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Inhibition of α-Chymotrypsin by Suc–L-Tyr–D-Leu–D-Phe–*p*NA, a Stereoisomer of a Specific Substrate[1)]

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Stereoisomers of specific chromogenic substrates for various enzymes were synthesized by a conventional solution method. Among them, Suc–L-Tyr–D-Leu–D-Phe–*p*NA was found to be an effective and specific inhibitor of α-chymotrypsin. However, Suc–L-Tyr–D-Leu–D-Phe–Pipe did not show any inhibitory effect on α-chymotrypsin. The role of the *p*NA moiety of the above stereoisomer was investigated, and it was found that the *p*NA moiety participated in binding with some part of the enzyme, resulting in the manifestation of the inhibitory activity.

Keywords——chromogenic substrate; stereoisomer; α-chymotrypsin; inhibition; *p*-nitroanilide moiety

Previously, it was reported that Suc–L-Tyr–L-Leu–L-Val–*p*NA[2,3)] was a specific substrate for human spleen fibrinolytic proteinase (SFP) and human leukocyte elastase (ELP) and that Suc–L-Tyr–D-Leu–D-Val–*p*NA[4)] was a specific and reversible inhibitor of SFP and ELP. This inhibitor was employed successfully for affinity column chromatography as a useful tool for simple and rapid purification of the enzymes described above.[5,6)] It is interesting that although Suc–L-Tyr–D-Leu–D-Val–*p*NA inhibited SFP and ELP, Suc–L-Tyr–D-Leu–D-Val–Pipe (Pipe = 4-methylpiperidine) and Boc–L-Tyr–D-Leu–D-Val–CH_2Cl[7)] did not inhibit these enzymes. Our hypothesis was that the *p*NA moiety of the L–D–D form could bind with some part (secondary binding site) of the enzymes to act as an effective inhibitor, while the Pipe and CH_2Cl groups in the L–D–D form could not bind with the enzymes.[4)]

This report deals with synthesis of specific substrates and the corresponding L–D–D forms for other enzymes, examination of their inhibitory activity and studies on their inhibitory mechanism by using circular dichroism (CD) spectrometry in order to find specific peptide inhibitors for other enzymes and to clarify the role of the *p*NA moiety of the L–D–D form in manifesting inhibitory activity. Substrate and the corresponding L–D–D forms synthesized by

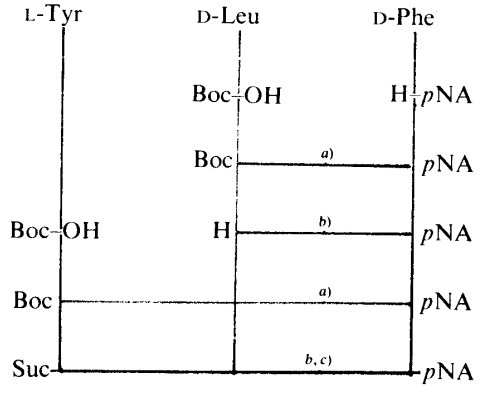

Fig. 1. Synthetic Scheme for Suc–L-Tyr–D-Leu–D-Phe–*p*NA

a) DCC–HOBt method; *b*) HCl–dioxane; *c*) Succinic anhydride.

TABLE I. Amino Acid Ratios in Acid Hydrolysates of Synthetic Peptides

Compound	Amino acid ratios			Average recovery (%)
	Tyr (Ile)	Leu (Phe)	Phe (Lys)	
Suc–L-Tyr–D-Leu–D-Phe–*p*NA	0.95	1.06	1.00	(74.3)
Suc–L-Tyr–D-Leu–L-Phe–*p*NA	0.80	0.97	1.00	(70.1)
H–L-Ile–D-Phe–D-Lys–*p*NA	0.87	1.00	1.00	(98.6)
Suc–L-Tyr–L-Leu–L-Phe–Pipe	0.91	0.99	1.00	(79.3)
Suc–L-Tyr–D-Leu–D-Phe–Pipe	0.84	0.98	1.00	(77.6)

TABLE II. Kinetic Parameters for the Amidolysis of Substrates by Various Enzymes

Substrate	Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)
Suc–L-Tyr–L-Leu–L-Val–*p*NA[3),a)]	ELP	0.21	3.70	17600
Suc–L-Tyr–L-Leu–L-Phe–*p*NA[a)]	α-Chymotrypsin	0.30	2.60	8700
H–L-Ile–L-Phe–L-Lys–*p*NA	Plasmin	0.22	11.4	51800
Suc–L-Ala–L-Ala–L-Ala–*p*NA[8)]	PPE	1.80	12.7	7100

a) Final dioxane concentration was 2.4%.

TABLE III. Inhibitory Effects on Amidolysis of Synthetic Substrates by Enzymes

Compound	Enzyme	Substrate[a)]	K_i (mM)	
			Method A[b)]	Method B[c)]
Suc–L-Tyr–D-Leu–D-Val–*p*NA[4)]	ELP	Suc–Tyr–Leu–Val–*p*NA	0.13 (2.4%)	—[e)]
Suc–L-Tyr–D-Leu–D-Phe–*p*NA	α-Chymotrypsin	Suc–Tyr–Leu–Phe–*p*NA	0.50 (1.6%)	0.42 (4.0%)
			0.71 (4.0%)	0.87 (9.0%)
		Suc–Ala–Leu–Phe–*p*NA[d)]	1.29 (4.0%)	0.65 (4.0%)
Suc–L-Tyr–D-Leu–L-Phe–*p*NA	α-Chymotrypsin	Suc–Tyr–Leu–Phe–*p*NA	N.D.[f)]	N.D.
Suc–L-Tyr–L-Leu–L-Phe–Pipe	α-Chymotrypsin	Suc–Tyr–Leu–Phe–*p*NA	N.D.	N.D.
Suc–L-Tyr–D-Leu–D-Phe–Pipe	α-Chymotrypsin	Suc–Tyr–Leu–Phe–*p*NA	N.D.	N.D.
H–L-Ile–D-Phe–D-Lys–*p*NA	Plasmin	H–Ile–Phe–Lys–*p*NA	N.D.	N.D.
Suc–L-Ala–D-Ala–D-Ala–*p*NA	PPE	Suc–Ala–Ala–Ala–*p*NA	N.D.	N.D.

