Synthesis of Tripeptide Chloromethyl Ketones and Examination of Their Inhibitory Effects on Plasmin and Plasma Kallikrein[1)]

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With the aim of obtaining selective synthetic inhibitors of plasmin and plasma kallikrein, D-Ile–Phe–Lys–CH_2Cl, Ile–Phe–Lys–CH_2Cl, D-Ile–Phe–Arg–CH_2Cl and Ile–Phe–Arg–CH_2Cl were synthesized and their inhibitory activity against plasmin, plasma kallikrein and other trypsin-like serine proteinases was examined. Among them, D-Ile–Phe–Arg–CH_2Cl exhibited a highly selective inhibitory activity against plasma kallikrein, yet D-Ile–Phe–Lys–CH_2Cl exhibited nearly the same order of inhibitory activity against plasmin as well as plasma kallikrein.

Keywords synthetic inhibitor; substrate-derived inhibitor; chloromethyl ketone derivative; selective inhibitor; chemical synthesis; plasma kallikrein; plasmin; trypsin-like serine proteinase

It is known that plasmin preferentially hydrolyzes the lysyl-peptide bonds in physiological substrates, while thrombin, plasma kallikrein, urokinase and factor Xa preferentially hydrolyze the arginyl-peptide bonds, whereas trypsin hydrolyzes both bonds.[2)] Because of their important physiological roles, these enzymes have been extensively studied. Nevertheless, their pathologic roles are not satisfactorily understood in detail. For advanced studies on these enzymes, specific synthetic substrates as well as inhibitors would represent very useful tools. The present report deals with the synthesis of substrate-derived chloromethyl ketone derivatives and their inhibitory effect on the trypsin-like serine proteinases, including plasmin and plasma kallikrein.

Prior to designing specific inhibitors, some specific substrates were designed and synthesized. It was reported that D-Ile–Phe–Lys–*p*NA and Ile–Phe–Lys–*p*NA were effective and specific substrates for plasmin ($K_m = 0.020$ and 0.33 mM, respectively),[3,4)] which indicated to us that in the substrates mentioned above, substitution of the Lys residue with Arg would offer some appropriate substrates. Therefore, we prepared D-Ile–Phe–Arg–*p*NA and Ile–Phe–Arg–*p*NA in the same manner as described for the synthesis of lysine *p*-nitroanilides[3)] and their efficiency as substrates for the enzymes was examined.

The results obtained are shown in Table I in comparison with those for D-Ile–Phe–Lys–*p*NA and Ile–Phe–Lys–*p*NA. D-Ile–Phe–Lys–*p*NA and Ile–Phe–Lys–*p*NA are specific substrates for plasmin and D-Ile–Phe–Arg–*p*NA and Ile–Phe–Arg–*p*NA are fairly specific substrates for plasma kallikrein. These results were suggestive for designing specific irreversible inhibitors against plasmin and plasma kallikrein. Such an approach had been reported in previous papers.[5,6)] By combination of the peptide moiety of the specific substrate with chloromethyl ketone, four kinds of irreversible inhibitors, D-Ile–Phe–Lys–CH_2Cl, Ile–Phe–Lys–CH_2Cl, D-Ile–Phe–Arg–CH_2Cl and Ile–Phe–Arg–CH_2Cl, were designed.

Peptide chloromethyl ketones were synthesized by the same procedure as described preivously.[6)] As an example, the synthetic route to Ile–Phe–Arg–CH_2Cl is shown in Fig. 1. The homogeneity of synthetic intermediates and final peptide chloromethyl ketones was ascertained by thin-layer chromatography (TLC) on silica gel and by amino acid and elemental analyses.

Next, the inhibitory effect on amidolytic activity of several kinds of trypsin-like enzymes was examined (Table II). The k_2/K_i value was used as the efficiency index of the chloromethyl ketones. The dissociation constants (K_i) and alkylation rate constants (k_2) were determined by the method of Kitz and Willson.[7)] As shown in Table II, D-Ile–Phe–Lys–CH_2Cl is an extremely potent inhibitor of plasmin ($k_2/K_i = 77000\ M^{-1}s^{-1}$), the value being 15 times that of Ile–Phe–Lys–CH_2Cl ($k_2/K_i = 5200$). This result seems to be compatible with the result obtained with the corresponding *p*-nitroanilides as substrates ($k_{cat}/K_m = 500000$ and 58000, respectively). However, the k_2/K_i values of D-Ile–Phe–Lys–CH_2Cl for plasmin and plasma kallikrein are similar (77000 and 49000, respectively), although the k_{cat}/K_m values of D-Ile–Phe–Lys–*p*NA for plasmin and plasma kallikrein are different (500000 and 43000 respectively). This indicates that the Lys–CH_2Cl group at the P_1 and P_1' positions[8)] does not discriminate the difference of topograpy at the S_1 and S_1' positions between plasmin and plasma kallikrein, despite the fact that the Lys–*p*NA moiety can do so.

