsin/PK in Table 1). The replacement of *t*-AMCHA with EACA (**1**) and Ac-Lys (**2**) remarkably reduced the inhibitory activity of PKSI-527, with IC_{50} values of 190 μM and 890 μM, respectively. Peptides **3** and **4**, which have an additional basic group, exhibited no inhibitory ability at the concentration of 1000 μM. The additional positive charge seems to interfere with the interaction between the enzyme and the peptide. A study on the crystal structure of the trypsin-PKSI-527 complex showed that the amino group of *t*-AMCHA moiety interacts with Asp-189 of trypsin, while the Phe and APAA residues were not defined on the electron density map.[7)] Many enzymes of the coagulation and fibrinolysis pathway are closely related to trypsin and have a highly conserved pocket, which has Asp-189 at the bottom. The basic side chain of *t*-AMCHA is expected to bind with PK in the similar manner to that of trypsin, so that one basic group is required for the peptide to bind with PK. However, an additional positive charge could interfere with the interaction between PK and the inhibitor. The above findings show that the *t*-AMCHA moiety, which has a structure bearing a hydrophobic backbone with a cationic charge, is critical for PKSI-527 to manifest inhibitory activity.

Evaluation of the importance of the Phe residue in PKSI-527 could be a second step to improve the affinity

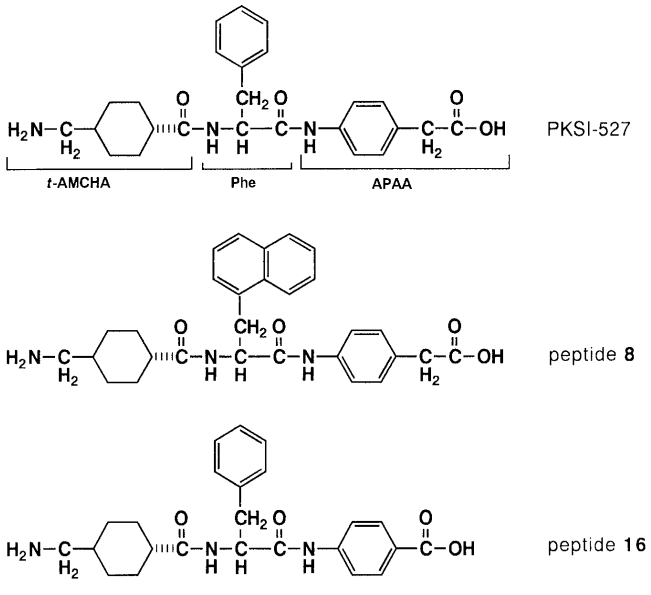

Fig. 1 Structures of PKSI-527, Peptide **8** and Peptide **16**

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Table 1. *In Vitro* IC_{50} Values (μM) and Selectivity Ratios of Peptide **1**—**16** against PK, PL, and Trypsin

Peptide	Peptide ID	PK	PL	UK	TH	Trypsin	Trypsin/PK	PL/PK
EACA-Phe-APAA	**1**	190	>1000	>1000	>1000	NT[a]	NA[b]	5.2
Ac-Lys-Phe-APAA	**2**	890	>1000	>1000	>1000	NT[a]	NA[b]	1.1
Lys-Phe-APAA	**3**	1200	>1000	>1000	>1000	>600	>0.5	>0.8
Arg-Phe-APAA	**4**	1600	>1000	>1000	>1000	>600	>0.4	>0.6
t-AMCHA-Cha-APAA	**5**	12	830	380	>1000	7.6	0.6	69
t-AMCHA-Phg-APAA	**6**	400	>500	>500	>500	120	0.3	>1.2
t-AMCHA-Hph-APAA	**7**	160	450	>500	>500	60	0.4	2.8
t-AMCHA-1-Nal-APAA	**8**	5.1	750	470	>500	9.8	1.9	147
t-AMCHA-2-Nal-APAA	**9**	2.0	95	>125	>200	5.8	2.9	48
t-AMCHA-Bpa-APAA	**10**	2.3	42	>125	>200	0.75	0.3	18
t-AMCHA-Phe-APAA-NH_2	**11**	4.3	180	320	>1000	3.7	0.9	42
t-AMCHA-Phe-NH_2	**12**	1300	1500	520	>1000	37	0.03	1.2
t-AMCHA-Phe-AP	**13**	1.3	130	53	>1000	4.4	3.4	100
t-AMCHA-Phe-AMBA	**14**	65	24	140	>100	6	0.1	0.4
t-AMCHA-Phe-AcHA	**15**	>1000	>1000	>1000	>1000	>600	NA[b]	NA[b]
t-AMCHA-Phe-ABA	**16**	2.7	1400	1200	>1000	26	9.6	519
t-AMCHA-Phe-APAA	PKSI-527	1.3	620	350	>1000	12	9.2	477

a) Not tested. *b*) Not available.

and the selectivity of PKSI-527. Therefore, substitution of the Phe moiety with analogues was undertaken.