a) All amino acids used are of L-configuration. *b*) Method A: in the presence of dioxane. Final dioxane concentration is indicated in parentheses. *c*) Method B: in the presence of MeOH. Final MeOH concentration is indicated in parentheses. *d*) K_m values (mM) of this peptide were 0.1 and 0.2 in the presence of dioxane (4%) and MeOH (4%), respectively. *e*) Not determined. *f*) Not detectable.

the solution method were as follows: Suc–L-Tyr–L-Leu–L-Phe–*p*NA and Suc–L-Tyr–D-Leu–D-Phe–*p*NA for α-chymotrypsin, H–L-Ile–L-Phe–L-Lys–*p*NA and H–L-Ile–D-Phe–D-Lys–*p*NA for plasmin and Suc–L-Ala–L-Ala–L-Ala–*p*NA[8)] and Suc–L-Ala–D-Ala–D-Ala–*p*NA for porcine pancreatic elastase (PPE). As an example, the synthetic scheme for Suc–L-Tyr–D-Leu–D-Phe–*p*NA is shown in Fig. 1. Z–D-Phe–*p*NA was prepared from Z–D-Phe–OH and *p*NA by the phosphazo method.[9)] After removal of the Z group by treatment with 25% HBr–AcOH, the resulting amine was coupled with Boc–D-Leu–OH by the DCC–HOBt method[10)] to give Boc–D-Leu–D-Phe–*p*NA. The Boc group was removed by HCl–dioxane treatment and the resulting dipeptide amine was condensed with Boc–D-Tyr–OH by the DCC–HOBt method to afford Boc–L-Tyr–D-Leu–D-Phe–*p*NA. After removal of the Boc group with HCl–dioxane,

the resulting amine was succinylated with succinic anhydride. The succinyl group on the hydroxy functional group of the Tyr residue was removed by hydrolysis with sodium hydroxide. The desired compound was purified by recrystallization and/or silica gel column chromatography. Other substrates were prepared by the same procedure as described above. All peptide thus obtained were homogeneous upon silica gel thin-layer chromatography (TLC). Amino acid ratios in acid hydrolysates were in good agreement with the expected values, as presented in Table I.

The amidolytic activity was assayed by measuring the *p*NA released from substrates (L-L-L-*p*NA, absorbancy at 410 nm). The kinetic constants were estimated from the initial rates of amidolysis at six distinct concentrations of substrates by the method of Lineweaver and Burk. The obtained kinetic parameters for amidolysis of the substrates are summarized in Table II.

The inhibitory activity of the synthetic peptides was measured in the same manner after adding peptide inhibitor (synthetic peptide inhibitor was dissolved in organic solvent-containing buffer). The results are summarized in Table III. As in the case of the Suc-Tyr-Leu-Val-*p*NA and ELP system, Suc-L-Tyr-D-Leu-D-Phe-*p*NA exhibited a competitive inhibitory effect on the amidolytic activity of α-chymotrypsin towards Suc-L-Tyr-L-Leu-L-Phe-*p*NA and Suc-L-Ala-L-Leu-L-Phe-*p*NA.[11] After the reaction of Suc-L-Tyr-L-Leu-L-Phe-*p*NA and α-chymotrypsin for 1 h, neither Suc-Tyr-OH nor H-Leu-Phe-*p*NA was detected by high-performance liquid chromatography (HPLC). Thus, it was concluded that only the Phe-*p*NA bond of the substrate was cleaved during measurement of the enzyme activity. In contrast, other peptides examined did not show any inhibitory activity towards the enzymes. Suc-L-Tyr-D-Leu-D-Phe-Pipe did not inhibit α-chymotrypsin activity towards Suc-L-Tyr-L-Leu-L-Phe-*p*NA. Thus, it was deduced that the *p*NA moiety of Suc-L-Tyr-D-Leu-D-Phe-*p*NA could bind with some part of the enzyme to manifest inhibitory activity, whereas the Pipe group of the peptide could not interact with the enzyme. In order to gain further insight into the role of the *p*NA moiety of the L-D-D form in the manifestation of the inhibitory activity, the CD spectra of the enzyme-Suc-L-D-D-*p*NA mixture were measured in 0.01 M NaCl in the presence of organic solvent at room temperature using α-chymotrypsin and PPE, which are commercially available in a pure form. The molar ratio of peptide to enzyme was 1 : 1. The ellipticity (θ) was obtained from

$$\theta = H \times S$$

where H is the measured value (cm) and S is the scale (deg/cm). The CD spectra of Suc-L-Tyr-D-Leu-D-Phe-*p*NA, α-chymotrypsin and their 1 : 1 mixture measured in the presence of 13% dioxane are shown in Fig. 2a. It can be seen that the CD spectrum of Suc-L-Tyr-D-Leu-D-Phe-*p*NA has a peak at around 300 nm due to the *p*NA moiety. Figure 2b illustrates the CD spectrum of the mixture of the peptide and α-chymotrypsin in the presence of 13% dioxane in comparison with the curve calculated from the CD spectra of Suc-L-D-D-*p*NA and the enzyme. The values of θ at around 330 nm are almost identical with the calculated values.