As also shown in Table II, D-Ile–Phe–Arg–CH_2Cl inhibits plasma kallikrein selectively. The k_2/K_i value is

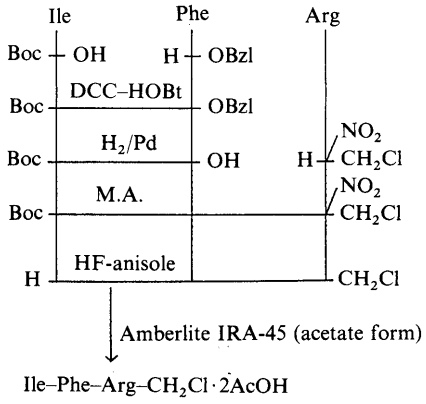

Fig. 1. Synthetic Route to Ile–Phe–Arg–CH_2Cl

This paper is dedicated to Professor Haruaki Yajima on the occasion of his retirement from Kyoto University in March, 1989.

TABLE I. Kinetic Parameters for the Amidolysis of Substrate by Trypsin-like Serine Proteinases

	Human plasmin	Bovine thrombin	Human plasma kallikrein	Human urokinase	Bovine factor Xa	Bovine trypsin
D-Ile–Phe–Lys–*p*NA						
k_{cat} (s^{-1})	10	—	43	—	ND	13
K_m (M) × 10^6	20	—	1000	—	ND	210
k_{cat}/K_m ($M^{-1}s^{-1}$)	500000	—	43000	—		61000
(Index)	(100)		(8.6)			(12)
Ile–Phe–Lys–*p*NA						
k_{cat} (s^{-1})	19	—	—	—	ND	5.9
K_m (M) × 10^6	330	—	—	—	ND	580
k_{cat}/K_m ($M^{-1}s^{-1}$)	58000	—	—	—		10000
(Index)	(100)					(17)
D-Ile–Phe–Arg–*p*NA						
k_{cat} (s^{-1})	22	2.4×10^{-8} *a)*	67	—	1.3×10^{-7} *b)*	74
K_m (M) × 10^6	120	250	57	—	630	130
k_{cat}/K_m ($M^{-1}s^{-1}$)	180000		1200000			570000
(Index)	(15)		(100)			(48)
Ile–Phe–Arg–*p*NA						
k_{cat} (s^{-1})	7	—	57	—	7.3×10^{-8} *b)*	35
K_m (M) × 10^6	450	—	78	—	1300	360
k_{cat}/K_m ($M^{-1}s^{-1}$)	16000		730000			97000
(Index)	(2)		(100)			(13)

—, not detectable; ND, not determined. *a)* V_{max}, mol·min^{-1}·U^{-1}. *b)* V_{max}, mol·min^{-1}·$ampoule^{-1}$.

TABLE II. Kinetic Constants of the Inhibition of Serine Proteinases by Peptide Chloromethyl Ketones