As a first approach, the effect of the benzyl group of Phe was examined by substitution of Phe with Cha (**5**), Phg (**6**) and Hph (**7**). Reduction of the phenyl ring (**5**) reduced the inhibitory activity of PKSI-527, giving an IC_{50} value of 12 μM, which represents a 9-fold decrease in potency. However, peptide **5** showed a high selectivity ratio (plasmin to PK) of 69. Removal of the methylene group of the Phe side chain (Phg) and extension of the methylene group (Hph) reduced the inhibitory activity of PKSI-527 by 220-fold and 90-fold, respectively. As well as reducing the potency of inhibition, substitution with Hph decreased the selectivity between plasmin and PK; peptide **7** exhibited a low selectivity ratio (plasmin to PK) of 2.8. Peptides containing phenylglycine have fewer rotational degrees of freedom than those which contain phenylalanine, since the phenyl ring is spatially closer to the peptide backbone in the case of Phg. The substitution of Phe with Phg was not favorable for PK-inhibitory activity. This finding shows that both of the existence of a phenyl ring and the distance between the phenyl ring and the peptide backbone are important when PKSI-527 binds to PK to manifest inhibitory activity.

Next, addition of steric bulkiness to the phenyl ring of Phe was examined. This modification could have the effect of decreasing conformational flexibility and creating new interactions between the inhibitor and the enzyme. A bicyclic aromatic ring might be physically constrained. The replacement of Phe with either 1-Nal or 2-Nal gave peptide **8** or **9**, which exhibited an IC_{50} value of 5.1 μM or 2.0 μM, respectively. Peptide **9** was 2.5-fold more potent than peptide **8**; however, it exhibited a 3-fold loss of enzyme selectivity (plasmin to PK), with a selectivity ratio of 48. Furthermore, addition of a benzoyl group at the 4-position of Phe gave peptide **10** with an equal potency to that of PKSI-527; however, peptide **10** exhibited some decrease in enzyme selectivity (plasmin to PK) with a selectivity ratio of 18, indicating that the benzoyl group increased the ability of the inhibitor to bind with some part of not only PK, but also plasmin. Among the substituted peptides, *t*-AMCHA-1-Nal-APAA (**8**) exhibited a relatively high inhibitory activity for PK (IC_{50} = 5.1 μM) with a selectivity ratio (plasmin to PK) of 147. This result implies that not only the phenyl ring of PKSI-527, but also the more bulky naphthyl ring of peptide **8** (structure shown in Fig.1) could bind with some part of PK more strongly than with plasmin. The site of PK and plasmin corresponding to the Phe residue of PKSI-527 should be a hydrophobic region.

Third, the APAA moiety was modified: a) amidation (**11**), b) elimination of APAA, and then amidation (**12**, **13**), c) instead of APAA, incorporation of 4-aminomethylbenzoic acid (**14**), 1-aminocyclohexane-1-carboxylic acid (**15**) and 4-aminobenzoic acid (**16**). Peptide **11**, which lacked the carboxyl group at the C-terminus, exhibited a ten-fold loss of selectivity (plasmin to PK), although it inhibited PK with a similar potency to PKSI-527. This suggests that the carboxyl group at the C-terminus contributes to the selectivity rather than the potency. Although the elimination of the APAA moiety diminished the inhibitory activity (**12**), further introduction of a pyridine ring improved the inhibitory activity against PK with an IC_{50} value of 1.3 μM, which is as potent as that of PKSI-527. It seems that a hydrophobic residue at the C-terminus binds to a corresponding site which differs in each enzyme. Interestingly, removal of the methylene group gave **16** (structure shown in Fig. 1) with an equal potency and selectivity to PKSI-527. Additionally, **16** exhibited the highest degree of trypsin to PK selectivity (9.6, as shown in Table 1), even though this is still not sufficient. Peptide **16** might be a candidate for further development to obtain potent and selective PK inhibitors.

In conclusion, this work has demonstrated the critical importance of the *t*-AMCHA moiety in the structure of PKSI-527. Further simple modification of Phe and APAA could have a beneficial effect on enzyme selectivity.