Figure 3 shows the CD spectra of Suc-L-Tyr-D-Leu-D-Phe-*p*NA, α-chymotrypsin and their mixture in the presence of 13% MeOH. From Fig. 3b, it can be seen that the peak at around 330 nm was shifted to the longer wavelength and θ values were increased significantly as compared with those of the calculated curve. The discrepancy of θ values at around 330 nm between in the presence of 13% dioxane and MeOH is presumably related to the difference in the inhibitory effect of Suc-L-Tyr-D-Leu-D-Phe-*p*NA on α-chymotrypsin in the presence of dioxane and MeOH. Suc-L-Tyr-D-Leu-D-Phe-*p*NA shows only slight inhibitory activity against the enzyme in the presence of 13% dioxane, but exhibits a marked inhibitory effect on the enzyme in the presence of 13% MeOH. The effects of organic solvents on the amidolytic activity of α-chymotrypsin and the inhibitory activity of Suc-L-Tyr-D-Leu-D-Phe-*p*NA

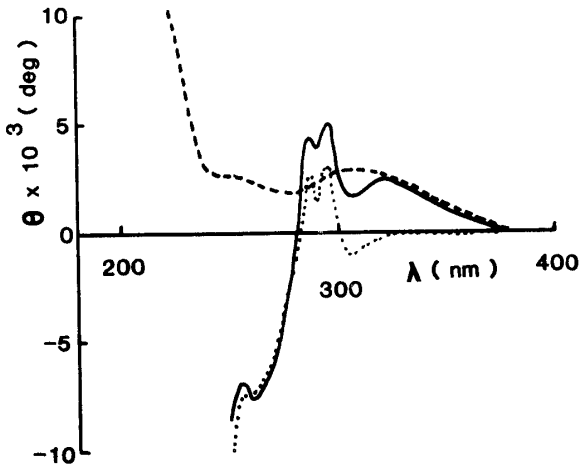

Fig. 2a. CD Spectra of Suc–L-Tyr–D-Leu–D-Phe–*p*NA, α-Chymotrypsin and Their Mixture in the Presence of 13% Dioxane at pH 7.8

– – –, Suc-L–D–D–*p*NA (0.24 mg/ml); ------, α-chymotrypsin (9.0 mg/ml); ——, Suc-L–D–D–*p*NA (0.24 mg/ml) + α-chymotrypsin (9.0 mg/ml).

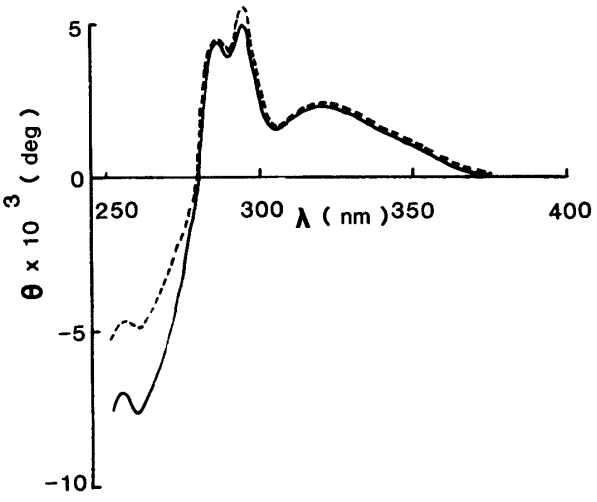

Fig. 2b. Comparison of the Observed and Calculated CD Spectra for the Same Mixture as in Fig. 2a

——, measured curve, – – – –, calculated from the CD spectra of Suc–L–D–D–*p*NA and α-chymotrypsin.

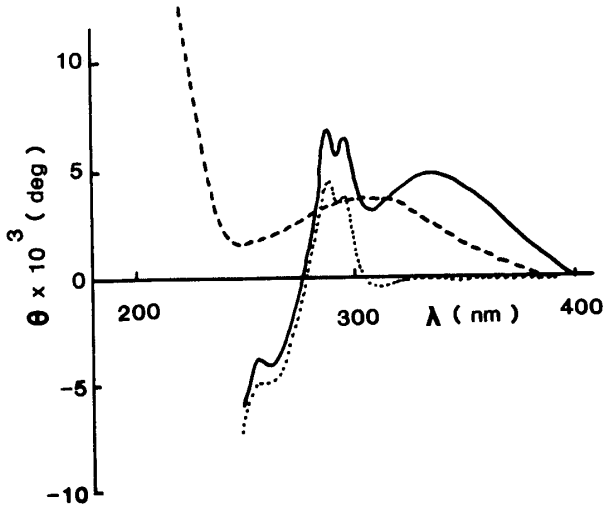

Fig. 3a. CD Spectra of Suc–L-Tyr–D-Leu–D-Phe–*p*NA, α-Chymotrypsin and Their Mixture in the Presence of 13% MeOH at pH 7.8

– – –, Suc-L–D–D–*p*NA (0.24 mg/ml); ------, α-chymotrypsin (9.0 mg/ml); ——, Suc-L–D–D–*p*NA (0.24 mg/ml) + α-chymotrypsin (9.0 mg/ml).

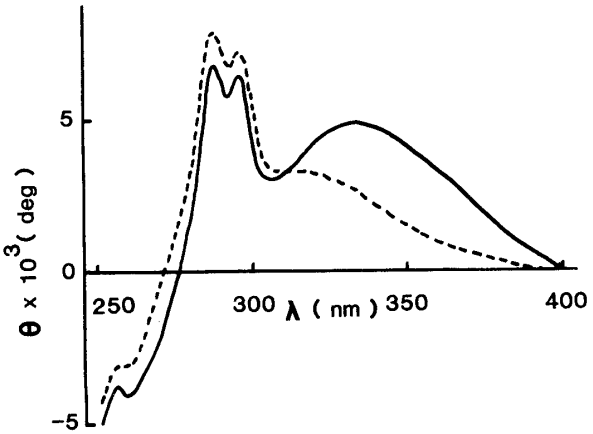

Fig. 3b. Comparison of the Observed and Calculated CD Spectra for the Same Mixture as in Fig. 3a

——, measured curve; – – – –, calculated from the CD spectra of Suc–L–D–D–*p*NA and α-chymotrypsin.

towards the enzyme are illustrated in Fig. 4.

