[Chem. Pharm. Bull. 35(9)3576—3584(1987)]

Synthesis of Peptide Chloromethyl Ketones and Examination of Their Inhibitory Effects on Human Spleen Fibrinolytic Proteinase (SFP) and Human Leukocyte Elastase (LE)[1]

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(Received February 12, 1987)

Various substrate-derived chloromethyl ketones were synthesized by a conventional method for the purpose of obtaining specific and potent irreversible inhibitors for human spleen fibrinolytic proteinase (SFP) and human leukocyte elastase (LE) in order to compare the properties of SFP with those of LE. It was found that Boc–Ala–Tyr–Leu–Val–CH_2Cl among the peptide chloromethyl ketones exhibited the most effective and specific inhibition of SFP and LE. The two enzymes were inhibited by peptide chloromethyl ketones having a Val residue at the C-terminus in a similar manner, demonstrating a similarity between SFP and LE.

Keywords——human spleen fibrinolytic proteinase; human leukocyte elastase; peptide chloromethyl ketone; chemical synthesis; specific inhibition

Human spleen fibrinolytic proteinase (SFP)[2] and human leukocyte elastase (LE) have recently attracted our interest as non-plasmin fibrinolytic proteinases. The latter enzyme is responsible for the tissue destruction that occurs in pulmonary emphysema[3] and in inflammation.[4] Previously, we reported that Suc–Tyr–Leu–Val–*p*NA and Suc–Ala–Tyr–Leu–Val–*p*NA were specific substrates for SFP (k_{cat}/K_m=22600 and 84000 $M^{-1}s^{-1}$, respectively)[5-7] as well as LE (k_{cat}/K_m=17600 and 48500 $M^{-1}s^{-1}$, respectively). It was also indicated that the substrate specificities of these enzymes are similar. In addition, it was shown that stereoisomers of Suc–Tyr–Leu–Val–*p*NA, except for Suc–D-Tyr–Leu–Val–*p*NA, exhibited reversible inhibitory activity on both enzymes in a similar manner. In order to clarify further the enzymatic properties and physiological roles of SFP and LE, more potent and selective inhibitors were required.

Specific inhibitors are useful tools in understanding the properties and physiological roles of enzymes. For example, diisopropylphosphofluoridate (DIPF)[8] reacts stoichiometrically with the active site serine residue of serine proteases, thus making it useful in the initial characterization of an enzyme as a serine protease. On the other hand, Tos–Phe–CH_2Cl and Tos–Lys–CH_2Cl, developed by Shaw and his coworkers,[9] have proved to be inhibitors of chymotrypsin and trypsin, respectively. Substrate-derived chloromethyl ketones appeared to be candidates for use as potent and selective inhibitors against the corresponding enzymes.

In order to obtain effective and specific inhibitors against SFP and LE, we planned the synthesis of peptides having valine chloromethyl ketones at the C-terminus, because our previous studies had shown that SFP and LE cleaved valyl bonds most rapidly and specifically.[7] First of all, we designed and synthesized Boc–Tyr–Leu–Val–CH_2Cl [I], Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] and peptide chloromethyl ketones [III—VI] with substitution of Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] at the P_3 or P_5 position. Next, we prepared 8 kinds of stereoisomeric Boc–Tyr–Leu–Val–CH_2Cl [I, VII—XIII] on the basis of our previous

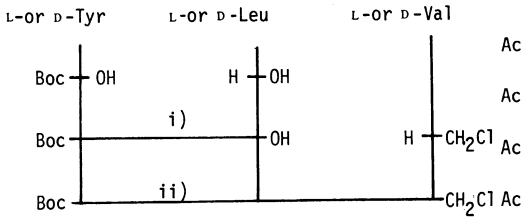

Fig. 1a. Synthetic Scheme for Tripeptide Chloromethyl Ketones

i) azide method; ii) DCC–HOBt method.

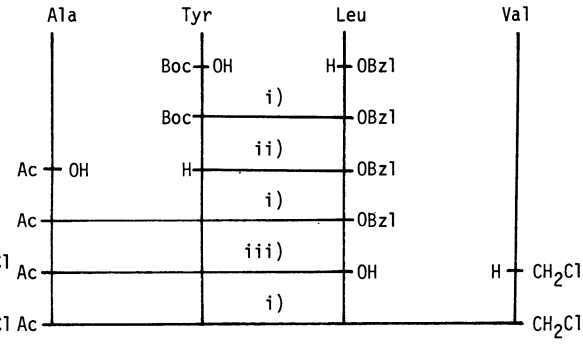

Fig. 1b. Synthetic Scheme for Tetrapeptide Chloromethyl Ketones

i) DCG–HOBt method; ii) HCl/dioxane; iii) H_2–Pd.

studies.[10] This paper deals with the synthesis of the series of peptide chloromethyl ketones, as well as their inhibitory effects on SFP in comparison with those on LE.