	Human plasmin	Bovine thrombin	Human plasma kallikrein	Human urokinase	Bovine factor Xa	Bovine trypsin
D-Ile–Phe–Lys–CH_2Cl						
k_2 (s^{-1}) × 10^2	4.4	3.8	5.9	—	89	9.0
K_i (M) × 10^6	0.57	14	1.2	—	830	4.8
k_2/K_i ($M^{-1}s^{-1}$)	77000	2700	49000	<1	1100	19000
(Index)	(100)	(3.5)	(64)	(<1)	(1.3)	(25)
Ile–Phe–Lys–CH_2Cl						
k_2 (s^{-1}) × 10^2	3.2	5.0	3.9	—	8.2	8.4
K_i (M) × 10^6	6.2	400	7.7	—	1800	40
k_2/K_i ($M^{-1}s^{-1}$)	5200	130	5100	<1	46	2100
(Index)	(100)	(2.5)	(98)	(<1)	(<1)	(40)
D-Ile–Phe–Arg–CH_2Cl						
k_2 (s^{-1}) × 10^2	1.4	1.4	2.7	—	2.6	1.3
K_i (M) × 10^6	33	32	0.48	—	110	3.1
k_2/K_i ($M^{-1}s^{-1}$)	420	440	56000	<1	240	4200
(Index)	(<1)	(<1)	(100)	(<1)	(<1)	(7.5)
Ile–Phe–Arg–CH_2Cl						
k_2 (s^{-1}) × 10^2	2.4	2.0	3.6	—	3.6	1.9
K_i (M) × 10^6	180	170	4.1	—	410	20
k_2/K_i ($M^{-1}s^{-1}$)	130	120	8800	<1	88	950
(Index)	(1.5)	(1.4)	(100)	(<1)	(1)	(11)

—, not detectable.

56000, while the value for plasmin is 420. Thus, the difference in the k_2/K_i values is more than 100-fold, while the difference in k_2/K_i values for Pro–Phe–Lys–CH_2Cl reported previously,[9] was 60-fold. This selectivity is much greater than that between the k_{cat}/K_m values of D-Ile–Phe–Arg–*p*NA for plasma kallikrein and plasmin (1200000 and 180000, respectively), indicating that the Arg–CH_2Cl moiety at the P_1 and P_1' positions could distinguish the topographies of the active centers of plasma kallikrein and plasmin more precisely than the Arg–*p*NA moiety. No significant inhibition by these inhibitors was observed toward bovine thrombin, human urokinase and bovine factor Xa.

In conclusion, the combination of the D-Ile–Phe–Lys moiety and the CH_2Cl group produced a potent irreversible inhibitor of plasmin and plasma kallikrein. This inhibitor apparently did not inhibit bovine thrombin, human urokinase or bovine factor Xa. D-Ile–Phe–Arg–CH_2Cl is a very potent and selective inhibitor of plasma kallikrein and might be a useful tool for research on plasma kallikrein.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DPI-360 (Japan Spectroscopic Co.,

Ltd.). Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 18 h) were determined with an amino acid analyzer (K-101AS, Kyowa Seimitsu). The preparative high performance liquid chromatography (HPLC) was performed with a Waters Associates model 440, using a column on YMC R-ODS-5 (20 × 250 mm) with MeOH–water (7.5 : 2.5) as the eluant. On TLC (Kieselgel G, 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), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase) and *n*-BuOH, AcOH, pyridine and H_2O (4 : 1 : 1 : 2), respectively.

Boc–Phe–Arg(NO_2)–*p*NA A mixed anhydride[10] [prepared from Boc–Phe–OH (2.0 g, 7.4 mmol), *N*-methylmorpholine (0.81 ml, 7.4 mmol) and isobutyl chloroformate (0.97 ml, 7.4 mmol) at −15 °C] in THF (20 ml) was added to a solution of H–Arg(NO_2)–*p*NA·HBr [prepared from Z–Arg(NO_2)–*p*NA[11] (4.7 g, 10 mmol) and 25% HBr–AcOH (9.6 ml, 30 mmol)] in DMF (20 ml) containing Et_3N (1.0 ml, 7.4 mmol). The reaction mixture was stirred at −15 °C for 1 h and at 4 °C for 15 h. After removal of the solvent, the residue was extracted with AcOEt and the extract was washed with 5% $NaHCO_3$, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a precipitate, which was collected by filtration. The gelatinous product was recrystallized from EtOH, yield 2.5 g (43%), mp 154—157 °C, $[\alpha]_D^{25}$ −18.7° (*c* = 0.8, MeOH), Rf^1 0.40, Rf^2 0.36. *Anal.* Calcd for $C_{26}H_{34}N_8O_8 \cdot 0.5H_2O$: C, 52.4; H, 5.92; N, 18.8. Found: C, 52.7; H, 5.83; N, 19.1.