It is clear that the amidolytic activity of α-chymotrypsin towards Suc–L-Tyr–L-Leu–L-Phe–*p*NA as well as the inhibitory activity of Suc–L-Tyr–D-Leu–D-Phe–*p*NA towards the enzyme decreased much more profoundly with increase of dioxane concentration than with increase of MeOH concentration. It can be deduced that the *p*NA moiety of Suc–L-Tyr–D-Leu–D-Phe–*p*NA interact with some part of the enzyme, resulting in a bathochromic shift and increase of θ value of the peak at around 330 nm, as well as causing the manifestation of inhibitory activity against α-chymotrypsin.

The role of the *p*NA moiety of the L–D–D form in the manifestation of the inhibitory activity was further investigated. Suc–L-Ala–D-Ala–D-Ala–*p*NA and Suc–L-Tyr–D-Leu–L-

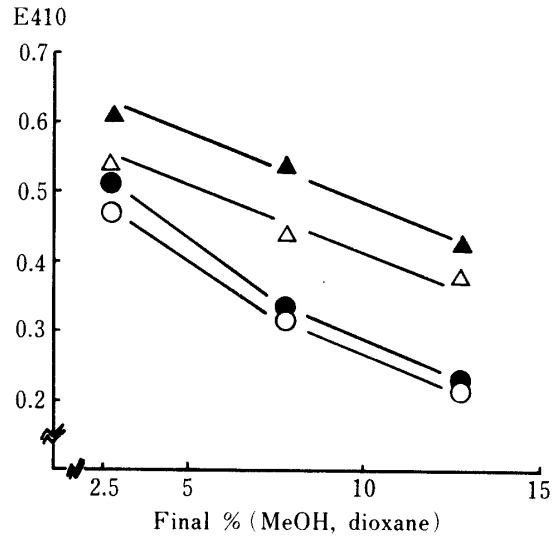

Fig. 4. Effects of Organic Solvents on Amidolytic Activity of α-Chymotrypsin towards Suc-L-Tyr-L-Leu-L-Phe-*p*NA and Inhibitory Activity of Suc-L-Tyr-D-Leu-D-Phe-*p*NA towards α-Chymotrypsin

—▲—, MeOH without Suc-L-D-D-*p*NA; —△—, MeOH with Suc-L-D-D-*p*NA; —●—, dioxane without Suc-L-D-D-*p*NA; —○—, dioxane with Suc-L-D-D-*p*NA.

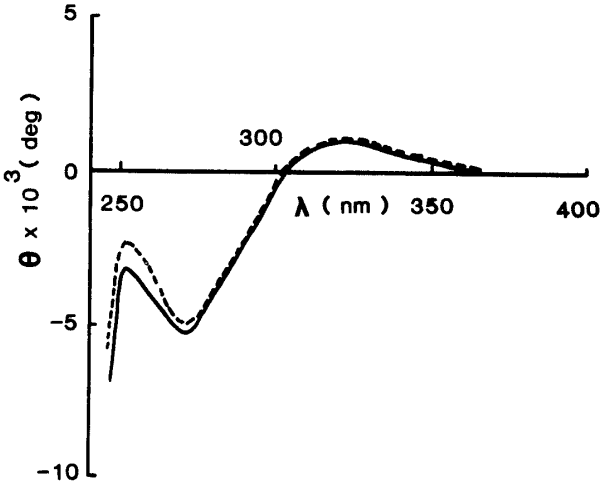

Fig. 5a. CD Spectrum of the Mixture of Suc-L-Ala-D-Ala-D-Ala-*p*NA and PPE

——, measured curve of the mixture of Suc-L-D-D-*p*NA (0.087 mg/ml) and PPE (5.0 mg/ml); – – – –, calculated from the CD spectra of Suc-L-D-D-*p*NA (0.087 mg/ml) and PPE (5.0 mg/ml).
Spectra were taken at pH 8.5 in the presence of 13% MeOH.

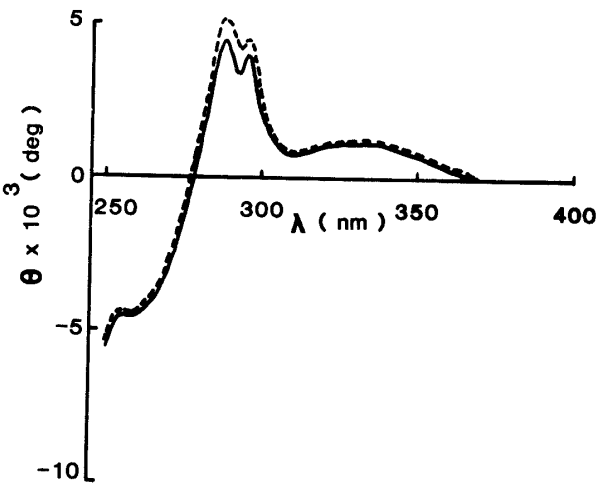

Fig. 5b. CD Spectrum of the Mixture of Suc-L-Tyr-D-Leu-L-Phe-*p*NA and α-Chymotrypsin

——, measured curve of the mixture of Suc-L-D-L-*p*NA (0.24 mg/ml) and α-chymotrypsin (9.0 mg/ml); – – – –, calculated from the CD spectra of Suc-L-D-D-*p*NA (0.24 mg/ml) and α-chymotrypsin (9.0 mg/ml).
Spectra were taken at pH 7.8 in the presence of 13% MeOH.

Phe-*p*NA did not inhibit PPE and α-chymotrypsin, respectively. In these cases, it is predicted that the *p*NA moiety in these peptides does not bind with the enzymes. The CD spectra of the mixtures of Suc-L-Ala-D-Ala-D-Ala-*p*NA and PPE and Suc-L-Tyr-D-Leu-L-Phe-*p*NA and α-chymotrypsin are shown in Figs. 5a and 5b, respectively. As expected, in both CD spectra (5a and 5b), the θ values around 330 nm were identical with the calculated values, suggesting that the *p*NA moieties could not interact with enzymes.