In earlier studies,[11-14] amino acid chloromethyl ketone was coupled with various kinds of N-protected peptides by the mixed anhydride method[15] to produce the corresponding peptide chloromethyl ketones. With regard to fragment condensation, the azide method[16] or the DCC–HOBt method[17] is generally employed in order to minimize racemization and avoid formation of the urethan-type derivative which was reported to occur during the coupling reaction between the mixed anhydride formed and the amino group of a bulky amino acid such as valine.[18] Thus, we attempted to prepare peptides having Val chloromethyl ketone at the C-terminus by the azide method. However, the resultant products were a mixture of the desired product and the urea derivative formed through Curtius rearrangement[19] judging from elemental analysis (data not shown). Therefore, the DCC–HOBt method was used to prepare peptides having valine chloromethyl ketone at the C-terminus, and purification by silica gel column chromatography was carried out, if necessary. As a typical experiment, two synthetic routes to peptide chloromethyl ketones are shown in Fig. 1a, b. The homogeneity of peptide chloromethyl ketones obtained was ascertained by thin-layer chromatography (TLC) on silica gel and amino acid and elemental analysis. The results are summarized in the experimental section.

The inhibitory effect of synthetic peptides was assayed by measuring the *p*-nitroaniline (E_{410}) released by the enzyme in the presence of the inhibitor. The kinetics of inhibition of SFP or LE by the peptide chloromethyl ketones were determined at three or four different concentrations. It appears that during the period employed, the reaction follows first-order kinetics, as shown in Fig. 2. From the slope of the curve, the inactivation rate constant (k) was calculated by using the equation, $k = 0.693/T_{1/2}$ (where $T_{1/2}$ is the apparent half life in seconds) according to Ardelt *et al.*[20] Since the k values thus obtained approximate to k_{obsd},[11-13] we employed these k values described above as k_{obsd}.

The inhibitory effects of Boc–Tyr–Leu–Val–CH_2Cl [I], Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] and its analogs [III—VI] modified at the P_3 or P_5 position of II on SFP and LE are summarized in Table I. Boc–Tyr–Leu–Val–CH_2Cl [I] was found to be a potent inhibitor of SFP and LE. Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] and its analogs [III—VI] are more reactive and selective than Boc–Tyr–Leu–Val–CH_2Cl, while the shorter analogs, Boc–Val–CH_2Cl and Boc–Leu–Val–CH_2Cl, are incapable of inhibiting SFP and LE under the same conditions. From these results, it can be deduced that the inhibitory effect of peptide chloromethyl ketones on SFP and LE is strongly influenced by the peptide chain length, as shown in the case of substrates.[5]

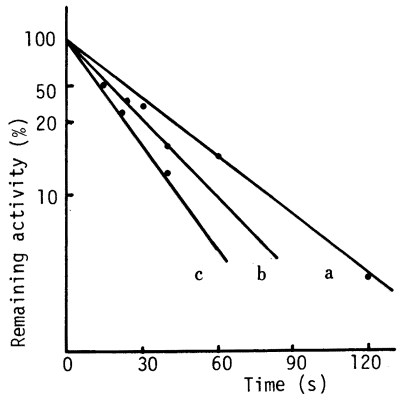

Fig. 2. Kinetics of Inactivation of SFP by Boc–Ala–Tyr–Leu–Val–CH_2Cl

The final concentrations were as follows: dioxane, 0.6% (v/v); SFP, 0.35 μM; inhibitor, 0.01 mM (a) or 0.015 mM (b) or 0.02 mM (c).

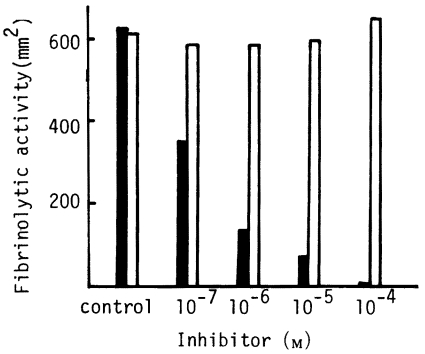

Fig. 3. Inhibitory Effects of Boc–Ala–Tyr–Leu–Val–CH_2Cl on the Fibrinolytic Activities of LE and PPE

■, LE; □, PPE (porcine pancreatic elastase). Boc–Ala–Tyr–Leu–Val–CH_2Cl was mixed with a fibrinogen solution before thrombin was added. The final concentrations of the inhibitor in the plates were 10^{-7} to 10^{-4} M.