Boc–Ile–Phe–Arg(NO_2)–*p*NA The title compound was prepared from Boc–Ile–OH (0.25 g, 1.1 mmol) and H–Phe–Arg(NO_2)–*p*NA·HCl [prepared from Boc–Phe–Arg(NO_2)–*p*NA (0.64 g, 1.1 mmol] by the mixed anhydride method, yield 0.33 g (44%), mp 195—199 °C, $[\alpha]_D^{25}$ −8.2° (*c* = 1.0, DMF), Rf^1 0.52, Rf^2 0.52. Anal. Calcd for $C_{32}H_{45}N_9O_9$: C, 54.9; H, 6.47; N, 18.0. Found: C, 54.5; H, 6.38; N, 18.1.

Boc–D-Ile–Phe–Arg(NO_2)–*p*NA The title compound was prepared from Boc–D-Ile–OH (0.27 g, 1.2 mmol) and H–Phe–Arg(NO_2)–*p*NA·HCl [prepared from Boc–Phe–Arg(NO_2)–*p*NA (0.7 g, 1.2 mmol)] by the mixed anhydride method. The crude material was purified by column chromatography on silica gel, yield 0.25 g (39%), mp 139—142 °C, $[\alpha]_D^{25}$ +2.9° (*c* = 0.6, DMF), Rf^1 0.51, Rf^2 0.31. *Anal.* Calcd for $C_{32}H_{45}N_9O_9 \cdot H_2O$: C, 53.5; H, 6.60; N, 17.6. Found: C, 53.6; H, 6.50; N, 17.5.

H–Ile–Phe–Arg–*p*NA A solution of Boc–Ile–Phe–Arg(NO_2)–*p*NA (100 mg, 0.15 mmol) in HF (5 ml) containing anisole (0.5 ml) was stirred at 0 °C for 60 min. After removal of HF, the residue was dried over KOH pellets *in vacuo*. The residue was dissolved in H_2O (20 ml). The solution was treated with Amberlite IRA-45 (acetate form). After removal of the resin by filtration, the filtrate was washed with ether and lyophilized to afford an amorphous powder, yield 90 mg (90%), $[\alpha]_D^{25}$ −7.6° (*c* = 0.8, H_2O), Rf^4 0.14, Rf^5 0.56. *Anal.* Calcd for $C_{27}H_{38}N_8O_5 \cdot 2CH_3COOH \cdot H_2O$: C, 53.7; H, 6.98; N, 16.2. Found: C, 53.7; H, 6.77; N, 16.4. Amino acid ratios in an acid hydrolysate: Ile 0.76, Phe 0.91, Arg 1.00 (average recovery 95.5%).

H–D-Ile–Phe–Arg–*p*NA The title compound was prepared from Boc–D-Ile–Phe–Arg(NO_2)–*p*NA (50 mg, 0.073 mmol) and HF (5 ml) containing anisole (0.5 ml) in the same manner as described for the synthesis of H–Ile–Phe–Arg–*p*NA, yield 35 mg (70%), $[\alpha]_D^{25}$ −30.5° (*c* = 0.4, H_2O), Rf^4 0.26, Rf^5 0.52. *Anal.* Calcd for $C_{27}H_{38}N_8O_5 \cdot 2CH_3COOH \cdot H_2O$: C, 53.7; H, 6.98; N, 16.2. Found: C, 53.7; H, 6.86; N, 16.0. Amino acid ratios in an acid hydrolysate: Ile 0.77, Phe 0.92, Arg 1.00 (average recovery 80.9%).

Boc–Lys(Z)–CH_2Cl Diazomethane [prepared from nitrosomethylurea (9.0 g, 48 mmol)] was added to a mixed anhydride [prepared from Boc–Lys(Z)–OH (18.3 g, 48 mmol), Et_3N (6.7 ml, 48 mmol) and ethyl chloroformate (4.3 ml, 48 mmol)] in THF (200 ml) at −15 °C and the reaction mixture was stirred at 4 °C for 15 h. Then 7.2 N HCl–dioxane (20 ml, 140 mmol) was added to the above solution at −15 °C and the reaction mixture was stirred at −15 °C for 30 min. After neutralization of the solution with Et_3N and removal of the solvent, the residue was dissolved in AcOEt. This solution was washed with 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give crystals, which were recrystallized from EtOH, yield 12.6 g (63.6%), mp 101—103 °C, $[\alpha]_D^{25}$ −21.9° (*c* = 1.1, MeOH), Rf^1 0.7. *Anal.* Calcd for $C_{28}H_{29}ClN_2O_5$: C, 58.2; H, 7.03; N, 6.79. Found: C, 58.3; H, 7.15; N, 6.71.