In conclusion, the results obtained here provided a clue to solve a question why Suc-L-Tyr-D-Leu-D-Val-*p*NA can inhibit human leukocyte elastase-like proteinase (ELP) and human spleen fibrinolytic proteinase (SFP), while Suc-L-Tyr-D-Leu-D-Val-Pipe and Boc-L-Tyr-D-Leu-D-Val-CH_2Cl can not, and supported our hypothesis that the *p*NA moiety of the peptide inhibitors must bind with some part of the enzymes for the manifestation of inhibitory activity, and that the Pipe or CH_2Cl group can not bind with these enzymes.[3)]

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 (110 °C, 18 h, 6 N HCl) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co., Ltd.). For column chromatography, a Toyo SF-160K fraction collector was used. HPLC was conducted with a Waters Associate model 440, using a column of Cosmosil (5C18, 4.6 × 150 mm) with MeOH–water (8 : 2) as the eluant. On TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3 and Rf^4 values refer to the systems of $CHCl_3$–MeOH–AcOH (90 : 8 : 2), $CHCl_3$–MeOH–water (8 : 3 : 1, lower phase), *n*-BuOH–AcOH–water (4 : 1 : 5, upper phase) and *n*-BuOH–AcOH–pyridine–water (4 : 1 : 1 : 2), respectively.

Z-D-Phe-*p*NA——The title compound was prepared from Z-D-Phe-OH (7.8 g, 0.026 mol) and *p*NA (3.6 g, 0.026 mol) by the phosphazo method[9] in a pure form. Yield 3.8 g (35%), mp 138—141 °C, $[\alpha]_D^{28}$ −75.6° (*c* = 0.8, MeOH), Rf^1 0.68. *Anal.* Calcd for $C_{23}H_{21}N_3O_5$: C, 65.9; H, 5.05; N, 10.0. Found: C, 65.9; H, 5.00; N, 10.0.

Boc-D-Leu-D-Phe-*p*NA——Boc-D-Leu-OH (2.0 g, 8.9 mmol), H-D-Phe-*p*NA·HBr (prepared from 3.7 g, 10 mmol of Z-D-Phe-*p*NA and 9.7 ml, 30 mmol of 25% HBr–AcOH) and HOBt (1.2 g, 8.9 mmol) were dissolved in DMF (40 ml) containing Et_3N (1.2 ml, 8.9 mmol) and cooled with ice-salt. DCC (2.2 g, 11 mmol) was added to the cold solution, and the reaction mixture was stirred at room temperature overnight. 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 water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford white crystals, which were recrystallized from EtOH and ether, yield 3.7 g (84%), mp 98—101 °C, $[\alpha]_D^{25}$ +11.1° (*c* = 0.9, MeOH), Rf^1 0.56. *Anal.* Calcd for $C_{26}H_{34}N_4O_6$: C, 62.6; H, 6.87; N, 11.2; Found: C, 62.5; H, 6.84; N, 11.3.

Boc-L-Tyr-D-Leu-D-Phe-*p*NA——Boc-L-Tyr-OH (1.2 g, 4.0 mmol), H-D-Leu-D-Phe-*p*NA [prepared from 1.7 g (3.4 mmol) of Boc-D-Leu-D-Phe-*p*NA and 4.8 ml (24 mmol) of 5 N HCl–dioxane] and HOBt (0.58 g, 4.0 mmol) were dissolved in DMF (20 ml) containing Et_3N (0.47 ml, 3.4 mmol) and cooled with ice-salt. DCC (1.0 g, 4.8 mmol) was added to the solution, and the reaction mixture was stirred at room temperature overnight. 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 water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH, yield 1.4 g (58%), mp 200—202 °C, $[\alpha]_D^{25}$ +90.2° (*c* = 1.1, MeOH), Rf^1 0.39, Rf^2 0.68. *Anal.* Calcd for $C_{35}H_{43}N_5O_8$: C, 63.5; H, 6.54; N, 10.6. Found: C, 63.4; H, 6.60; N, 10.6.

Suc-L-Tyr-D-Leu-D-Phe-*p*NA——H-L-Tyr-D-Leu-D-Phe-*p*NA·HCl [prepared from 0.70 g (1.1 mmol) of Boc-L-Tyr-D-Leu-D-Phe-*p*NA and 0.64 ml (3.2 mmol) of 5 N HCl–dioxane] was dissolved in water (10 ml) and the pH of the solution was adjusted to 8 with Na_2CO_3. The oily precipitate was dissolved in AcOEt then the solution was washed with water and dried over Na_2SO_4. Succinic anhydride (0.11 g, 1.1 mmol) was added to the above solution containing Et_3N (0.15 ml, 1.1 mmol) in five equal portions over a period of 1 h. During the reaction, the pH of the solution was maintained at 8—9 by adding Et_3N. After 3 h, the AcOEt layer was washed with 5% AcOH and water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the solution to afford cystals. This crude material was dissolved in MeOH (10 ml) containing 1 N NaOH (3.0 ml). The reaction mixture was stirred at room temperature for 1 h. After neutralization of the solution by adding AcOH, the solvent was removed by evaporation. The residue was extracted with AcOEt, and the extract was washed with water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to give crystals, which were recrystallized from AcOEt, yield 0.42 g (61%), mp 233.5—235 °C, $[\alpha]_D^{25}$ +113.8° (*c* = 0.9, MeOH), Rf^1 0.12, Rf^2 0.20. *Anal.* Calcd for $C_{34}H_{39}N_5O_9$: C, 61.7; H, 5.94; N, 10.6. Found: C, 61.3; H, 6.10; N, 10.4.