TABLE I. Inhibitory Effects of Peptide Chloromethyl Ketones on the Amidolytic Activities of SFP and LE

Inhibitor	Enzyme	[I] (M) × 10^4	$10^4 \times k_{obsd}$ (s^{-1})	Half life (s)	k_{obsd}/[I] ($M^{-1}s^{-1}$)	k_{obsd}/[I] (rel.)
Boc–Val–CH_2Cl	SFP	0.25[a]	—	—	—	—
	LE	0.25[a]	—	—	—	—
Boc–Leu–Val–CH_2Cl	SFP	0.25[a]	—	—	—	—
	LE	0.25[a]	—	—	—	—
Boc–Tyr–Leu–Val–CH_2Cl [I]	SFP	0.1	224	31	2240	1.0
	LE	0.1	117	59	1170	0.52
Boc–Ala–Tyr–Leu–Val–CH_2Cl [II]	SFP	0.1	495	14	4950	2.2
	LE	0.1	330	21	3300	1.5
Boc–Ala–Phe–Leu–Val–CH_2Cl [III]	SFP			N.D.[b]		
	LE	0.05	165	42	3300	1.5
Ac–Ala–Tyr–Leu–Val–CH_2Cl [IV]	SFP	0.1	248	28	2480	1.1
	LE	0.1	151	46	1510	0.67
DNS–Ala–Tyr–Leu–Val–CH_2Cl [V]	SFP	0.1	301	23	3010	1.3
	LE	0.1	267	26	2670	1.2
DNS–Ala–Phe–Leu–Val–CH_2Cl [VI][c]						

The final concentrations were as follows: dioxane, 1.5% (v/v); enzyme, 0.35 μM, inhibitor, 0.1 or 0.05 mM. *a*) This compound did not show any inhibitory effect on the enzyme after preincubation for more than 10 min at this concentration. *b*) Not determined. *c*) The inhibitory activity was not determined, because this compound was not sufficiently soluble to give 0.1 mM concentration.

In order to determine the specificity of Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] against SFP and LE, we measured its inhibitory effect on fibrinolysis by porcine pancreatic elastase (PPE), plasmin, trypsin and α-chymotrypsin. As an example, the results for PPE are shown in Fig. 3. As expected, Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] did not inhibit PPE, plasmin, trypsin or α-chymotrypsin, indicating that Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] is a specific inhibitor of SFP and LE.

Next, the inhibitory effects of 8 kinds of stereoisomers [I, VII—XIII] of Boc–Tyr–Leu–

TABLE II. Inhibitory Effects of Stereoisomers of Boc–Tyr–Leu–Val–CH_2Cl on the Amidolytic Activity of SFP and LE

Inhibitor Boc–Tyr–Leu–Val–CH_2Cl	[I] (mM)	SFP	LE
		Inhibition (%)	
L–L–L [I]	0.0025	40	20
	0.005	80	55
	0.05	100	100
D–L–L [VII]	0.05	60	30
D–D–L [VIII]	0.05	10	8
L–D–L [IX]	0.05	<5	<5
L–L–D [X]	0.05	10	5
D–L–D [XI]	0.05	10	5
D–D–D [XII]	0.05	0	0
L–D–D [XIII]	0.05	0	0

The preincubation was performed at 37 °C for 2 min. The final concentrations were as follows: dioxane, 1.5% (v/v); enzyme, 0.35 μM.

Val–CH_2Cl on the amidolytic activity of SFP and LE are summarized in Table II. From the results, it can be seen that SFP and LE are inhibited in a similar manner by those peptide chloromethyl ketones, demonstrating a further similarity between SFP and LE. In addition, the potency of the inhibitory activity of peptide chloromethyl ketones on SFP and LE is in inverse proportion to that of the corresponding *p*NA derivatives. Although it is reasonable that Boc–Tyr–D-Leu–D-Val–CH_2Cl [XIII] did not inhibit the enzymes because the chloromethyl ketone functional group might be facing away from the active site histidine residue, it is interesting that Boc–Tyr–D-Leu–D-Val–CH_2Cl [XIII] did not inhibit either enzyme, while Suc–Tyr–D-Leu–D-Val–*p*NA inhibited the amidolytic activity of SFP and LE toward Suc–Tyr–Leu–Val–*p*NA. The fact that Boc–Tyr–D-Leu–D-Val–CH_2Cl [XIII] did not show any inhibitory effect on SFP or LE supports our previous hypothesis[21] that the *p*NA moiety in Suc–Tyr–D-Leu–D-Val–*p*NA is required to interact with some part of the enzymes for manifestation of inhibitory activity.

In conclusion, the results presented in this paper demonstrate that peptide chloromethyl ketones having a Val residue at the P_1 position are potent and specific inhibitors of SFP and LE. The similar modes of action of SFP and LE toward those peptide chloromethyl ketones suggest that the two enzymes have quite similar three-dementional structures around the active center. These substrate-derived chloromethyl ketones should be useful tools for the clarification of the roles of SFP and LE and for distinguishing these enzymes from other enzymes in the complex physiological environment.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-180 (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). For column chromatography, a Toyo SF-160K fraction collector was used. For TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5 and Rf^6 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), $CHCl_3$, MeOH and H_2O (89 : 10 : 1), $CHCl_3$, MeOH and H_2O (97 : 2.8 : 0.2), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase), *n*-BuOH, AcOH, pyridine and H_2O (4 : 1 : 1 : 2), respectively. Mass spectra (MS) were measured with a Hitachi RMU-7MG mass spectrometer by the field desorption (FD) technique.