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.). On TLC (Kieselgel 60 F_{254}, Merck), Rf^1, Rf^2, Rf^3, Rf^4 and Rf^5 values refer to the systems of $CHCl_3$, MeOH and AcOH (90:8:2), $CHCl_3$, MeOH and H_2O (89:10:1), $CHCl_3$ and ether (4:1), *n*-BuOH, AcOH and H_2O (4:1:5, upper phase) and *n*-BuOH, AcOH, pyridine and H_2O (4:1:1:2), respectively. Final products were each obtained as a powder or an amorphous powder with 95% or higher purity by analytical HPLC. Analytical HPLC was performed on a YMC R&D R-ODS-5A column (0.46 × 25 cm, 10—50% acetonitrile in 0.05% TFA, 1.0%/min gradient, 1.0 ml/min flow rate). The elution profile was monitored in terms of the absorbance at 220 nm. Mass spectra were determined on a SCIEX API III mass spectrometer.

Materials Boc-β-1-Naphthylalanine (Boc-1-Nal-OH), Boc-homophenylalanine (Boc-Hph-OH), Boc-phenylglycine (Boc-Phg-OH) and Boc-*p*-benzoylphenylalanine (Boc-Bpa-OH) were purchased from Bachem BioScience Inc. Boc-β-2-Naphthylalanine (Boc-2-Nal-OH) and β-cyclohexylalanine (Cha) were obtained from Advanced Chem Tech and Fulka, respectively. The enzymes used were as follows: human plasma kallikrein and plasmin (Chromogenix AB.), bovine thrombin (Mochida Seiyaku Co.), human urokinase (Midorijuji Co.) and bovine trypsin (Sigma Chemical Co.). All chromogenic substrates were purchased from Chromogenix AB.

General Procedure for Synthesis of Boc-X-Phe-APAA-OBzl [X: EACA, Lys(2-Cl-Z), Arg(NO_2)] A mixed anhydride of Boc-X-OH [prepared from Boc-X-OH (1.0 mmol), isobutyl chloroformate (0.13 ml, 1.0 mmol) and Et_3N (0.14 ml, 1.0 mmol) as usual] in THF (15 ml) was added to a solution of H-Phe-APAA-OBzl·TFA [prepared from Boc-Phe-APAA-OBzl[5] (0.50 g, 1.0 mmol) and TFA (2.35 ml, 30 mmol)–anisole (0.30 ml, 2.7 mmol) as usual] in THF (15 ml) containing Et_3N (0.14 ml, 1.0 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 crystals [X: EACA, Lys(2-Cl-Z)] or an amorphous powder [X: Arg(NO_2)]. The crystals were recrystallized from AcOEt, and the amorphous powder was purified by silica gel column chromatography (1% MeOH in $CHCl_3$). Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 3.

H-EACA-Phe-APPA-OH Boc-EACA-Phe-APAA-OBzl (0.13 g, 0.22 mmol) was treated with TFA (0.50 ml, 6.5 mmol)–anisole (0.050 ml, 0.46 mmol) as usual. Ether was added to the reaction mixture to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. The resulting TFA salt was dissolved in MeOH (5 ml) containing 1 N NaOH (1.0 ml, 1.0 mmol). The reaction mixture was stirred at 0 °C and at room temperature for 120 min. It was neutralized with AcOH, and the solvent was removed. To the residue, AcOEt was added to give a white precipitate, which was recrystallized from EtOH. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 4.

Ac-Lys-Phe-APAA-OH Boc-Lys(2-Cl-Z)-APAA-OBzl (0.15 g, 0.20 mmol) was treated with TFA (0.43 ml, 5.6 mmol)–anisole (0.040 ml, 0.37 mmol) as usual. Ether was added to the reaction mixture to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. The resulting TFA salt was dissolved in DMF (5 ml) containing acetic anhydride (0.052 ml, 0.51 mmol) and Et_3N (0.071 ml, 0.51 mmol). The reaction mixture was stirred at room temperature for 1 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give a white powder. The resulting Ac-Lys(2-Cl-Z)-Phe-APAA-OBzl was hydrogenated over a palladium catalyst. After removal of palladium and the solvent, ether was added to the residue to give a white precipitate, which was collected by centrifugation. The title compound was obtained by purification on Sephadex LH-20. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 4.

General Procedure for Synthesis of H-X-Phe-APAA-OH [X: Lys, Arg] Boc-X-Phe-APAA-OBzl [X: Lys(2-Cl-Z), Arg(NO_2)] (0.25 mmol) in MeOH (10 ml) was hydrogenated over a palladium catalyst. After removal of palladium and the solvent, ether was added to the residue to give a white powder, which was collected by filtration. The resulting powder was treated with TFA (0.62 ml, 8.1 mmol)–anisole (0.060 ml, 0.56 mmol) as usual. Ether was added to the mixture to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 4.

