Synthesis of Peptide Chloromethyl Ketones and Examination of Their Inhibitory Effects on Human Spleen Fibrinolytic Proteinase (SFP) and Human Leukocyte Elastase (LE)\textsuperscript{1)}

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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$_2$Cl 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)\textsuperscript{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\textsuperscript{3)} and in inflammation.\textsuperscript{4)} Previously, we reported that Suc-Tyr-Leu-Val-pNA and Suc-Ala-Tyr-Leu-Val-pNA were specific substrates for SFP ($k_{cat}/K_m=22600$ and $84000 \text{ M}^{-1} \text{s}^{-1}$, respectively)\textsuperscript{5–7)} as well as LE ($k_{cat}/K_m=17600$ and $48500 \text{ M}^{-1} \text{s}^{-1}$, 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-pNA, except for Suc-D-Tyr-Leu-Val-pNA, 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)\textsuperscript{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$_2$Cl and Tos–Lys–CH$_2$Cl, developed by Shaw and his coworkers,\textsuperscript{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.\textsuperscript{7)} First of all, we designed and synthesized Boc–Tyr–Leu–Val–CH$_2$Cl [I], Boc–Ala–Tyr–Leu–Val–CH$_2$Cl [II] and peptide chloromethyl ketones [III—VI] with substitution of Boc–Ala–Tyr–Leu–Val–CH$_2$Cl [II] at the P$_3$ or P$_5$ position. Next, we prepared 8 kinds of stereoisomeric Boc–Tyr–Leu–Val–CH$_2$Cl [I, VII—XIII] on the basis of our previous...
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, amino acid chloromethyl ketone was coupled with various kinds of N-protected peptides by the mixed anhydride method to produce the corresponding peptide chloromethyl ketones. With regard to fragment condensation, the azide method or the DCC–HOBt method 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. 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 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 (E410) 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. Since the k values thus obtained approximate to $k_{\text{obsd}}$, we employed these k values described above as $k_{\text{obsd}}$.

The inhibitory effects of Boc–Tyr–Leu–Val–CH₂Cl[I], Boc–Ala–Tyr–Leu–Val–CH₂Cl[II] and its analogs [III—VI] modified at the P₃ or P₅ position of II on SFP and LE are summarized in Table I. Boc–Tyr–Leu–Val–CH₂Cl[I] was found to be a potent inhibitor of SFP and LE. Boc–Ala–Tyr–Leu–Val–CH₂Cl[II] and its analogs [III—VI] are more reactive and selective than Boc–Tyr–Leu–Val–CH₂Cl, while the shorter analogs, Boc–Val–CH₂Cl and Boc–Leu–Val–CH₂Cl, 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.
In order to determine the specificity of Boc–Ala–Tyr–Leu–Val–CH$_2$Cl [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$_2$Cl [II] did not inhibit PPE, plasmin, trypsin or α-chymotrypsin, indicating that Boc–Ala–Tyr–Leu–Val–CH$_2$Cl [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 were investigated. The results are shown in Table I. As expected, Boc–Tyr–Leu–Val–CH$_2$Cl did not inhibit PPE, plasmin, trypsin or α-chymotrypsin, indicating that Boc–Tyr–Leu–Val–CH$_2$Cl is a specific inhibitor of SFP and LE.

In order to determine the specificity of Boc–Ala–Tyr–Leu–Val–CH$_2$Cl [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$_2$Cl [II] did not inhibit PPE, plasmin, trypsin or α-chymotrypsin, indicating that Boc–Ala–Tyr–Leu–Val–CH$_2$Cl [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—...
Val–CH₂Cl 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 pNA derivatives. Although it is reasonable that Boc-Tyr-D-Leu-D-Val–CH₂Cl [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–CH₂Cl [XIII] did not inhibit either enzyme, while Suc-Tyr-D-Leu-D-Val–pNA inhibited the amidolytic activity of SFP and LE toward Suc-Tyr-Leu-Val–pNA. The fact that Boc-Tyr-D-Leu-D-Val–CH₂Cl [XIII] did not show any inhibitory effect on SFP or LE supports our previous hypothesis that the pNA moiety in Suc-Tyr-D-Leu-D-Val–pNA 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 P₁ 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-dimensional 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 were determined with a Hitachi RMU-7MG mass spectrometer by the field desorption (FD) technique.