Boc–Arg(NO_2)–CH_2Cl The title compound was prepared from Boc–Arg(NO_2)–OH (3.2 g, 10 mmol) in the same manner as described above. The crude material was purified by column chromatography on silica gel, yield 0.90 g (26%), mp 78—83 °C, $[\alpha]_D^{25}$ −25.8° (*c* = 1.0, MeOH), Rf^1 0.27, Rf^2 0.34. *Anal.* Calcd for $C_{12}H_{22}ClN_5O_5$: C, 41.0; H, 6.30; N, 19.9. Found: C, 41.3; H, 6.47; N, 19.8.

Boc–Ile–Phe–OBzl Boc–Ile–OH (2.3 g, 10 mmol), HOBt[12] (1.4 g 10 mmol) and H–Phe–OBzl [prepared from H–Phe–OBzl·TosOH (4.3 g, 10 mmol) and Et_3N (1.4 ml, 10 mmol)] were dissolved DMF (20 ml). DCC (2.3 g, 11 mmol) was added to the above cold solution and the reaction mixture was stirred at −15 °C for 1 h and at 4 °C for 15 h. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 3.2 g (68%), mp 133—134 °C, $[\alpha]_D^{25}$ −35.0° (*c* = 0.8, MeOH), Rf^1 0.79, Rf^2 0.80. *Anal.* Calcd for $C_{27}H_{36}N_2O_5$: C, 69.2; H, 7.74; N, 5.98. Found: C, 69.2; H, 7.89; N, 6.13.

Boc–D-Ile–Phe–OBzl The title compound was prepared from Boc–D-Ile–OH (1.0 g, 4.3 ml) and H–Phe–OBzl·TosOH (1.8 g, 4.3 mmol) in the same manner as described above, yield 1.6 g (53%), mp 135—137 °C, $[\alpha]_D^{25}$ −0.4° (*c* = 0.9, MeOH), Rf^1 0.84, Rf^2 0.78. *Anal.* Calcd for $C_{27}H_{36}N_2O_5$: C, 69.2; H, 7.74; N, 5.98. Found: C, 69.2; H, 7.76; N, 5.98.

Boc–Ile–Phe–OH Boc–Ile–Phe–OBzl (2.0 g, 4.3 mmol) in MeOH (40 ml) was hydrogenated over Pd catalyst. After removal of Pd and the solvent, ether was added to the oily residue to give a precipitate, which was collected by filtration, yield 1.1 g (69%), mp 131—132.5 °C, $[\alpha]_D^{25}$ −15.7° (*c* = 0.9, MeOH), Rf^1 0.60, Rf^2 0.11. *Anal.* Calcd for $C_{20}H_{30}N_2O_5$: C, 63.5; H, 7.99; N, 7.40. Found: C, 63.5; H, 8.06; N, 7.35.

Boc–D-Ile–Phe–OH The title compound was prepared from Boc–D-Ile–Phe–OBzl (0.52 g, 1.0 mmol) in the same manner as described above, yield 0.33 g (82%), mp 136.5—138 °C, $[\alpha]_D^{25}$ +11.3° (*c* = 0.5, MeOH), Rf^1 0.60, Rf^2 0.13. *Anal.* Calcd for $C_{20}H_{30}N_2O_5$: C, 63.5; H, 7.99; N, 7.40. Found: C, 63.3; H, 7.85; N, 7.33.

Boc–Ile–Phe–Lys(Z)–CH_2Cl The title compound was prepared from Boc–Ile–Phe–OH (0.43 g, 1.2 mmol) and H–Lys(Z)–CH_2Cl·HCl [prepared from Boc–Lys(Z)–CH_2Cl (0.60 g, 1.5 mmol)] by DCC–HOBt method.[12] The crude material was purified by column chromatography on silica gel, yield 0.12 g (16%), mp 154—157 °C, $[\alpha]_D^{25}$ −42.2° (*c* = 1.0, DMF), Rf^1 0.65, Rf^2 0.42. *Anal.* Calcd for $C_{35}H_{49}ClN_4O_7$: C, 62.4; H, 7.33; N, 8.33. Found: C, 62.4; H, 7.47; N, 8.41. Amino acid ratios in an acid hydrolysate: Ile 0.93, Phe 1.00, Lys 0.06 (recovery of Phe 86%).

