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Amino Acids and Peptides. XXII. Synthesis of Substrates and Inhibitors of Human Leukocyte Cathepsin G[1,2]

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Suc–Tyr–Leu–Phe–*p*NA is a good substrate for human leukocyte cathepsin G and α-chymotrypsin but not for human leukocyte elastase (HLE). However, Suc–Tyr–D-Leu–D-Phe–*p*NA inhibited not only cathepsin G and α-chymotrypsin but also HLE (K_i values, 1.1, 0.94 and 0.16 mM, respectively). The *p*-nitroanilide (*p*NA) moiety of Suc–Tyr–Leu–Phe–*p*NA and Suc–Tyr–D-Leu–D-Phe–*p*NA was substituted with *p*-benzoylaniline (BZA), *p*-acetylaniline (ACA), 4-benzylpiperidine (BPP) and 4-methylpiperidine (Pipe). The relationship between the structure and inhibitory effect on HLE, cathepsin G and α-chymotrypsin was studied. Suc–Tyr–Leu–Phe–BZA inhibited HLE, cathepsin G and α-chymotrypsin with K_i values of 0.027, 0.1 and 0.01 mM, respectively.

Keywords——human leukocyte cathepsin G; human leukocyte elastase; α-chymotrypsin; tripeptide amide derivative; *p*-nitroanilide; *p*-benzoylanilide; *p*-acetylanilide; 4-benzylpiperidine amide; 4-methylpiperidine amide; structure–activity relationship

Human leukocyte cathepsin G (chymotrypsin-like proteinase) and human leukocyte elastase (HLE) have attracted our interest in recent years due to their possible involvement in connective tissue turnover and in diseases such as inflammation, rheumatoid arthritis and emphysema.[3] Both cathepsin G and HLE have major plasma inhibitors, α_1-antichymotrypsin and α_1-protease inhibitor, respectively.[4] Imbalances between the enzymes and their natural inhibitors cause serious diseases such as those mentioned above. Previously, we synthesized selective substrates[5] and inhibitors[6] for HLE and contributed to studies on the enzymatic properties of HLE, but the enzymatic properties of cathepsin G are not yet clear, and specific substrates and inhibitors for cathepsin G are now required in order to elucidate its physiological and pathological roles.

This paper deals with the synthesis of substrates and substrate-derived inhibitors for cathepsin G and the comparison of the enzymatic properties of cathepsin G with those of chymotrypsin, the enzymatic similarity of which to cathepsin G has been reported previously.[7] The inhibitory activities of the synthetic peptides toward HLE are also described.

Previously, it was reported that Suc–Tyr–Leu–Phe–*p*NA (**3**) was a good substrate for α-chymotrypsin and its stereoisomer, Suc–Tyr–D-Leu–D-Phe–*p*NA (**7**), inhibited the enzyme. In the latter case, the *p*NA moiety of the peptide significantly participated in binding of the peptide with α-chymotrypsin, resulting in the manifestation of the inhibitory activity.[8] Thus, kinetic parameters for the amidolysis of Suc–Tyr–Leu–Phe–*p*NA (**3**) and newly synthesized Suc–Ala–Leu–Phe–*p*NA (**5**) by cathepsin G were determined and the results are summarized in Table I in comparison with those of α-chymotrypsin. As can be seen in the table, k_{cat}/K_m values with cathepsin G are much smaller than those with α-chymotrypsin. This tendency is compatible with data reported previously.[7,9]

The hydrolysis of Suc–Phe–*p*NA (**1**) and Suc–Leu–Phe–*p*NA (**2**) by both enzymes was negligible. For hydrolysis of the Phe–*p*NA bond, a three amino acids sequence was required.