Boc-D-Leu-L-Phe-*p*NA——The title compound was prepared by the same method as described for the synthesis of Boc-D-Leu-D-Phe-*p*NA, yield 26%, mp 178—180 °C, $[\alpha]_D^{25}$ −38.1° (*c* = 0.9, MeOH), Rf^1 0.77, Rf^2 0.85. *Anal.* Calcd for $C_{26}H_{34}N_4O_6$: C, 62.6; H, 6.87; N, 11.2. Found: C, 62.2; H, 6.69; N, 11.5.

Boc-L-Tyr-D-Leu-L-Phe-*p*NA——The title compound was prepared in the same way as described for the synthesis of Boc-L-Tyr-D-Leu-D-Phe-*p*NA, yield 62%, mp 115—120 °C, $[\alpha]_D^{25}$ −25.0° (*c* = 0.5, MeOH), Rf^1 0.20, Rf^2 0.54. *Anal.* Calcd for $C_{35}H_{43}N_5O_8$: C, 63.5; H, 6.54; N, 10.6. Found: C, 63.3; H, 6.78; N, 10.6.

Suc-L-Tyr-D-Leu-L-Phe-*p*NA——The title compound was prepared in the same way described for the synthesis of Suc-L-Tyr-D-Leu-D-Phe-*p*NA, yield (32%), mp 129—132 °C, $[\alpha]_D^{25}$ +7.2° (*c* = 0.6, MeOH), Rf^1 0.20, Rf^2 0.33. *Anal.* Calcd for $C_{34}H_{39}N_5O_9 \cdot 1/2H_2O$: C, 60.9; H, 6.01; N, 10.4. Found: C, 60.8; H, 6.04; N, 10.3.

Boc-D-Lys(Z)-*p*NA——Boc-D-Lys(Z)-OH (7.6 g, 20 mmol) and *p*NA (2.7 g, 20 mmol) were dissolved in CH_3CN (80 ml) and cooled with ice-salt. DCC (4.4 g, 21 mmol) was added to the solution, and the reaction mixture was stirred at room temperature overnight. After removal of the dicyclohexylurea and the solvent, the desired compound was isolated in the same way as described previously.[12] Yield 1.2 g (12%), amorphous powder, $[\alpha]_D^{25}$ +11.7° (*c* = 0.9, MeOH), Rf^1 0.53, Rf^2 0.58. *Anal.* Calcd for $C_{25}H_{32}N_4O_7$: C, 60.0; H, 6.44; N, 11.2; Found: C, 60.2; H, 6.58; N, 11.2.

Boc-D-Phe-D-Lys(Z)-*p*NA——Boc-D-Phe-OH (0.24 g, 0.90 mmol) and H-D-Lys(Z)-*p*NA·HCl [prepared from 0.45 g (0.90 mmol) of Boc-D-Lys(Z)-*p*NA and 0.5 ml (2.5 mmol) of 5 N HCl–dioxane] and HOBt (0.13 g, 0.90 mmol) were dissolved in DMF (20 ml) containing Et_3N (0.13 ml, 0.90 mmol) and cooled with ice-salt. DCC (0.22 g,

1.1 mmol) was added to the solution, and the reaction mixture was stirred at room temperature overnight. 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 water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford crystals, which were recrystallized from AcOEt and ether, yield 0.26 g (45%), mp 152—153 °C, $[\alpha]_D^{25}$ −1.5° (c=0.5, DMF), Rf^1 0.56. *Anal.* Calcd for $C_{34}H_{41}N_5O_8$: C, 63.1; H, 6.36; N, 10.8. Found: C, 63.3; H, 6.61; N, 10.9.

Boc-L-Ile-D-Phe-D-Lys(Z)-*p*NA——Boc-L-Ile-OH (74 mg, 0.32 mmol), H-D-Phe-D-Lys(Z)-*p*NA·HCl [prepared from 210 mg (0.32 mmol) of Boc-D-Phe-D-Lys(Z)-*p*NA and 0.38 ml (1.9 mmol) of 5 N HCl-dioxane] and HOBt (44 mg, 0.32 mmol) were dissolved in DMF (15 ml) containing Et_3N (0.04 ml, 0.29 mmol) and cooled with ice-salt. DCC (79 mg, 0.38 mmol) was added to the cold solution, and the reaction mixture was stirred at room temperature overnight. 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 water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford crystals, which were recrystallized from EtOH, yield 70 mg, (32%), mp 201—202.5 °C, $[\alpha]_D^{25}$ +2.1° (c=0.6, DMF), +6.7° (c=0.5, MeOH), Rf^1 0.50, Rf^2 0.96. *Anal.* Calcd for $C_{40}H_{52}N_6O_9$: C, 63.1; H, 6.88; N, 11.0. Found: C, 63.1; H, 7.09; N, 11.0.

H-L-Ile-D-Phe-D-Lys-*p*NA·2HBr——Boc-L-Ile-D-Phe-D-Lys(Z)-*p*NA (0.35 g, 0.46 mmol) was treated with 1.0 ml (3.1 mmol) of 25% HBr-AcOH as usual. The resulting crude material was purified by gel-filtration on Sephadex LH-20 using EtOH as an eluant, yield 0.19 g (60%), amorphous powder, $[\alpha]_D^{25}$ +27.4° (c=0.7, MeOH), Rf^3 0.10, Rf^4 0.58. *Anal.* Calcd for $C_{27}H_{38}N_6O_5 \cdot 2HBr \cdot 2H_2O$: C, 44.8; H, 6.12; N, 11.6. Found: C, 44.9; H, 5.91; N, 11.7.