General Procedure for Synthesis of Boc-X-APAA-OBzl [X: Cha, Phg, Hph, 1-Nal, 2-Nal, Bpa] A mixed anhydride of Boc-X-OH [prepared from Boc-X-OH (3.0 mmol), isobutyl chloroformate (0.39 ml, 3.0 mmol)

Table 2. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Protected Derivatives

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (DMF)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^2
Boc-Cha-APAA-OBzl	18.4	Amorphous powder	−0.70 (*c*=0.81)	$C_{29}H_{38}N_2O_5$	70.42 (70.35	7.74 7.70	5.66 5.69)	0.79	0.88
Boc-Phg-APAA-OBzl	70.0	95—100	+43.8 (*c*=0.97)	$C_{28}H_{30}N_2O_5$	70.86 (70.69	6.37 6.36	5.90 5.75)	0.76	0.83
Boc-Hph-APAA-OBzl	66.0	94—108	−3.20 (*c*=0.94)	$C_{30}H_{34}N_2O_5 \cdot 0.25H_2O$	71.05 (71.19	6.85 6.83	5.52 5.23)	0.78	0.87
Boc-1-Nal-APAA-OBzl	43.3	159—162	+55.9 (*c*=0.99)	$C_{33}H_{34}N_2O_5$	73.58 (73.56	6.32 6.40	5.20 5.16)	0.70	0.83
Boc-2-Nal-APAA-OBzl	42.1	155—156	+47.5 (*c*=0.16)	$C_{33}H_{34}N_2O_5$	73.58 (73.61	6.32 6.18	5.20 5.23)	0.36	0.92
Bco-Bpa-APAA-OBzl	59.7	141—142.5	+39.5 (*c*=0.97)	$C_{36}H_{36}N_2O_6$	72.95 (72.73	6.12 5.98	4.73 4.72)	0.80	0.88
Boc-Phe-APAA-NH_2	79.7	182—183	+36.0 (*c*=0.76)	$C_{22}H_{27}N_3O_4$	66.48 (66.24	6.85 6.86	10.6 10.4)	0.55	0.75
Boc-Phe-NH_2	72.7	142—144	−1.13 (*c*=1.06)	$C_{14}H_{20}N_2O_3$	63.61 (63.42	7.63 7.67	10.6 10.6)	0.72	0.72
Boc-Phe-AP	24.9	75—78	+45.8 (*c*=0.71)	$C_{19}H_{23}N_3O_3 \cdot 0.2H_2O$	66.14 (66.18	6.83 6.89	12.2 12.0)	0.48	0.83
Boc-Phe-AMBA-OBzl	86.1	148—149	−4.76 (*c*=0.63)	$C_{29}H_{32}N_2O_5$	71.29 (71.36	6.60 6.59	5.73 5.74)	0.82	0.93
Boc-Phe-AcHA-OBzl	58.2	110—111	−17.8[a] (*c*=0.98)	$C_{28}H_{36}N_2O_5$	69.97 (69.68	7.55 7.59	5.83 5.77)	0.77	0.90
Boc-Phe-ABA-OMe	43.1	222—224	+39.6 (*c*=0.95)	$C_{30}H_{39}N_3O_6$	67.02 (67.07	7.31 7.20	7.82 7.68)	0.65	0.63

a) MeOH.

Table 3. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Protected Derivatives