Z–Val–CH_2Cl——Diazomethane [prepared from nitrosomethylurea (6.1 g, 60 mmol)] was added to a mixed anhydride [prepared from Z–Val–OH (7.5 g, 30 mmol), Et_3N (4.2 ml, 30 mmol) and ethyl chloroformate (2.8 ml,

30 mmol)] in THF (100 ml) at −15 °C and the reaction mixture was stirred for 15 h at 4 °C. After addition of 8.4 N HCl/dioxane (8.0 ml, 67 mmol) at −15 °C, the reaction mixture was stirred for 3 h at −15 °C. After neutralization of the solution with Et_3N and removal of the solvent, the residue was dissolved in AcOEt. This solution was washed with 0.1 N HCl, 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 a crystalline meterial, which was recrystallized from EtOH, yield 6.2 g (73%), mp 69—74 °C, $[\alpha]_D^{25}$ −24.3° (c=1.0, MeOH), Rf^1 0.73. *Anal.* Calcd for $C_{14}H_{18}ClNO_3$: C, 59.3; H, 6.39; N, 4.93. Found: C, 59.6; H, 6.49; N, 4.83.

Z-D-Val-CH_2Cl——The title compound was prepared from Z-D-Val-OH (7.5 g) in the same manner as described above, yield 5.0 g (59%), mp 74—76 °C, $[\alpha]_D^{25}$ +21.7° (c=1.0, MeOH), Rf^1 0.73. *Anal.* Calcd for $C_{14}H_{18}ClNO_3$: C, 59.3; H, 6.39; N, 4.93. Found: C, 59.4; H, 6.41; N, 4.98.

Boc-Val-CH_2Cl——The title compound was prepared from Boc-Val-OH (6.3 g) in the same manner as described above, yield 4.8 g (64%), mp 68—69 °C, $[\alpha]_D^{25}$ −34.4° (c=1.0, MeOH), Rf^1 0.81. *Anal.* Calcd for $C_{11}H_{20}ClNO_3$: C, 52.9; H, 8.07; N, 5.60. Found: C, 52.6; H, 8.16; N, 5.65.

Boc-D-Val-CH_2Cl——The title compound was prepared from Boc-D-Val-OH (6.3 g) in the same manner as described above, yield 2.4 g (32%), mp 67—69 °C, $[\alpha]_D^{25}$ +33.9° (c=1.0, MeOH), Rf^1=0.81. *Anal.* Calcd for $C_{11}H_{20}ClNO_3$: C, 52.9; H, 8.07; N, 5.60. Found: C, 52.7; H, 8.21; N, 5.78.

Boc-Leu-Val-CH_2Cl——Boc-Leu-OH (2.5 g, 0.01 mol), HOBt (1.4 g, 0.01 mol) and H-Val-CH_2Cl·HBr [prepared from Z-Val-CH_2Cl (2.8 g, 0.01 mol) and 25% HBr/AcOH (9.7 ml, 0.03 mol)] were dissolved in DMF (20 ml) containing Et_3N (1.4 ml). DCC (2.3 g, 0.011 mol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 18 h at 4 °C. 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 an oily material. The crude product in $CHCl_3$ was applied to a silica gel column (1.5×20 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (400—500 ml) was removed by evaporation. Petroleum ether was added to the residue to provide the purified material, yield 0.59 g (17%), mp 159—161.5 °C, $[\alpha]_D^{25}$ −54.6° (c=1.1, MeOH), Rf^1 0.81, Rf^3 0.79. *Anal.* Calcd for $C_{17}H_{31}ClN_2O_3 \cdot H_2O$: C, 56.0; H, 9.11; N, 7.67. Found: C, 56.4; H, 8.90; N, 7.87.

General Procedure for the Synthesis of Stereoisomeric Boc-Tyr-Leu-OH——Boc-Tyr-N_3 [prepared from Boc-Tyr-N_2H_3 (2.0 g, 6.8 mmol), 8.4 N HCl/dioxane (1.6 ml, 14 mmol) and isoamyl nitrite (0.94 ml, 6.8 mmol) at −40 °C] in DMF (10 ml) were added to a solution of H-Leu-OH (0.88 g, 6.8 mmol) in H_2O (20 ml) and DMF (10 ml) containing Et_3N (0.95 ml, 6.8 mmol). The reaction mixture was stirred for 30 min at −15 °C and for 48 h at 4 °C. The solvent was removed by evaporation under reduced pressure. The residue was dissolved in 5% $NaHCO_3$ and this solution was washed with AcOEt. The aqueous layer was acidified with citric acid and the resultant oily material was extracted with AcOEt and washed with H_2O. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Petroleum ether was added to the residue to give an amorphous powder. The yield, melting point, $[\alpha]_D$ value, Rf values and analytical data are summarized in Table III.