Z-D-Ala-*p*NA——*p*NA (4.1 g, 30 mmol) was dissolved in dry pyridine (50 ml) and cooled with ice-salt. PCl_3 (1.4 ml, 15 mmol) was added to the solution in several portions over a period of 20 min. Z-D-Ala-OH (6.7 g, 30 mmol) was added to the above solution at room temperature, and the reaction mixture was stirred at 70—80 °C for 3 h and at room temperature for 10 h. After removal of the solvent, the residue was dissolved in AcOEt and 1 N HCl (50 ml + 50 ml). The organic layer was washed with water, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. The residual oil in $CHCl_3$ (5 ml) was applied to a silica gel column (4 × 45 cm), equilibrated and eluted with $CHCl_3$. The solvent of the effluent (1400—1800 ml) was removed by evaporation. Petroleum ether was added to the residue to afford crystals, yield 5.5 g (52%), mp 65—69 °C, $[\alpha]_D^{25}$ +49.8° (c=1.0, MeOH), Rf^1 0.63. *Anal.* Calcd for $C_{17}H_{17}N_3O_5$: C,59.5; H, 4.99; N, 12.2. Found: C, 59.8; H, 5.35; N, 12.1.

Boc-D-Ala-D-Ala-*p*NA——Boc-D-Ala-OH (1.9 g, 10 mmol) and H-D-Ala-*p*NA·HBr [prepared from 3.4 g (10 mmol) of Z-D-Ala-*p*NA and 9.6 ml (30 mmol) of 25% HBr-AcOH] and HOBt (1.35 g, 10 mmol) were dissolved in DMF (50 ml) containing Et_3N (1.4 ml, 10 mmol) and cooled with ice-salt. DCC (2.5 g, 12 mmol) was added to the solution, and the reaction mixture was stirred at room temperature overnight. 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 water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to give crystals, which were recrystallized from AcOEt and ether, yield 2.2 g (58%), mp 197.5—200 °C, $[\alpha]_D^{25}$ +90.4° (c=0.6, MeOH), Rf^1 0.49. *Anal.* Calcd for $C_{17}H_{24}N_4O_6$: C, 53.7; H, 6.35; N, 14.7. Found: C, 53.9; H, 6.41; N, 14.9.

Boc-L-Ala-D-Ala-D-Ala-*p*NA——Boc-L-Ala-OH (0.94 g, 5.0 mmol), H-D-Ala-D-Ala-*p*NA·HCl [prepared from 1.9 g (5.0 mmol) of Boc-D-Ala-D-Ala-*p*NA and 2.7 ml (15 mmol) of 5.4 N HCl-dioxane] and HOBt (0.69 g, 5.0 mmol) were dissolved in DMF (20 ml) containing Et_3N (0.7 ml, 5.0 mmol) and cooled with ice-salt. DCC (1.2 g, 6.0 mmol) was added to the above solution, and the reaction mixture was stirred at room temperature overnight. 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 water, dried over Na_2SO_4 and concentrated. Petroleum ether was added to the residue to yield crystals, which were recrystallized from EtOH, yield 1.2 g (53%), mp 211—214 °C, $[\alpha]_D^{25}$ +1.0° (c=0.7, MeOH), Rf^1 0.40, Rf^2 0.56. *Anal.* Calcd for $C_{20}H_{29}N_5O_7$: C, 53.2; H, 6.47; N, 15.5. Found: C, 53.4; H, 6.45; N, 15.8.

Suc-L-Ala-D-Ala-D-Ala-*p*NA——H-L-Ala-D-Ala-D-Ala-*p*NA·HCl [prepared from 0.71 g (1.6 mmol) of Boc-L-Ala-D-Ala-D-Ala-*p*NA and 1.5 ml (7.8 mmol) of 5.4 N HCl-dioxane] was dissolved in pyridine (15 ml) containing Et_3N (0.22 ml, 1.6 mmol) and cooled with ice. Succinic anhydride (0.31 g, 31 mmol) was added to the above solution in five equal portions over a period of 1 h. After removal of the solvent, the residue was dissolved in AcOEt and 10% AcOH (15 ml + 15 ml) and the organic layer was washed with water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford crystals, which were recrystallized from AcOEt, yield 0.45 g (64%), mp 235—242 °C, $[\alpha]_D^{25}$ +25.0° (c=0.7, MeOH), Rf^1 0.10, Rf^2 0.55. *Anal.* Calcd for $C_{19}H_{25}N_5O_8$: C, 50.6; H, 5.56; N, 15.5. Found: C, 50.3; H, 5.62; N, 15.4.

Synthesis of Stereoisomeric Boc-Leu-Phe-Pipe——A mixed anhydride was prepared from Z-Phe-OH (6.0 g, 20 mmol) with Et_3N (2.8 ml, 20 mmol) and ethyl chloroformate (1.9 ml, 20 mmol) at −15 °C in tetrahydrofuran (THF, 80 ml). 4-Methylpiperidine (2.0 ml, 20 mmol) was added to the solution, and the reaction mixture was stirred at 0 °C for 1 h and at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 1 N HCl and water, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give an oily precipitate (Rf^1 0.64). Boc-Leu-OH (2.2 g, 9.5 mmol), H-Phe-Pipe [prepared from 3.5 g (9.2 mmol) of Z-Phe-Pipe by hydrogenolysis over a Pd catalysis] and HOBt (1.3 g,

9.5 mmol) were dissolved in DMF (50 ml) and cooled with ice-salt. DCC (2.0 g, 10 mmol) was added to the cold solution, and the reaction mixture was stirred at room temperature overnight. 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 water, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to afford crystals, which were recrystallized from AcOEt and ether, L-L; yield 1.9 g (59%), mp 142—144 °C, $[\alpha]_D^{25}$ −40.8° (c = 0.8, MeOH), Rf^1 0.71. *Anal.* Calcd for $C_{26}H_{41}N_3O_4$: C, 67.5; H, 8.99; N, 9.1. Found: C, 68.0; H, 9.07; N, 9.2. D-D; yield 1.3 g (39%), mp 146—149 °C, $[\alpha]_D^{25}$ +40.0° (c = 1.0, MeOH), Rf^1 0.79. *Anal.* Calcd for $C_{26}H_{41}N_3O_4$: C, 67.5; H, 8.99; N, 9.1. Found: C, 67.8; H, 9.23; N, 9.2.