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (DMF)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	*Rf*[1]	*Rf*[2]
Boc-EACA-Phe-APAA-OBzl	38.2	134—135	+27.3 (*c*=0.24)	$C_{35}H_{43}N_3O_6$	69.86 (69.59	7.20 7.18	6.98 6.75)	0.66	0.80
Boc-Lys(2-Cl-Z)-Phe-APAA-OBzl	37.5	143—145	+5.18 (*c*=0.81)	$C_{44}H_{50}ClN_4O_8 \cdot 1.3H_2O$	65.58 (65.70	6.57 6.27	6.95 7.14)	0.60	0.64
Boc-Arg(NO_2)-Phe-APAA-OBzl	32.0	Amorphous powder	+12.1 (*c*=0.78)	$C_{35}H_{43}N_7O_8 \cdot 2.3H_2O$	60.62 (60.52	6.30 6.37	14.1 13.7)	0.45	
Boc-*t*-AMCHA-Cha-APAA-OBzl	67.0	177—179	−12.3[a] (*c*=0.8)	$C_{37}H_{51}N_3O_6$	70.11 (69.98	8.11 8.11	6.63 6.63)	0.66	0.81
Boc-*t*-AMCHA-Phg-APAA-OBzl	27.1	213—218	+44.4 (*c*=0.45)	$C_{36}H_{43}N_3O_6$	70.43 (70.19	7.06 7.02	6.85 6.88)	0.56	0.16[b]
Boc-*t*-AMCHA-Hph-APAA-OBzl	13.5	189—192	−0.91 (*c*=0.44)	$C_{38}H_{47}N_3O_6$	71.11 (70.92	7.38 7.38	6.55 6.57)	0.63	0.55[b]
Boc-*t*-AMCHA-1-Nal-APAA-OBzl	18.5	218—220	+46.3 (*c*=0.70)	$C_{41}H_{46}N_3O_6$	72.68 (72.78	6.94 6.43	6.20 6.35)	0.72	0.59[b]
Boc-*t*-AMCHA-2-Nal-APAA-OBzl	55.1	214—215.5	+24.8 (*c*=0.81)	$C_{41}H_{46}N_3O_6$	72.68 (72.68	6.94 6.93	6.20 6.17)	0.72	0.84
Boc-*t*-AMCHA-Bpa-APAA-OBzl	61.0	171—177	+13.0 (*c*=0.90)	$C_{44}H_{49}N_3O_7$	72.20 (71.90	6.74 6.62	5.74 5.76)	0.58	0.88
Boc-*t*-AMCHA-Phe-APAA-NH_2	42.7	174—176	+24.6 (*c*=0.48)	$C_{30}H_{40}N_4O_5 \cdot 0.1H_2O$	66.91 (66.66	7.52 7.49	10.4 10.4)	0.60	0.47
Boc-*t*-AMCHA-Phe-NH_2	71.7	221—222	−7.77 (*c*=0.71)	$C_{22}H_{33}N_3O_4$	65.48 (65.03	8.24 8.28	10.4 10.4)	0.59	0.76
Boc-*t*-AMCHA-Phe-AP	41.6	178—182	+34.2 (*c*=0.71)	$C_{27}H_{36}N_4O_6$	67.47 (67.21	7.55 7.49	11.7 11.6)	0.48	0.79
Boc-*t*-AMCHA-Phe-AMBA-OBzl	75.0	210—211	−10.3 (*c*=0.69)	$C_{37}H_{45}N_3O_6$	70.79 (70.73	7.23 7.22	6.69 6.62)	0.72	0.95
Boc-*t*-AMCHA-Phe-AcHA-OBzl	46.8	164.5—165.5	−26.6 (*c*=0.68)	$C_{36}H_{49}N_3O_6$	69.76 (69.51	7.97 7.78	6.75 6.64)	0.72	0.93
Boc-*t*-AMCHA-Phe-ABA-OMe	43.1	222—224	+39.6 (*c*=0.95)	$C_{30}H_{39}N_3O_6$	67.02 (67.07	7.31 7.20	7.82 7.68)	0.65	0.63

a) 10% DMF/MeOH. *b*) *Rf*[3].

and Et_3N (0.42 ml, 3.0 mmol) as usual] in THF (20 ml) was added to a solution of H-APAA-OBzl·TosOH (1.3 g, 3.0 mmol) in DMF (15 ml) containing Et_3N (0.39 ml, 3.0 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 powder. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 2.

General Procedure for Synthesis of Boc-*t*-AMCHA-X-APAA-OBzl [X: Cha, Phg, Hph, 1-Nal, 2-Nal, Bpa] A mixed anhydride of Boc-*t*-AMCHA-OH [prepared from Boc-*t*-AMCHA-OH (0.30 g, 1.2 mmol), isobutyl chloroformate (0.16 ml, 1.2 mmol) and Et_3N (0.17 ml, 1.2 mmol) as usual] in THF (15 ml) was added to a solution of H-X-APAA-OBzl·TFA [prepared from Boc-X-APAA-OBzl (1.2 mmol) and TFA (2.8 ml, 36 mmol)–anisole (0.30 ml, 2.7 mmol) as usual] in THF (15 ml) containing Et_3N (0.17 ml, 1.2 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. Ether was added to the residue to give a precipitate, which was collected by filtration. The powder was suspended in hot AcOEt. After cooling, the precipitate was collected by filtration to give a white powder. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 3.

General Procedure for Synthesis of H-*t*-AMCHA-X-APAA-OH [X: Cha, Phg, Hph, 1-Nal, 2-Nal, Bpa] Boc-X-APAA-OBzl (0.10 mmol) was treated with TFA (1.0 ml, 13 mmol)–anisole (0.10 ml, 1.0 mmol) as usual. Ether was added to the mixture to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. The resulting powder was hydrogenated over a palladium catalyst. After removal of palladium and the solvent, ether was added to the residue to give a white precipitate, which was collected by centrifugation. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 4.