Z-Val-CH₂Cl—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), Et₃N (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 Et3N and removal of the solvent, the residue was dissolved in AcOEt. This solution was washed with 0.1 N HCl, 5% Na2CO3 and H2O, dried over Na2SO4 and concentrated to a small volume. Petroleum ether was added to the residue to give a crystalline material, which was recrystallized from EtOH, yield 6.2 g (73%), mp 69-74 °C, [α]D + 24.3 ° (c = 1.0, MeOH), Rf1 0.73. Anal. Calcd for C14H18C1NO3: C, 59.3; H, 6.39; N, 4.93. Found: C, 59.6; H, 6.49; N, 4.83.

Z-D-Val-CH2Cl 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, [α]D + 21.7 ° (c = 1.0, MeOH), Rf1 0.73. Anal. Calcd for C14H18C1NO3: C, 59.3; H, 6.39; N, 4.93. Found: C, 59.4; H, 6.41; N, 4.98.

Boc-Val-CH2Cl 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, [α]D + 34.4 ° (c = 1.0, MeOH), Rf1 0.81. Anal. Calcd for C14H18C1NO3: C, 59.3; H, 6.39; N, 4.93. Found: C, 59.4; H, 6.41; N, 4.98.

Boc-D-Val-CH2Cl 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, [α]D + 33.9 ° (c = 1.0, MeOH), Rf1 0.81. Anal. Calcd for C14H18C1NO3: C, 59.3; H, 6.39; N, 4.93. Found: C, 59.4; H, 6.41; N, 4.98.

Boc-Leu-Val-CH2Cl Boc-Leu-OH (2.5 g, 0.01 mol), HOBt (1.4 g, 0.01 mol) and H-Val-CH2Cl.HBr [prepared from Z-Val-CH2Cl (2.8 g, 0.01 mol) and 25% HBr/AcOH (9.7 ml, 0.03 mol)] were dissolved in DMF (20 ml) containing Et3N (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% Na2CO3, 10% citric acid and H2O, dried over Na2SO4 and concentrated to a small volume. Petroleum ether was added to the residue to give an oily material. The crude product in CHCl3 was applied to a silica gel column (1.5 x 20 cm) equilibrated and eluted with CHCl3. 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, [α]D - 54.6 ° (c = 1.1, MeOH), Rf1 0.81, Rf3 0.79. Anal. Calcd for C17H31C1N2O3.H2O: 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-N3 [prepared from Boc-Tyr-N2H3 (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 H2O (20 ml) and DMF (10 ml) containing Et3N (0.95 mol). DCC (2.3 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% Na2CO3, 10% citric acid and H2O, dried over Na2SO4 and concentrated to a small volume. Petroleum ether was added to the residue to give an amorphous powder. The yield, melting point, [α]D value, Rf values and analytical data are summarized in Table III.

General Procedures for the Synthesis of Stereoisomeric Boc-Tyr-Leu-Val-CH2Cl [I, VII-XIII] Boc-Tyr-Leu-OH (0.39 g, 1.0 mmol), HOBt (0.13 g, 1.0 mmol) and H-Val-CH2Cl.HBr [prepared from Z-Val-CH2Cl (0.28 g, 1.0 mmol) and 25% HBr/AcOH (1.0 ml, 3.0 mmol)] were dissolved in DMF (10 ml) containing Et3N (0.14 mol). 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% Na2CO3, 10% citric acid and H2O, dried over Na2SO4 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 CHCl3 was applied to a silica gel column (1.5 x 35.5 cm) equilibrated and eluted with CHCl3. The solvent of the effluent (500-700 ml) was removed by evaporation. Ether was added to the residue to provide the purified material.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
<th>mp (°C)</th>
<th>[α]D (MeOH)</th>
<th>Formula</th>
<th>Elemental analysis</th>
<th>TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc-Tyr-Leu-OH</td>
<td>37</td>
<td>Amorphous</td>
<td>-5.8 (c = 1.0)</td>
<td>C20H36N2O6</td>
<td>60.9</td>
<td>0.17</td>
</tr>
<tr>
<td>Boc-Tyr-Leu-Cl</td>
<td>60</td>
<td>Amorphous</td>
<td>+12.6 (c = 0.5)</td>
<td>C20H36N2O6</td>
<td>60.9</td>
<td>0.18</td>
</tr>
<tr>
<td>Boc-Tyr-Leu-Val-OH</td>
<td>56</td>
<td>Amorphous</td>
<td>-13.5 (c = 0.9)</td>
<td>C20H36N2O6</td>
<td>60.9</td>
<td>0.19</td>
</tr>
<tr>
<td>Boc-Tyr-Leu-Val-Cl</td>
<td>56</td>
<td>Amorphous</td>
<td>+6.3 (c = 1.0)</td>
<td>C20H36N2O6</td>
<td>60.9</td>
<td>0.16</td>
</tr>
</tbody>
</table>
The yield, melting point, \([\alpha]_D\) value, \(R_f\) values and analytical data are summarized in Table IV.