TABLE I. Kinetic Parameters for the Amidolysis of Substrates by Human Leukocyte Cathepsin G and α-Chymotrypsin

Substrate	Cathepsin G			α-Chymotrypsin		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Suc-Phe-*p*NA (**1**)	n.d.			n.d.		
Suc-Leu-Phe-*p*NA (**2**)	n.d.			n.d.		
Suc-Tyr-Leu-Phe-*p*NA (**3**)[a]	0.2	0.66	3300	0.11	1.3	12400
Suc-Ala-Tyr-Leu-Phe-*p*NA (**4**)	n.d.			n.d.		
Suc-Ala-Leu-Phe-*p*NA (**5**)	2.3	1.33	580	0.09	7.4	82200
Suc-Ala-Ala-Leu-Phe-*p*NA (**6**)	n.d.			n.d.		

n.d.: values could not be calculated because of low hydrolysis rates. *a*) See ref. 9.

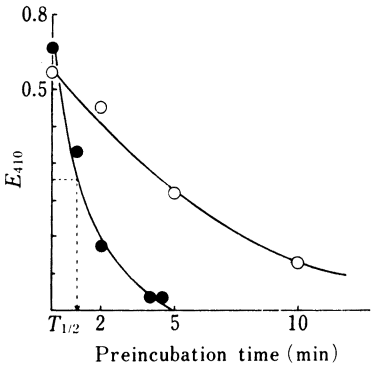

Fig. 1. Inhibitory Effect of Boc-Tyr-Leu-Phe-CH_2Cl on Human Leukocyte Cathepsin G and α-Chymotrypsin

○, cathepsin G; ●, α-chymotrypsin. The reaction was carried out in Tris-HCl buffer (0.1 M, pH 8.0) containing 5% MeOH. The concentration of inhibitor was 25 μM and that of enzyme was 0.2 μM.

The extension of the tripeptide substrates (**3**, **5**) at the N-terminus, Suc-Ala-Tyr-Leu-Phe-*p*NA (**4**) and Suc-Ala-Ala-Leu-Phe-*p*NA (**6**), resulted in markedly decreased hydrolysis rates of the Phe-*p*NA bond by both enzymes. Tripeptide substrates are the most favorable for both enzymes when the Leu-Phe-*p*NA moiety was employed because the k_{cat}/K_m values of Suc-Val-Pro-Phe-*p*NA and Suc-X-Val-Pro-Phe-*p*NA (X: Met, Leu, Phe, Ala, Lys and Glu) were similar.[10]

Although the substrate specificities of cathepsin G and α-chymotrypsin were reported to be similar,[7] the stereogeometry of the active centers of the two enzymes might be quite different. This difference was confirmed by using an irreversible inhibitor, Boc-Tyr-Leu-Phe-CH_2Cl. The inhibitory activity of Boc-Tyr-Leu-Phe-CH_2Cl against cathepsin G and α-chymotrypsin was examined and the results are shown in Fig. 1. Boc-Tyr-Leu-Phe-CH_2Cl exhibited an irreversible inhibitory effect on the amidolytic activity of cathepsin G and α-chymotrypsin toward Suc-Val-Pro-Phe-*p*NA[10] with half lives ($T_{1/2}$) of 225 and 70 s, respectively.

Next, substrate-derived peptidyl inhibitors were prepared by substitution with D-amino acids and/or modification of the *p*NA moiety with compounds such as *p*-acetylaniline (ACA), *p*-benzoylaniline (BZA), 4-methylpiperidine (Pipe) and 4-benzylpiperidine (BPP) with reference to our previous finding that the L-D-D form derived from a substrate (L-L-L form) converted the substrate to an inhibitor, and the *p*NA moiety in the L-D-D form had an important role in increasing the affinity of peptide inhibitors for HLE and α-chymotrypsin.[8,11]

Although HLE did not hydrolyze Suc-Tyr-Leu-Phe-*p*NA (**3**), Suc-Tyr-D-Leu-D-Phe-*p*NA (**7**) inhibited HLE competitively with a K_i value of 0.16 mM. Therefore, the inhibitory effects of synthetic peptides having Tyr-Leu-Phe and Tyr-D-Leu-D-Phe sequences toward

TABLE II. Inhibitory Effects of Peptides (7—19) on the Amidolysis of Synthetic Substrates[a] by HLE, Cathepsin G and α-Chymotrypsin