Boc-Phe-X [X: APAA-NH_2, NH_2, AP, AMBA-OBzl, AcHA-OBzl, ABA-OMe] A mixed anhydride of Boc-Phe-OH [prepared from Boc-Phe-OH (0.32 g, 1.2 mmol), isobutyl chloroformate (0.16 ml, 1.2 mmol) and Et_3N (0.17 ml, 1.2 mmol) as usual] in THF (10 ml) was added to a solution of H-APAA-NH_2·TFA [prepared from Boc-APAA-NH_2[9] (0.30 g, 1.2 mmol) and TFA (2.8 ml, 36 mmol)–anisole (0.30 ml, 2.7 mmol) as usual], 28% NH_4OH (0.30 ml, 5.0 mmol), AP (0.11 g, 1.2 mmol), H-AMBA-OBzl·TFA [prepared from Boc-AMBA-OBzl[10] (0.41 g, 1.2 mmol) and TFA (2.8 ml, 36 mmol)–anisole (0.30 ml, 2.7 mmol) as usual], H-AcHA-OBzl·TFA [prepared from Boc-AcHA-OBzl[11] (0.40 g, 1.2 mmol) and TFA (2.8 ml, 36 mmol)–anisole (0.30 ml, 2.7 mmol) as usual], or H-ABA-OMe·HCl (0.22 g, 1.2 mmol), respectively, in DMF (15 ml) containing Et_3N (0.17 ml, 1.2 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. Ether was added to the residue to give a precipitate, which was collected by filtration. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 2.

General Procedure for Synthesis of Boc-*t*-AMCHA-Phe-X [X: APAA-NH_2, NH_2, AP, AMBA-OBzl, AcHA-OBzl, ABA-OMe] A mixed anhydride of Boc-*t*-AMCHA-OH [prepared from Boc-*t*-AMCHA-OH (0.26 g, 1.0 mmol), isobutyl chloroformate (0.13 ml, 1.0 mmol) and Et_3N (0.14 ml, 1.0 mmol) as usual] in THF (10 ml) was added to a solution of H-Phe-X·TFA [prepared from Boc-Phe-X (1.0 mmol) and TFA (1.5 ml, 20 mmol)–anisole (0.15 ml, 1.4 mmol) as usual] in THF (15 ml) containing Et_3N (0.14 ml, 1.0 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. Ether was added to the residue to give a precipitate, which was collected by filtration. The resulting powder was recrystallized from EtOH or AcOEt. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 3.