General Procedures for the Synthesis of Stereoisomeric Boc-Tyr-Leu-Val-CH_2Cl [I, VII—XIII]——Boc-Tyr-Leu-OH (0.39 g, 1.0 mmol), HOBt (0.13 g, 1.0 mmol) and H-Val-CH_2Cl·HBr [prepared from Z-Val-CH_2Cl (0.28 g, 1.0 mmol) and 25% HBr/AcOH (1.0 ml, 3.0 mmol)] were dissolved in DMF (10 ml) containing Et_3N (0.14 ml). DCC (0.25 g, 1.2 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 18 h at 4 °C. 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. The crude material in $CHCl_3$ was applied to a silica gel column (1.5×35.5 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (500—700 ml) was removed by evaporation. Ether was added to the residue to provide the purified material.

TABLE III. Yield, Melting Point, Optical Rotation, Rf Values and Analytical Data of Boc-Tyr-Leu-OH

Compound Boc-Tyr-Leu-OH	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis			TLC	
					C	H	N	Rf^1	Rf^2
L-L	37	Amorphous	−5.8 (c=1.0)	$C_{20}H_{30}N_2O_6$	60.9 (61.0	7.66 7.87	7.10 6.96)	0.17	0.26
L-D	60	Amorphous	+12.6 (c=0.5)	$C_{20}H_{30}N_2O_6$	60.9 (60.9	7.66 7.86	7.10 6.99)	0.18	0.25
D-L	56	Amorphous	−13.5 (c=0.9)	$C_{20}H_{30}N_2O_6$	60.9 (60.9	7.66 7.93	7.10 6.87)	0.19	0.27
D-D	56	Amorphous	+6.3 (c=1.0)	$C_{20}H_{30}N_2O_6$	60.9 (60.8	7.66 7.82	7.10 6.94)	0.16	0.26

TABLE IV. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Boc-Tyr-Leu-Val-CH_2Cl

Compound Boc-Tyr-Leu-Val-CH_2Cl	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis			TLC	
					C	H	N	Rf^1	Rf^2
L-L-L	9.4	125—127	−42.5 (c=0.4)	$C_{26}H_{40}ClN_3O_6 \cdot 1/2H_2O$	58.4 (58.5	7.72 7.63	7.85 7.90)	0.40	0.70
D-L-L	12	169—170	−80.6 (c=0.3)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.7	7.66 7.98	7.98 8.33)	0.41	0.69
D-D-L	13	88—89	−18.0 (c=0.1)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.5	7.66 7.87	7.98 7.84)	0.43	0.66
L-D-L	8.6	80—87	+5.5 (c=0.4)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.9	7.66 8.04	7.98 7.61)	0.44	0.67
L-L-D	28	89—95	+18.1 (c=0.5)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.6	7.66 7.91	7.98 7.75)	0.45	0.68
D-L-D	14	74—80	−6.8 (c=0.6)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.5	7.66 7.87	7.98 7.74)	0.45	0.69
D-D-D	10	119—121	+39.4 (c=0.7)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.1	7.66 7.79	7.98 7.69)	0.48	0.69
L-D-D	6.1	169—170	+76.0 (c=0.4)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.4	7.66 7.83	7.98 7.98)	0.48	0.70

The yield, melting point, $[\alpha]_D$ value, *Rf* values and analytical data are summarized in Table IV.

Boc-Ala-Tyr-N_2H_3——H-Tyr-OMe·HCl (6.9 g, 30 mmol), Boc-Ala-OH (5.6 g, 30 mmol) and HOBt (4.0 g, 30 mmol) were dissolved in DMF (30 ml) containing Et_3N (4.2 ml). DCC (8.2 g, 40 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 18 h at room temperature. 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 white precipitate, which was collected by filtration. The crude Boc-Ala-Tyr-OMe was dissolved in MeOH (30 ml), and 80% hydrazine hydrate (4.6 ml, 74 mmol) was added. The reaction mixture was allowed to stand for 15 h at room temperature. The resultant precipitate was collected by filtration, and recrystallized from MeOH, yield 4.4 g (40%), mp 184—186 °C, $[\alpha]_D^{25}$ −16.0° (c=1.0, AcOH), Rf^1 0.20. *Anal.* Calcd for $C_{17}H_{26}N_4O_5$: C, 55.7; H, 7.15; N, 15.3. Found: C, 55.5; H, 7.14; N, 15.2.