Boc–D-Ile–Phe–Lys(Z)–CH_2Cl The title compound was prepared from Boc–D-Ile–Phe–OH (0.3 g, 0.79 mmol) and H–Lys(Z)–CH_2Cl·HCl [prepared from Boc–Lys(Z)–CH_2Cl (0.41 g, 1.0 mmol)] in the same manner as described above. The crude material was purified by gel-filtration on Sephadex LH-20, yield 0.07 g (13%), mp 178—180 °C, $[\alpha]_D^{25}$ −36.9° (*c* = 0.6, DMF), Rf^1 0.66, Rf^2 0.55. *Anal.* Calcd for $C_{35}H_{49}ClN_4O_7$: C, 62.4; H, 7.33: N, 8.32. Found: C, 62.7; H, 7.50; N, 8.43. Amino acid ratios in an acid hydrolysate: Ile 0.89, Phe 1.00, Lys 0.04 (recovery of Phe 60%).

Boc–Ile–Phe–Arg(NO_2)–CH_2Cl The title compound was prepared from Boc–Ile–Phe–OH (0.76 g, 2.0 mmol) and H–Arg(NO_2)–CH_2Cl·HCl [prepared from Boc–Arg(NO_2)–CH_2Cl (0.70 g, 2.0 mmol)] by the mixed anhydride method. The crude material was purified by gel-filtration on Sephadex LH-20, yield 0.51 g (41%), mp 159—162 °C, $[\alpha]_D^{25}$ −39.2° (*c* = 0.3, DMF), Rf^1 0.40, Rf^2 0.19. *Anal.* Calcd for $C_{27}H_{42}ClN_2O_7 \cdot 0.5H_2O$: C, 52.2; H, 6.97; N, 15.8. Found: C, 52.4; H, 6.92; N, 15.8. Amino acid ratios in an acid hydrolysate: Ile 1.00, Phe 1.00, Arg 0 (recovery of Phe 80%).

Boc–D-Ile–Phe–Arg(NO_2)–CH_2Cl The title compound was prepared from Boc–D-Ile–Phe–OH (0.76 g, 2.0 mmol) and H–Arg(NO_2)–CH_2Cl·HCl [prepared from Boc–Arg(NO_2)–CH_2Cl (0.70 g, 2.0 mmol)]. The crude material was purified by preparative HPLC on a column (20 × 250 mm) of YMC R-ODS-5 using MeOH and H_2O (7.5 : 2.5), yield 0.036 g (4.1%), mp 151—154 °C, $[\alpha]_D^{25}$ −28.7° (*c* = 0.1, DMF), Rf^1 0.41, Rf^2 0.24. *Anal.* Calcd for $C_{27}H_{42}ClN_4O_7$: C, 53.0; H, 6.92; N, 16.0. Found: C, 53.0; H, 7.02; N, 16.0. Amino acid ratios in an acid hydrolysate: Ile 1.00, Phe 1.00, Arg 0.23 (recovery of Phe 74%).

H–Ile–Phe–Lys–CH_2Cl Boc–Ile–Phe–Lys(Z)–CH_2Cl (90 mg, 0.13 mmol) was dissolved in 25% HBr–AcOH (1.0 ml, 1.0 mmol) containing anisole (0.10 ml) and the solution was kept at room temperature for 1 h. Ether was added to the solution to form an amorphous powder, which was collected by centrifugation and dried over KOH pellets *in vacuo*, yield 47 mg (78%), $[\alpha]_D^{25}$ +81.2° (*c* = 0.6, H_2O), Rf^4 0.27, Rf^5 0.43. *Anal.* Calcd for $C_{22}H_{35}ClN_4O_3 \cdot 2HBr \cdot 1.5H_2O$: C, 42.1; H, 6.42; N, 8.92. found: C, 42.1; H, 6.55; N, 8.67. Amino acid ratios in an acid hydrolysate: Ile 0.84, Phe 1.00, Lys 0.06 (recovery of Phe 79%).

H–D-Ile–Phe–Lys–CH_2Cl The title compound was prepared from Boc–D-Ile–Phe–Lys(Z)–CH_2Cl (0.12 g, 0.18 mmol) in the same manner as described above, yield 0.033 g (55%), $[\alpha]_D^{25}$ −29.0° (*c* = 1.1, H_2O), Rf^4 0.33,

Rf^5 0.44. *Anal.* Calcd for $C_{22}H_{35}ClN_4O_3 \cdot 2HBr \cdot 2H_2O$: C, 41.5; H, 6.48; N, 8.79. Found: C, 41.4; H, 6.15; N, 8.35. Amino acid ratios in an acid hydrolysate: Ile 0.80, Phe 1.00, Lys 0.07 (recovery of Phe 98%).