General Procedure for Synthesis of H-*t*-AMCHA-Phe-X [APAA-NH_2,

Table 4. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Desired Peptides

Peptide	Peptide ID	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (10% AcOH)	MS (*m/z*)	Formula	Elemental analysis Calcd (Found)			TLC	
							C	H	N	Rf^4	Rf^5
H-EACA-Phe-APAA	**1**	47.3	238—241	+33.7 (c=0.64)	NT[a]	$C_{23}H_{29}N_3O_4 \cdot H_2O$	64.31 (64.47	7.27 7.00	9.77 9.76)	0.22	
Ac-Lys-Phe-APAA	**2**	18.0	Amorphous powder	−1.08 (c=0.37)	NT[a]	$C_{25}H_{32}N_4O_5 \cdot TFA \cdot 2H_20$	52.42 (52.12	6.02 5.52	9.05 8.95)	0.11	0.33
H-Lys-Phe-APAA	**3**	15.8	Amorphous powder	+62.3 (c=0.68)	NT[a]	$C_{23}H_{30}N_4O_4 \cdot 2TFA \cdot 1.8H_2O$	47.20 (47.04	5.22 4.99	8.15 8.16)		0.34
H-Arg-Phe-APAA	**4**	29.3	Amorphous powder	+51.8 (c=0.49)	NT[a]	$C_{23}H_{30}N_6O_4 \cdot 2.3TFA$	46.24 (46.14	4.54 4.78	11.7 11.9)		0.42
H-*t*-AMCHA-Cha-APAA	**5**	86.0	149—154	−12.2 (c=0.88)	444.7	$C_{25}H_{37}N_3O_4 \cdot TFA \cdot 0.5H_2O$	57.23 (57.17	6.93 6.87	7.41 7.55)	0.43	0.36
H-*t*-AMCHA-Phg-APAA	**6**	98.0	155—162	+68.6 (c=0.83)	424.3	$C_{24}H_{29}N_3O_4 \cdot TFA \cdot 0.75H_2O$	56.67 (59.27	5.76 5.69	7.62 7.40)	0.45	0.15
H-*t*-AMCHA-Hph-APAA	**7**	44.2	138—146	−0.90 (c=0.50)	452.3	$C_{26}H_{33}N_3O_4 \cdot TFA \cdot 0.1H_2O$	59.27 (59.48	6.07 6.40	7.40 7.64)	0.53	0.12
H-*t*-AMCHA-1-Nal-APAA	**8**	49.9	147—149	+50.4[b] (c=0.45)	488.3	$C_{29}H_{33}N_3O_4 \cdot TFA \cdot H_2O$	60.09 (60.12	5.85 5.76	6.78 6.71)	0.48	0.16
H-*t*-AMCHA-2-Nal-APAA	**9**	78.3	158—160	+32.0 (c=0.50)	488.4	$C_{29}H_{33}N_3O_4 \cdot TFA \cdot H_2O$	60.09 (63.05	5.85 5.70	6.78 6.56)	0.45	0.40
H-*t*-AMCHA-Bpa-APAA	**10**	74.4	130—135	+18.0 (c=0.89)	NA[c]	$C_{32}H_{35}N_3O_5 \cdot 0.8TFA \cdot 0.4H_2O$	63.16 (65.03	6.09 8.28	6.62 10.4)	0.38	0.33
H-*t*-AMCHA-Phe-APAA-NH_2	**11**	98.0	214—216	+17.6 (c=0.82)	437.7	$C_{25}H_{32}N_4O_3 \cdot 1.3TFA$	56.68 (56.87	5.73 5.81	9.58 9.81)	0.17	0.50
H-*t*-AMCHA-Phe-NH_2	**12**	95.7	108—112	+5.19 (c=1.0)	304.3	$C_{17}H_{25}N_3O_2 \cdot 1.6TFA$	49.93 (49.77	5.51 5.53	8.64 8.87)	0.16	0.47
H-*t*-AMCHA-Phe-AP	**13**	98.0	109—111	+25.8 (c=0.95)	NA[c]	$C_{22}H_{28}N_4O_2 \cdot 2.5TFA$	48.72 (49.01	4.61 4.68	8.41 8.98)	0.20	0.50
H-*t*-AMCHA-Phe-AMBA	**14**	87.8	228—231	−8.95 (c=0.86)	438.0	$C_{25}H_{31}N_3O_4 \cdot TFA \cdot 0.4H_2O$	58.04 (58.31	5.91 5.78	7.52 7.80)	0.28	0.63
H-*t*-AMCHA-Phe-AcHA	**15**	87.9	113—120	−16.1 (c=1.1)	430.0	$C_{24}H_{35}N_3O_4 \cdot HCl \cdot H_2O$	59.55 (59.35	7.91 7.59	8.68 8.29)	0.31	0.59
H-*t*-AMCHA-Phe-ABA	**16**	64.1	Amorphous powder	+32.1[d] (c=0.62)	424.6	$C_{24}H_{29}N_3O_4 \cdot H_2O$	62.26 (62.20	7.03 7.00	8.37 8.55)	0.24	0.22

a) Not tested. *b*) MeOH. *c*) Not available. *d*) AcOH.

NH_2, AP] Boc-*t*-AMCHA-Phe-X was treated with TFA (1.0 ml, 13 mmol)–anisole (0.10 ml, 1.0 mmol) as usual. Ether was added to the mixture to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 4.

H-*t*-AMCHA-Phe-AMBA Boc-*t*-AMCHA-Phe-AMBA-OBzl (0.31 g, 0.51 mmol) was treated with TFA (1.2 ml, 15 mmol)–anisole (0.20 ml, 1.8 mmol) as usual. Ether was added to the mixture to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. The resulting powder was hydrogenated over a palladium catalyst. After removal of palladium and the solvent, ether was added to the residue to give a white precipitate, which was collected by centrifugation. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 4.

H-*t*-AMCHA-Phe-AcHA Boc-*t*-AMCHA-Phe-AcHA-OBzl (0.20 g, 0.32 mmol) in MeOH (10 ml) was hydrogenated over a palladium catalyst. After removal of palladium and the solvent, ether and petroleum ether were added to the residue to give a white powder, which was collected by filtration. The resulting powder was treated with 3.4 N HCl (1.0 ml, 3.4 mmol) as usual. Ether was added to the mixture to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 4.

H-*t*-AMCHA-Phe-ABA Boc-*t*-AMCHA-Phe-ABA-OMe (0.54 g, 1.0 mmol) was treated with TFA (3.8 ml, 50 mmol)–anisole (0.40 ml, 3.6 mmol) as usual. Ether was added to the mixture to give a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. The resulting TFA salt was dissolved in DMSO (7 ml) containing 2 N NaOH (5.0 ml, 10 mmol). The reaction mixture was stirred at room temperature for 90 min. The pH of the solution was adjusted to 5—6 with acetic acid to give a white precipitate, which was collected by centrifugation. The resulting product was dissolved in 20% AcOH. The solution was lyophilized to afford an amorphous powder. Yield, mp, $[\alpha]_D^{25}$ values, elemental analysis and *Rf* values are summarized in Table 4.