Boc-Ala-Tyr-Leu-OH——Boc-Ala-Tyr-N_3 [prepared from Boc-Ala-Tyr-N_2H_3 (3.6 g, 10 mmol), 8.4 N HCl/dioxane (2.4 ml, 20 mmol) and isoamyl nitrite (1.4 ml, 10 mmol) at −40 °C] in DMF (30 ml) was added to a solution of H-Leu-OH (1.4 g, 10 mmol) in H_2O (60 ml) and DMF (30 ml) containing Et_3N (1.4 ml, 10 mmol). The reaction mixture was stirred for 30 min at −40 °C and for 48 h at 4 °C. After removal of the solvent by evaporation, the residue was dissolved in 5% $NaHCO_3$ and this solution was washed with AcOEt. The aqueous layer was acidified with citric acid and the resultant oily material was extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4 and evaporated under reduced pressure. Petroleum ether was added to the residue to give a precipitate, which was collected by filtration. The crude material in $CHCl_3$ was applied to a silica gel column (2.5 × 33 cm) equilibrated with $CHCl_3$, and eluted with $CHCl_3$ (600 ml), 1% MeOH/$CHCl_3$ (600 ml) and then 2% MeOH/$CHCl_3$ (2000 ml). The solvent of the 2% MeOH/$CHCl_3$ effluent (700—2000 ml) was removed by evaporation. Ether was added to the residue to give a white powder, yield 1.8 g (39%), mp 113—115 °C, $[\alpha]_D^{25}$ −29.5° (c=1.0, MeOH), Rf^1 0.20. *Anal.* Calcd for $C_{23}H_{35}N_3O_7$: C, 59.3; H, 7.57; N, 9.02. Found: C, 59.7: H, 7.89; N, 8.57.

Boc-Ala-Tyr-Leu-Val-CH_2Cl [II]——Boc-Ala-Tyr-Leu-OH (0.54 g, 1.2 mmol), HOBt (0.16 g, 1.2 mmol) and H-Val-CH_2Cl·HCl [prepared from Boc-Val-CH_2Cl (0.25 g, 1.0 mmol) and 2.7 N HCl/dioxane (1.8 ml, 5.0 mmol)] were dissolved in DMF (10 ml) containing Et_3N (0.14 ml). DCC (0.30 g, 1.5 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 48 h at 4 °C. 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 and ether were added to the residue to give a light yellow precipitate, which was recrystallized from $CHCl_3$–ether, yield 0.10 g (17%), mp 172—175 °C, $[\alpha]_D^{25}$ −63.1° (c=0.3, MeOH), Rf^1 0.29. *Anal.* Calcd for $C_{29}H_{45}ClN_4O_7$: C, 58.3; H, 7.60; N, 9.38. Found: C, 58.2; H, 7.57; N, 9.47. FD-MS m/z: 597 (M^+).

Boc-Ala-Phe-Leu-Val-CH_2Cl [III]——The title compound was prepared from Boc-Ala-Phe-Leu-OH[22,23] (0.90 g) and H-Val-CH_2Cl [prepared from Z-Val-CH_2Cl (0.70 g)] in the same manner as described for the preparation of II, yield 0.36 g (31%), mp 114—118 °C, $[\alpha]_D^{25}$ −59.6° (c=1.0, MeOH), Rf^1 0.56, Rf^2 0.85. *Anal.* Calcd

for $C_{29}H_{45}ClN_4O_6$: C, 59.9; H, 7.80; N, 9.64. Found: C, 59.7; H, 7.89; N, 9.43.

Boc–Tyr–Leu–OBzl——Boc–Tyr–OH (2.8 g, 10 mmol), H–Leu–OBzl·TosOH (3.9 g, 10 mmol) and HOBt (1.3 g, 10 mmol) were dissolved in DMF (50 ml) containing Et_3N (1.4 ml, 10 mmol). DCC (2.3 g, 11 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 18 h at 4 °C. 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. The crude material in $CHCl_3$ was applied to a silica gel column (2.5 × 30 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (750—1200 ml) was removed by evaporation. Petroleum ether was added to the residue to give an amorphous powder, yield 2.3 g (49%), $[\alpha]_D^{25}$ −18.2° (c=1.0, MeOH), Rf^1 0.68, Rf^2 0.64. *Anal.* Calcd for $C_{27}H_{36}N_2O_6$: C, 66.9; H, 7.49; N, 5.79. Found: C, 66.6; H, 7.53; N, 6.07.

Ac–Ala–Tyr–Leu–OBzl——Ac–Ala–OH (0.20 g, 1.6 mmol), H–Tyr–Leu–OBzl·HCl [prepared from Boc–Tyr–Leu–OBzl (0.94 g, 2.0 mmol) and 3.6 N HCl/dioxane (2.8 ml, 10 mmol)] and HOBt (0.22 g, 1.6 mmol) were dissolved in DMF (10 ml) containing Et_3N (0.28 ml). DCC (0.41 g, 2.0 mmol) was added to the above cold solution and the reaction mixture was stirred for 18 h at 4 °C. 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 recrystallized from AcOEt, yield 0.61 g (61%), mp 140—144 °C, $[\alpha]_D^{25}$ −47.4° (c=1.0, MeOH), Rf^1 0.22, Rf^3 0.20. *Anal.* Calcd for $C_{27}H_{35}N_3O_6$: C, 65.2; H, 7.09; N, 8.45. Found: C, 65.1; H, 7.19; N, 8.66.

Ac–Ala–Tyr–Leu–OH——Ac–Ala–Tyr–Leu–OBzl (0.56 g, 1.1 mmol) was dissolved in MeOH (50 ml) and hydrogenated over a 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 0.35 g (78%), mp 224—229 °C, $[\alpha]_D^{25}$ −44.3° (c=0.9, MeOH), Rf^1 0.10, Rf^5 0.69, Rf^6 0.63. *Anal.* Calcd for $C_{20}H_{29}N_3O_6$: C, 59.0; H, 7.17; N, 10.3. Found: C, 59.0; H, 7.36; N, 9.97.