Synthesis of Stereoisomeric Boc-Tyr-Leu-Phe-Pipe——Boc-Tyr-OH (0.48 g, 1.7 mmol), H-Leu-Phe-Pipe·HCl [prepared from 0.78 g (1.7 mmol) of Boc-Leu-Phe-Pipe and 1.6 ml (8.5 mmol) of 5.4 N HCl-dioxane] and HOBt (0.24 g, 1.7 mmol) were dissolved in DMF (15 ml) containing Et_3N (0.24 ml, 1.7 mmol) and cooled with ice-salt. DCC (0.38 g, 1.9 mmol) was added to the solution, and the reaction mixture was stirred at room temperature overnight. 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 water, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to afford a white powder, which was reprecipitated from AcOEt, ether and petroleum ether, L-L-L; yield 0.47 g (44%), mp 117—120 °C, $[\alpha]_D^{25}$ −25.6° (c = 1.0, MeOH), Rf^1 0.55, Rf^2 0.75. Amino acid ratios in an acid hydrolysate; Leu 1.10; Tyr 1.00; Phe 1.00 (average recovery 87.6%). *Anal.* Calcd for $C_{35}H_{50}N_4O_6$: C, 67.5; H, 8.09; N, 9.0. Found: C, 67.0; H, 8.24; N, 8.7. L-D-D; yield 0.82 g (78%), mp 113—119 °C, $[\alpha]_D^{25}$ +34.8° (c = 0.9, MeOH), Rf^1 0.54, Rf^2 0.75. Amino acid ratios in an acid hydrolysate: Leu 1.05; Tyr 0.98; Phe 1.00 (average recovery 76.5%). *Anal.* Calcd for $C_{35}H_{50}N_4O_6$: C, 67.5; H, 8.09; N, 9.0. Found: C, 67.0; H, 8.20; N, 8.8.

Synthesis of Stereoisomeric Suc-Tyr-Leu-Phe-Pipe——H-Tyr-Leu-Phe-Pipe·HCl (0.35 g, 0.64 mmol) (prepared from Boc-Tyr-Leu-Phe-Pipe and 5.4 N HCl-dioxane) was dissolved in AcOEt (40 ml) containing Et_3N (0.18 ml, 1.3 mmol) and cooled with ice. Succinic anhydride (0.12 g, 1.2 mmol) was added to the solution, and the reaction mixture was stirred at 0 °C for 1 h. During the reaction, the pH of the solution was maintained at 8—9 by adding Et_3N. The solution was washed with 10% AcOH and water, dried over Na_2SO_4 and concentrated to a small volume. Ether and petroleum ether were added to the residue to afford crystals. This crude material was dissolved in MeOH (30 ml) containing 1 N NaOH (1 ml). The reaction mixture was stirred at room temperature for 1 h. After neutralization of the solution, the solvent was removed by evaporation. The residue was extracted with AcOEt. The extract was washed with 10% AcOH and water, dried over Na_2SO_4 and concentrated to a small volume. Ether and petroleum ether were added to the residue to give crystals. This material in $CHCl_3$ (5 ml) was applied to a silica gel column (2 × 29 cm), equilibrated and eluted with $CHCl_3$, MeOH and H_2O (95 : 4.6 : 0.4). Individual fractions (50 ml each) were collected. After removal of the solvent of the appropriate effluent (fraction Nos. 3—7), ether and petroleum ether were added to the residue to give crystals. This product was further purified by gel-filtration on Sephadex LH-20 using EtOH as an eluant, L-L-L; yield 0.060 g (15%), mp 120—130 °C, $[\alpha]_D^{25}$ −23.2° (c = 0.8, MeOH), Rf^1 0.46, Rf^2 0.80. *Anal.* Calcd for $C_{34}H_{46}N_4O_7 \cdot 1/2H_2O$: C, 64.6; H, 7.49; N, 8.9. Found: C, 64.3; H, 7.55; N, 8.7. L-D-D; yield 0.040 g (10%), mp 150—160 °C, $[\alpha]_D^{25}$ +31.0° (c = 0.7, MeOH), Rf^1 0.46, Rf^2 0.80. *Anal.* Calcd for $C_{34}H_{46}N_4O_7 \cdot 3/2H_2O$: C, 62.8; H, 7.60; N, 8.6. Found: C, 62.7; H, 7.32; N, 8.2.

Assay Procedure——Enzymes used were α-chymotrypsin (Miles, Erkhart), plasmin (KABI, Stockholm) and porcine pancreatic elastase (Sigma, St. Louis). The amidolytic activity and the inhibitory activity of the synthetic compounds were measured in essentially the same manner as described previously.[4] The assay procedure for α-chymotrypsin was as follows: 0.1—0.7 mM peptide substrate dissolved in Tris-HCl buffer (0.1 M, pH 8.0) containing dioxane or MeOH and 0.02—0.2 μM α-chymotrypsin were mixed in an ice bath, and this mixture was immediately incubated at 37 °C for 5 min (concentrations of the peptide and the enzyme are final ones). The *p*NA released was determined by measuring the absorbancy at 410 nm. The inhibitory activity of the synthetic compounds was measured in the same manner after adding peptide inhibitor (synthetic inhibitor was dissolved in dioxane- or MeOH-containing buffer). In Table III, the final concentrations (%) of dioxane and MeOH are indicated for each compound. Kinetic parameters (K_m, k_{cat}, K_i values) were calculated from Lineweaver-Burk plots at substrate concentrations of 0.1—0.7 mM.

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References and Notes

1) 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; *p*NA, *p*-nitroanilide; Pipe, 4-methylpiperidine; HOBt, 1-hydroxybenzotriazole; DCC, *N*,*N'*-dicyclohexylcarbodiimide; Et_3N, triethylamine; AcOH, acetic acid; DMF, dimethylformamide; AcOEt, ethyl acetate; *n*-BuOH, *n*-butanol;

PPE, porcine pancreatic elastase.
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