Amidolytic Assay Amidolytic assay for the synthetic peptides was carried out at 37 °C in 0.05 M Tris–HCl buffer (pH 7.8 for PK, pH 7.4 for plasmin, pH 8.3 for thrombin or pH 8.8 for urokinase) or Tris–imidazole buffer (pH 8.1 for trypsin). The rate of reaction was determined from the hydrolysis rate by measuring the absorbance at 405 nm of *p*-nitroaniline released from D-Pro-Phe-Arg-*p*NA (s-2302, at the final concentration of 0.2 mM with PK), D-Val–Leu-Lys-*p*NA (s-2251, at the final concentration of 0.3 mM with plasmin), D-Phe-Pip-Arg-*p*NA (s-2238, at the final concentration of 0.02 mM with thrombin), Glp-Gly-Arg-*p*NA (s-2444, at the final concentration of 0.1 mM with urokinase) and D-Phe-Pip-Arg-*p*NA (s-2238, at the final concentration of 0.1 mM with trypsin). The IC_{50} values were determined, after incubation of various concentrations of each peptide with each enzyme, as the concentration of peptide which inhibited the enzyme activity by 50%.

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References and Notes

1) The customary L indication for amino acid residues is omitted; only the D isomer is indicated. Standard abbreviations for amino acid and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485—2489 (1966); *ibid.*, **6**, 362—364 (1967); *ibid.*, **11**, 1726—1732

(1976). Other abbreviations used are: ABA, 4-aminobenzoic acid; Ac, acetyl; AcHA, 1-amino-1-cyclohexanecarboxylic acid; AcOEt, ethyl acetate; AcOH, acetic acid; AMBA, 4-aminomethylbenzoic acid; *t*-AMCHA, *trans*-aminomethylcyclohexanecarboxylic acid; AP, 4-aminopyridine; APAA, 4-aminophenylacetic acid; Bpa, *p*-benzoylphenylalanine; Boc, *tert*-butyloxycarbonyl; *n*-BuOH, *n*-butanol; Cha, β-cyclohexylalanine; 2-Cl-Z, 2-chlorobenzyloxycarbonyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EACA, ε-aminocaproic acid; Et_3N, triethylamine; Glp, pyroglutamic acid; Hph, homophenylalanine; 1-Nal, β-1-naphthylalanine; 2-Nal, β-2-naphthylalanine; OBzl, benzyl ester; OMe, methyl ester; Phg, phenylglycine; Pip, pipecolinic acid; PK, plasma kallikrein; PL, plasmin; TFA, trifluoroacetic acid; TH, thrombin; THF, tetrahydrofuran; TosOH, *p*-toluenesulfonic acid; UK, urokinase.

2) DeLa Cadena R. A., Wachtfogel Y. T., Colman R. W., "Hemostasis and Thrombosis; Basic Principles and Clinical Practice," 3rd Ed., eds. by Colman R. W., Hirsh J., Marder V. J., Salzman E. W., J. B. Lippincott Company, Philadelphia, 1994, pp. 219—240.
3) Bhoola K. D., Figueroa C. D., Worthy K., *Pharmacol. Rev.*, **44**, 1—80 (1992).
4) Bhoola K. D., Elson C. J., Dieppe P. A., *Br. J. Rheumatol.*, **31**, 509—518 (1992).
5) Wanaka K., Okada Y., Tsuda Y., Okamoto U., Hijikata-Okunomiya A., Okamoto S., *Chem. Pharm. Bull.*, **40**, 1814—1817 (1992).
6) Wanaka K., Okamoto S., Bohgaki M., Hijikata-Okunomiya A., Naito T., Okada Y., *Thromb. Res.*, **57**, 889—895 (1990).
7) Katsuura Y., Okamoto S., Ohno N., Wanaka K., *Thromb. Res.*, **82**, 361—368 (1996).
8) Nakamura M., Tomoo K., Ishida T., Taguchi H., Tsuda Y., Okada Y., Okunomiya A., Wanaka K., Okamoto S., *Biochem. Biophys. Res. Commun.*, **213**, 583—587 (1995).
9) mp 159—161 °C. *Anal.* Calcd for $C_{13}H_{18}N_2O_3 \cdot 0.1H_2O$: C, 61.9; H, 7.27; N, 11.1. Found: C, 61.8; H, 7.16; N, 11.1.
10) mp 84.5—85.5 °C. *Anal.* Calcd for $C_{20}H_{23}NO_4$: C, 70.4; H, 6.79; N, 4.10. Found: C, 70.4; H, 6.86; N, 3.90.
11) mp 95—96 °C. *Anal.* Calcd for $C_{19}H_{27}NO_4$: C, 68.4; H, 8.16; N, 4.21. Found: C, 68.4; H, 8.25; N, 4.18.