Ac–Ala–Tyr–Leu–Val–CH_2Cl [IV]——Ac–Ala–Tyr–Leu–OH (0.20 g, 0.50 mmol), H–Val–CH_2Cl·HBr [prepared from Z–Val–CH_2Cl (0.18 g, 0.63 mmol) and 25% HBr/AcOH (0.60 ml, 1.9 mmol)] and HOBt (0.070 g, 0.50 mmol) were dissolved in DMF (40 ml) containing Et_3N (0.088 ml). DCC (0.12 g, 0.60 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 18 h at 4 °C. 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 evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration. The crude material in $CHCl_3$ was applied to a silica gel column (2.0 × 31 cm) equilibrated with $CHCl_3$. After elution with $CHCl_3$ (900 ml) and 3% MeOH/$CHCl_3$ (900 ml), the solvent of the latter effluent (300—900 ml) was removed by evaporation. Ether was added to the residue to give a white powder, yield 0.040 g (12%), mp 183—185 °C, $[\alpha]_D^{25}$ −40.2° (c=0.2, MeOH), Rf^1 0.53, Rf^3 0.42. *Anal.* Calcd for $C_{26}H_{39}ClN_4O_6 \cdot 1/4H_2O$: C, 57.5; H, 7.32; N, 10.3. Found: C, 57.3; H, 7.39; N, 10.4.

DNS–Ala–Tyr–Leu–OBzl——The title compound was prepared from DNS–Ala–OH (0.80 g) and H–Tyr–Leu–OBzl [prepared from Boc–Tyr–Leu–OBzl (1.2 g)] in the same manner as described for the preparation of Ac–Ala–Tyr–Leu–OBzl, yield 1.1 g (64%), mp 125—130 °C, $[\alpha]_D^{25}$ −37.5° (c=0.9, MeOH), Rf^1 0.64, Rf^3 0.70. *Anal.* Calcd for $C_{37}H_{44}N_4O_7S$: C, 64.5; H, 6.43; N, 8.13. Found: C, 64.4; H, 6.48; N, 7.90.

DNS–Ala–Tyr–Leu–OH——The title compound was prepared from DNS–Ala–Tyr–Leu–OBzl (1.0 g) in the same manner as described for the preparation of Ac–Ala–Tyr–Leu–OH, yield 0.72 g (80%), mp 220—222.5 °C, $[\alpha]_D^{25}$ −37.6° (c=1.0, DMF), Rf^1 0.32, Rf^3 0.08. *Anal.* Calcd for $C_{30}H_{38}N_4O_7S$: C, 60.2; H, 6.39; N, 9.35. Found: C, 60.0; H, 6.29; N, 9.06.

DNS–Ala–Tyr–Leu–Val–CH_2Cl [V]——The title compound was prepared from DNS–Ala–Tyr–Leu–OH (0.30 g) and H–Val–CH_2Cl [prepared from Z–Val–CH_2Cl (0.18 g)] in the same manner as described for the preparation of IV. The crude material in $CHCl_3$ was applied to a silica gel column (2.0 × 29.5 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (500—800 ml) was removed by evaporation. Ether was added to the residue to give a precipitate, which was collected by filtration, yield 0.076 g (17%), mp 203—206 °C, $[\alpha]_D^{25}$ −41.0° (c=1.0, DMF), Rf^1 0.70, Rf^3 0.70. *Anal.* Calcd for $C_{36}H_{38}ClN_5O_7S$: C, 59.2; H, 6.62; N, 9.59. Found: C, 59.0; H, 6.80; N, 9.67.

Boc–Phe–Leu–OBzl——The title compound was prepared from Boc–Phe–OH (2.7 g) and H–Leu–OBzl·TosOH (3.9 g) in the same manner as described for the preparation of Boc–Tyr–Leu–OBzl, yield 1.7 g (36%), mp 87.5—89.5 °C, $[\alpha]_D^{25}$ −23.0° (c=0.9, MeOH), Rf^1 0.84, Rf^3 0.72. *Anal.* Calcd for $C_{27}H_{36}N_2O_5$: C, 69.2; H, 7.74; N, 5.98. Found: C, 69.0; H, 7.81; N, 6.21.

Boc–Ala–Phe–Leu–OBzl——The title compound was prepared from Boc–Ala–OH (0.57 g) and H–Phe–Leu–OBzl [prepared from Boc–Phe–Leu–OBzl (1.4 g)] in the same manner as described for the preparation of Ac–Ala–Tyr–Leu–OBzl, yield 0.53 g (33%), amorphous powder, $[\alpha]_D^{25}$ −24.8° (c=1.0, DMF), Rf^1 0.57, Rf^3 0.58. *Anal.* Calcd for $C_{30}H_{41}N_3O_6$: C, 66.8; H, 7.61; N, 7.79. Found: C, 66.8; H, 7.58; N, 7.93.