Boc-Ala-Tyr-N$_2$H$_3$ H Tyr-OMe \[\text{HCl}\] (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$_3$N (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$_2$CO$_3$, 10% citric acid and H$_2$O, dried over Na$_2$SO$_4$ and concentrated to a small volume. Re crystallization of the crude material was achieved with acetone. The crystals were 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, \([\alpha]_D\) -16.0° (c = 1.0, AcOH), \(R_f\) 0.20. Anal. Calcd for C$_{17}$H$_{21}$N$_2$O$_5$: 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$_3$ \[\text{prepared from Boc-Ala-Tyr-N$_2$H$_3$ (3.6 g, 10 mmol), 8.4 N HC1/dioxane (2.4 ml, 20 mmol) and isoamyl nitrite (1.4 ml, 10 mmol) at -40 °C}\] in DMF (30 ml) was added to a solution of H-Leu-OH (1.4 g, 10 mmol) in H$_2$O (60 ml) and DMF (30 ml) containing Et$_3$N (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$_2$O, dried over Na$_2$SO$_4$ 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 x 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, \([\alpha]_D\) -29.5 ° (c = 1.0, MeOH), \(R_f\)' 0.20. Anal. Calcd for C$_{23}$H$_{35}$N$_3$O$_7$: 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$_2$Cl
Boc-Ala-Tyr-Leu-OH (0.54 g, 1.2 mmol), HOBt (0.16 g, 1.2 mmol) and H-Val-CH$_2$Cl \[\text{HCl}\] \[\text{prepared from Boc-Val-CH$_2$Cl (0.25 g, 1.0 mmol) and 2.7 N HC1/dioxane (1.8 ml, 5.0 mmol)\] were dissolved in DMF (10 ml) containing Et$_3$N (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$_2$CO$_3$, 10% citric acid and H$_2$O, dried over Na$_2$SO$_4$ 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, \([\alpha]_D\) -63.1 ° (c = 0.3, MeOH), \(R_f\) 0.29. Anal. Calcd for C$_{29}$H$_{45}$ClN$_4$O$_7$: 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$_2$Cl
The title compound was prepared from Boc-Ala-Phe-Leu-OH and H-Val-CH$_2$Cl \[\text{prepared from Z-Val-CH$_2$Cl (0.70 g)\] in the same manner as described for the preparation of II, yield 0.36 g (31%), mp 114-118 °C, \([\alpha]_D\) -59.6 ° (c = 1.0, MeOH), \(R_f\) 0.56, \(R_f\)' 0.85. Anal. Calcd for C$_{27}$H$_{41}$N$_3$O$_7$: C, 58.0; H, 7.57; N, 9.38. Found: C, 58.2; H, 7.57; N, 9.47.
for C$_2$H$_4$ClN$_2$O$_5$: 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$_3$N (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 $-15\,^\circ$C and for 18 h at $4\,^\circ$C. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na$_2$CO$_3$, 10% citric acid and H$_2$O, dried over Na$_2$SO$_4$ 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 x 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%), $d_3^{19}$ = 18.2° (c = 1.0, MeOH), $R_f^1$ 0.68, $R_f^2$ 0.64. Anal. Calcd for C$_{29}$H$_{36}$N$_2$O$_5$: C, 60.0; H, 6.39; N, 9.70.

**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$_3$N (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$_2$CO$_3$, 10% citric acid and H$_2$O, dried over Na$_2$SO$_4$ and concentrated to a small volume. Petroleum ether was added to the residue to give a precipitate, which was recrystallized from AcOEt, yield 0.66 g (61%), mp 140–144°C, $[\alpha]_D^{25}$ = -47.4° (c = 1.0, MeOH), $R_f^1$ 0.22, $R_f^2$ 0.20. Anal. Calcd for C$_{20}$H$_{29}$N$_3$O$_6$: C, 59.0; H, 7.17; N, 10.3. Found: C, 59.0; H, 7.36; N, 9.97.