Compound	K_i value (mM)		
	HLE	Cathepsin G	α-Chymotrypsin
Suc-Tyr-D-Leu-D-Phe-*p*NA (7)[b]	0.16	1.1	0.94
Suc-Phe-BZA (8)	2.0	2.0	0.68
Suc-Leu-Phe-BZA (9)	2.0	1.4	0.38
Suc-Tyr-Leu-Phe-BZA (10)	0.027	0.1	0.011
Suc-Tyr-D-Leu-D-Phe-BZA (11)	0.020	0.75	0.60
Suc-Tyr-Leu-Phe-ACA (12)	0.21	1.6	—
Suc-Tyr-D-Leu-D-Phe-ACA (13)	0.13	1.4	—
Suc-Phe-BPP (14)	2.0	2.0	2.0
Suc-Leu-Phe-BPP (15)	2.0	1.8	2.0
Suc-Tyr-Leu-Phe-BPP (16)	0.40	0.12	0.19
Suc-Tyr-D-Leu-D-Phe-BPP (17)	0.21	0.50	1.2
Suc-Tyr-Leu-Phe-Pipe (18)[c]	2.0	1.2	1.0
Suc-Tyr-D-Leu-D-Phe-Pipe (19)[c]	0.31	2.0	2.0

a) Substrate for HLE, Suc-Ala-Tyr-Leu-Val-*p*NA; substrate for cathepsin G and α-chymotrypsin, Suc-Val-Pro-Phe-*p*NA. *b*) See ref. 6. *c*) See ref. 9. —: not determined.

not only cathepsin G and α-chymotrypsin but also HLE were examined and the results are summarized in Table II.

HLE was inhibited by L-D-D-type peptides (**11**, **13**, **17** and **19**) more potently than by the corresponding L-L-L-type peptides (**10**, **12**, **16** and **18**), whereas α-chymotrypsin and cathepsin G were inhibited by L-L-L-type peptides (**10**, **12**, **16** and **18**) more potently than by the corresponding L-D-D-type peptides (**11**, **13**, **17** and **19**). This phenomenon is compatible with the known inhibitory activity of Suc-Tyr-Leu-Val-R (L-L-L and L-D-D, R=*p*NA, BZA, ACA, BPP and Pipe) against HLE and cathepsin G.[11]

Both HLE and α-chymotrypsin favored an anilide-type moiety at the P_1'[12] position rather than a piperidine amide-type moiety, whereas anilide-type peptides (**10** and **11**) and piperidine amide-type peptides (**16** and **17**) exhibited similar inhibitory activities toward cathepsin G, indicating a difference of stereogeometry of the active center between cathepsin G and α-chymotrypsin.

With regard to the relationship between the chain length and inhibitory activity, the three enzymes were inhibited by tripeptide derivatives (**10** and **16**) more potently than by amino acid derivatives (**8** and **15**) and dipeptide derivatives (**9** and **15**). It is clear that the amino acid at P_3 position is very important in manifestation of the inhibitory activity.

Interestingly, Suc-Tyr-Leu-Phe-BZA (**10**) inhibited HLE, cathepsin G and α-chymotrypsin fairly potently even though the substrate specificities of HLE and cathepsin G (or α-chymotrypsin were different. The Phe-BZA bond of the peptide (**10**) was resistant to hydrolysis by cathepsin G (1.7% in 30 min), although Suc-Tyr-Leu-Phe-ACA (**12**) was cleaved to release ACA (K_m=0.50 mM, k_{cat}=1.63 s^{-1}).

These results provide valuable information concerning the stereogeometry of HLE, cathepsin G and α-chymotrypsin and should be useful in the design of the peptide inhibitors of the above enzymes.

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-101 AS, Kyowa Seimitsu). For column chromatography, a Toyo SF-160 fraction collector was used. For thin layer chromatography (TLC) (Kieselgel G, Merck), Rf^1, Rf^2 and Rf^3 values refer to the systems of $CHCl_3$, MeOH and H_2O (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (89 : 10 : 1) and $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), respectively.