H–Ile–Phe–Arg–CH_2Cl A solution of Boc–Ile–Phe–Arg(NO_2)–CH_2Cl (0.10 g, 0.15 mmol) in HF (5 ml) containing anisole (0.5 ml) was stirred at 0 °C for 1 h. After removal of HF, the residue was dried over KOH pellets *in vacuo*. A solution of the product in H_2O (10 ml) was washed with ether and treated with Amberlite IRA-45 (acetate form). After removal of the resin by filtration, the filtrate was lyophilized to afford an amorphous powder, yield 0.055 g (59%), $[\alpha]_D^{25}$ +4.0° (c=0.7, H_2O), Rf^4 0.26, Rf^5 0.44. *Anal.* Calcd for $C_{22}H_{35}ClN_6O_3 \cdot 2CH_3COOH \cdot H_2O$: C, 51.9; H, 7.54; N, 14.0. Found: C, 51.5; H, 7.53; N, 14.1.

H–D-Ile–Phe–Arg–CH_2Cl The title compound was prepared from Boc-D-Ile–Phe–Arg(NO_2)–CH_2Cl (0.05 g, 0.073 mmol) in the same manner as described above, yield 0.034 g (78%), $[\alpha]_D^{25}$ −8.8° (c=0.5, H_2O), Rf^4 0.26, Rf^5 0.46. *Anal.* Calcd for $C_{22}H_{35}ClN_6O_3 \cdot 2CH_3COOH \cdot H_2O$: C, 52.4; H, 7.44; N, 14.1. Found: C, 52.3; H, 7.54; N, 14.5. Amino acid ratios in an acid hydrolysate: Ile 0.71, Phe 1.00, Arg 0.22 (recovery of Phe 75%).

Assay Procedure The enzymes used were as follows: human plasmin and plasma kallikrein (KABI Co.), bovine thrombin (Mochida Seiyaku Co.), human urokinase (Midorijyuji Co.), bovine factor Xa (Diagnostic Reagents) and bovine trypsin (Sigma Chemical Co.). Concentrations of plasmin and trypsin were determined by active-site titration using *p*-nitrophenyl *p*-guanidinobenzoate according to the procedure reported by Chase and Shaw.[13] Concentration of plasma kallikrein was cited from the data of KABI Co. The enzyme reaction was carried out in 0.05 M Tris–HCl buffer. The pH of the buffer was adjusted for each enzyme (7.4 for plasmin, 8.3 for thrombin and factor Xa, 7.8 for plasma kallikrein, 8.8 for urokinase and 8.2 for trypsin). Kinetic constants, K_m and k_{cat}, were determined by the method described previously[4] by measuring the *p*-nitroaniline (E_{405}) released from *p*-nitroanilides. The dissociation constants (K_i) and alkylation rate constants (k_2) were determined according to the method described Kitz and Willson.[7] Inactivation of the enzymes with chloromethyl ketone derivatives was performed at 25 °C, and remaining enzyme activities were assayed using the specific chromogenic substrates purchased from KABI Co. Plasmin activity was measured with D-Val–Leu–Lys–*p*NA (s-2251) at the final concentration of 0.3 mM, thrombin with D-Phe–Pip–Arg–*p*NA (s-2238) at final concentration of 0.08 mM, plasma kallikrein with D-Pro–Phe–Arg–*p*NA (s-2302) at final concentration of 0.2 mM, urokinase with Glp–Gly–Arg–*p*NA (s-2444) at final concentration of 0.3 mM, factor Xa and trypsin with Bz–Ile–Glu(γ-OR)–Gly–Arg–*p*NA (s-2222) at final concentration of 0.2 mM.

References and Notes

1) The customary L configuration for amino acid residues is omitted; only D isomers are indicated. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bz, benzoyl; *p*NA, *p*-nitroanilide; HOBt, *N*-hydroxybenzotriazole; AcOH, acetic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; AcOEt, ethyl acetate; THF, tetrahydrofuran; *n*-BuOH, 1-butanol.
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