**DNS–Ala–Tyr–Leu–OBzl**—DNS–Ala–OH (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), $R_f$ 0.10, $R_f^1$ 0.69, $R_f^2$ 0.63. Anal. Calcd for C$_{26}$H$_{39}$N$_3$O$_6$: C, 59.0; H, 7.17; N, 10.3. Found: C, 59.0; H, 7.36; N, 10.3.

**DNS–Ala–Tyr–Val–CH$_2$Cl** [IV]—DNS–Ala–Tyr–Leu–OH (0.20 g, 0.50 mmol), H–Val–CH$_2$Cl·HBr [prepared from Z–Val–CH$_2$Cl (0.18 g, 0.63 mmol) and 25% HBr/AcOH (0.60 ml, 1.9 mmol)] and HOBt (0.50 g, 0.50 mmol) were dissolved in DMF (40 ml) containing Et$_3$N (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 $-15\,^\circ$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$_2$CO$_3$, 10% citric acid and H$_2$O, dried over Na$_2$SO$_4$ 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 x 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), $R_f$ 0.53, $R_f^1$ 0.42. Anal. Calcd for C$_{23}$H$_{37}$ClN$_3$O$_6$: C, 60.0; H, 6.79; N, 10.4.

**DNS–Ala–Tyr–Leu–OBzl**—The title compound was prepared from DNS–Ala–OH (0.30 g) and H–Val–CH$_2$Cl [prepared from Z–Val–CH$_2$Cl (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 x 25.5 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), $R_f^1$ 0.32, $R_f^2$ 0.08. Anal. Calcd for C$_{25}$H$_{39}$ClN$_3$O$_6$: 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$_2$Cl** [V]—The title compound was prepared from DNS–Ala–Leu–OH (0.30 g) and H–Val–CH$_2$Cl [prepared from Z–Val–CH$_2$Cl (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 x 25.5 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), $R_f^1$ 0.70, $R_f^2$ 0.70. Anal. Calcd for C$_{25}$H$_{37}$ClN$_3$O$_6$: 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), $R_f^1$ 0.84, $R_f^2$ 0.72. Anal. Calcd for C$_{27}$H$_{36}$N$_2$O$_5$: C, 69.2; H, 7.74; N, 9.58. Found: C, 69.0; H, 7.81; N, 6.21.

**Boc–Ala–Phe–OBzl**—**Boc–Ala–Phe–OBzl** [prepared from Boc–Ala–OH (0.57 g) and H–Phe–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), $R_f^1$ 0.57, $R_f^2$ 0.58. Anal. Calcd for C$_{27}$H$_{36}$N$_2$O$_5$: C, 66.8; H, 7.61; N, 7.79. Found: C, 66.8; H, 7.58; N, 7.93.

**DNS–Ala–Phe–OBzl**—DNS–Cl (0.72 g, 2.7 mmol) was dropped into a solution of H–Ala–Phe–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$_3$N (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, [α]D 25° – 42.4° (c = 1.0, DMF), Rf 0.38, Rf 0.50. Anal. Caled for C37H44N4O6S: 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, [α]D 25° – 38.6° (c = 1.0, DMF), Rf 0.38, Rf 0.50. Anal. Caled for C30H38N4O6S 1/2H2O: C, 60.9; H, 6.64; N, 9.46. Found: C, 60.4; H, 6.87; N, 10.0.

**DNS-Ala–Phe–Leu–Val–CH2Cl [VI]**—A mixed anhydride [prepared from DNS-Ala–Phe–Leu–OH (1.0 g, 1.7 mmol), Et3N (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–CH2Cl–HBr [prepared from Z-Val–CH2Cl (0.59 g, 2.1 mmol) and 25% HBr/AcOH (0.24 ml, 6.3 mmol)] in DMF (10 ml) containing Et3N (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% NaHCO3, 0.1 N HCl and H2O, dried over Na2SO4 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 CHCl3 was applied to a silica gel column (2 × 27 cm) equilibrated and eluted with CHCl3. 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, [α]D 25° – 48.2° (c = 1.0, DMF), Rf 1.00, Rf 0.10. Anal. Caled for C36H48ClN5O6S: 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 and affinity chromatography. 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 NaClO4. 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 (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.

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**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-butylloxycarbonyl; Suc, succinyl; Ac, acetyl; DNS, dansyl; pNA, p-nitroanilide; OBzl, benzyl ester; Et3N, triethylamine; AcOH, acetic acid; DCC, N,N'-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole, DMF, dimethylformamide; AcOEt, ethyl acetate; THF, tetrahydrofuran; n-BuOH, n-butanol.