Boc-Ala-Tyr-Leu-Phe-*p*NA——Boc-Ala-OH (0.55 g, 2.9 mmol), H-Tyr-Leu-Phe-*p*NA·HCl [prepared from Boc-Tyr-Leu-Phe-*p*NA[9] (1.9 g, 2.9 mmol) and 2.8 N HCl/dioxane (10.3 ml, 29 mmol)] and HOBt (0.40 g, 2.9 mmol) were dissolved in DMF (40 ml) containing Et_3N (0.40 ml, 2.9 mmol). DCC (0.71 g, 3.4 mmol) was added to the above solution under cooling with ice-salt, and the reaction mixture was stirred at room temperature overnight. After removal of 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 evaporated down. Ether was added to the residue to give crystals, which were collected by filtration. The crude material in $CHCl_3$ (3 ml) was applied to a silica gel column (2 × 31 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (600—800 ml) was removed by evaporation. Petroleum ether was added to the residue to provide the purified material. Yield 0.97 g (45%), mp 179—183 °C, $[\alpha]_D^{25}$ +1.1 (c=0.9, DMF), Rf^1 0.53, Rf^2 0.47. *Anal.* Calcd for $C_{38}H_{48}N_6O_9 \cdot 2H_2O$: C, 59.4; H, 6.81; N, 10.9. Found: C, 59.0; H, 6.72; N, 10.6.

Boc-Ala-Leu-Phe-*p*NA——The title compound was prepared from Boc-Ala-OH (0.7 g, 3.7 mmol) and H-Leu-Phe-*p*NA·HCl [prepared from Boc-Leu-Phe-*p*NA[9] (2.0 g, 4.0 mmol) and 3.7 N HCl/dioxane (11 ml, 40 mmol)] in the same way as described for the synthesis of Boc-Ala-Tyr-Leu-Phe-*p*NA. Yield 1.7 g (77%), mp 110—112 °C, $[\alpha]_D^{25}$ −28.5° (c=0.6, MeOH), Rf^1 0.65, Rf^2 0.68. *Anal.* Calcd for $C_{29}H_{39}N_5O_7 \cdot 0.5H_2O$: C, 60.2; H, 6.96; N, 12.1. Found: C, 60.6; H, 7.04; N, 12.0.

Boc-Ala-Ala-Leu-Phe-*p*NA——The title compound was prepared from Boc-Ala-OH (0.55 g, 2.9 mmol) and H-Ala-Leu-Phe-*p*NA·HCl [prepared from Boc-Ala-Leu-Phe-*p*NA (1.6 g, 2.9 mmol) and 2.8 N HCl/dioxane (10.3 ml, 29 mmol)] in the same way as described for the synthesis of Boc-Ala-Tyr-Leu-Phe-*p*NA and recrystallized from EtOH. Yield 1.69 g (87%), mp 227—230 °C, $[\alpha]_D^{25}$ −6.2 °C (c=0.9, DMF), Rf^1 0.53, Rf^2 0.55. *Anal.* Calcd for $C_{32}H_{44}N_6O_8$: C, 60.0; H, 6.92; N, 13.1. Found: C, 59.8; H, 6.95; N, 13.3.

Suc-Phe-*p*NA (1)——H-Phe-*p*NA·HBr [prepared from Z-Phe-*p*NA[9] (0.23 g, 0.48 mmol) and 13% HBr-AcOH (1.0 ml)] was dissolved in pyridine (10 ml) containing Et_3N (0.07 ml, 0.48 mmol) and cooled with ice. Succinic anhydride (0.15 g, 1.5 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 extracted with AcOEt. The extract was washed with 10% AcOH and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford crystals, which were collected by filtration. Yield 0.13 g (71%), mp 178—181 °C, $[\alpha]_D^{25}$ −35.9° (c=1.0, DMF), Rf^1 0.47. *Anal.* Calcd for $C_{19}H_{19}N_3O_6 \cdot 0.25H_2O$: C, 58.6; H, 5.00; N, 10.82. Found: C, 58.6; H, 4.89; N, 10.6.