DNS–Ala–Phe–Leu–OBzl——DNS–Cl (0.72 g, 2.7 mmol) was dropped into a solution of H–Ala–Phe–Leu–OBzl·HCl [prepared from Boc–Ala–Phe–Val–OBzl (1.2 g, 2.2 mmol) and 3.7 N HCl/dioxane (6.0 ml, 22 mmol)] in DMF (20 ml) containing Et_3N (0.31 ml, 2.2 mmol) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and for 1 h

at room temperature. After removal of the solvent, AcOEt was added to the residue to give a precipitate, which was collected by filtration and recrystallized from EtOH, yield 1.5 g (100%), mp 224—226 °C, $[\alpha]_D^{25}$ −42.4° (c=1.0, DMF), Rf^1 0.56, Rf^2 0.87, Rf^4 0.40. *Anal.* Calcd for $C_{37}H_{44}N_4O_6S$: C, 66.0; H, 6.59; N, 8.32. Found: C, 66.0; H, 6.56; N, 8.55.

DNS–Ala–Phe–Leu–OH——The title compound was obtained from DNS–Ala–Phe–Leu–OBzl (1.3 g) by hydrogenation over a Pd catalyst in DMF (40 ml). After removal of Pd and the solvent, EtOH was added to the residue to give a yellow precipitate, which was collected by filtration, yield 1.1 g (95%), mp 216—220 °C, $[\alpha]_D^{25}$ −38.6° (c=1.0, DMF), Rf^1 0.38, Rf^2 0.50. *Anal.* Calcd for $C_{30}H_{38}N_4O_6S \cdot 1/2H_2O$: C, 60.9; H, 6.64; N, 9.46. Found: C, 60.4; H, 6.87; N, 10.0.

DNS–Ala–Phe–Leu–Val–CH_2Cl [VI]——A mixed anhydride [prepared from DNS–Ala–Phe–Leu–OH (1.0 g, 1.7 mmol), Et_3N (0.24 ml, 1.7 mmol) and ethyl chloroformate (0.24 ml, 1.7 mmol) at −15 °C] in DMF (10 ml) was added to a solution of H–Val–$CH_2Cl \cdot HBr$ [prepared from Z–Val–CH_2Cl (0.59 g, 2.1 mmol) and 25% HBr/AcOH (0.24 ml, 6.3 mmol)] in DMF (10 ml) containing Et_3N (0.24 ml, 1.7 mmol). The reaction mixture was stirred for 1 h at −15 °C and for 15 h at 4 °C. After removal of the solvent, the residue was dissolved in AcOEt and this solution was washed with 5% $NaHCO_3$, 0.1 N HCl 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 crude material in $CHCl_3$ was applied to a silica gel column (2×27 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (700—1100 ml) was removed by evaporation. Ether was added to the residue to provide the purified material, yield 0.14 g (9.3%), mp 215—218 °C, $[\alpha]_D^{25}$ −48.2° (c=1.0, DMF), Rf^1 0.48, Rf^4 0.10. *Anal.* Calcd for $C_{36}H_{48}ClN_5O_6S$: C, 60.5; H, 6.84; N, 9.80. Found: C, 60.3; H, 6.70; N, 9.85.

Assay Procedure——SFP and LE were purified by gel-filtration[2] and affinity chromatography.[24] SFP and LE eluted with 8 M urea from the affinity column were used after dialysis against 0.1 M Tris–HCl buffer (pH 8.0) containing 2 M $NaClO_4$. All synthetic substrates and inhibitors were dissolved in 0.1 M Tris–HCl buffer (pH 8.0) containing dioxane. The final concentrations of dioxane, enzyme and inhibitor were as indicated in Fig. 2 and Tables I and II. The enzyme solution was mixed with an equal volume of the inhibitor at 37 °C, and preincubation was continued until the addition of an excess of the substrate. The remaining amidolytic activity of the enzyme was measured at intervals by using Suc–Ala–Tyr–Leu–Val–pNA[5] (0.5 mM) as the substrate, and the inhibitory activity was estimated by comparison with the amidolytic activity after a preincubation time of 0 second. The fibrinolytic activity was estimated with plasminogen-free fibrin plates in essentially the same manner as described previously.[24]

Acknowledgment This work was supported in part by a Grant-in-Aid for Interdisciplinary Research from Kobe-Gakuin University. The authors thank Dr. A. Kato, Niigata College of Pharmacy, for valuable discussions throughout the course of this investigation.

References and Notes

1) All amino acid residues are of L-configuration unless otherwise 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; Suc, succinyl; Ac, acetyl; DNS, dansyl; *p*NA, *p*-nitroanilide; OBzl, benzyl ester; Et_3N, triethylamine; AcOH, acetic acid; DCC, *N*,*N*′-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; AcOEt, ethyl acetate; THF, tetrahydrofuran; *n*-BuOH, *n*-butanol.
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