Suc-Leu-Phe-*p*NA (2)——The title compound was prepared from H-Leu-Phe-*p*NA·HCl [prepared from Boc-Leu-Phe-*p*NA[9] (0.17 g, 0.33 mmol)] and succinic anhydride (0.10 g, 1.0 mmol) in the same way as described for the synthesis of **1**. Yield 0.091 g (55%), mp 195—197 °C, $[\alpha]_D^{25}$ +8.5° (c=0.7, DMF), Rf^1 0.80. *Anal.* Calcd for $C_{25}H_{30}N_4O_7 \cdot 0.25H_2O$: C, 59.7; H, 6.07; N, 11.1. Found: C, 59.8; H, 6.02; H, 11.0.

Suc-Ala-Tyr-Leu-Phe-*p*NA (4)——The title compound was prepared from H-Ala-Tyr-Leu-Phe-*p*NA·HCl [prepared from Boc-Ala-Tyr-Leu-Phe-*p*NA (0.7 g, 0.96 mmol) and 2.8 N HCl/dioxane (3.4 ml, 9.6 mmol)] and succinic anhydride (0.18 g, 1.8 mmol) in the same way as described for the synthesis of **1**. The crude product (0.40 g) was dissolved in MeOH (30 ml) containing 1 N NaOH (1.1 ml) and the solution was stirred at room temperature for 40 min. After neutralization of the solution with AcOH and removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% AcOH and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give crystals. Yield 0.31 g (45%), mp 206—211 °C, $[\alpha]_D^{25}$ +0.9° (c=1.0, DMF), Rf^1 0.29, Rf^2 0.65. *Anal.* Calcd for $C_{37}H_{44}N_6O_{10} \cdot 0.5H_2O$: C, 59.9; H, 6.13; N, 11.3. Found: C, 59.8; H, 5.98; N, 11.2.

Suc-Ala-Leu-Phe-*p*NA (5)——The title compound was prepared from H-Ala-Leu-Phe-*p*NA·HCl [prepared from Boc-Ala-Leu-Phe-*p*NA (0.39 g, 0.71 mmol) and 2.8 N HCl/dioxane (2.6 ml)] and succinic anhydride in the same way as described for the synthesis of **4**. Yield 0.27 g (69%), mp 208—217 °C, $[\alpha]_D^{25}$ −27.5° (c=0.8, MeOH), Rf^1 0.40, Rf^2 0.31. *Anal.* Calcd for $C_{28}H_{35}N_5O_8 \cdot H_2O$: C, 57.2; H, 6.35; N, 11.9. Found: C, 57.5; H, 6.07; N, 11.5.

Suc-Ala-Ala-Leu-Phe-*p*NA (6)——The title compound was prepared from H-Ala-Ala-Leu-Phe-*p*NA·TFA [prepared from Boc-Ala-Ala-Leu-Phe-*p*NA (0.62 g, 1.0 mmol), TFA (1.5 ml) and anisole (0.2 ml)] and succinic anhydride (0.3 g, 3.0 mmol) in the same way as described for the synthesis of **4**. Yield 0.50 g (79%), mp 249—251.5 °C, $[\alpha]_D^{25}$ −3.2° (c=1.1, MeOH), Rf^1 0.30, Rf^3 0.47. *Anal.* Calcd for $C_{31}H_{40}N_6O_9$: C, 58.1; H, 6.29; N, 13.1. Found: C, 58.0; H, 6.22; N, 13.1.

General Procedure for Preparation of Stereoisomeric Boc-Phe-R (R=BZA, ACA and BPP)——Mixed anhydride [prepared from Boc-Phe-OH (2.7 g, 10 mmol), isobutyl chloroformate (1.31 ml, 10 mmol) and *N*-methylmorpholine (1.1 ml, 10 mmol)] in THF (30 ml) was combined with H-R (10 mmol, R = BZA, ACA and BPP) in THF (20 ml) at 0 °C. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 5% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give the desired compound. This material was purified

TABLE III. Yields, Melting Points, $[\alpha]_D$ Values, Elemental Analyses and *Rf* Values of Boc-L-Phe-R and Boc-D-Phe-R (R = BZA, ACA and BPP)

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$	Formula	Elemental analysis Calcd (Found) C H N	TLC *Rf*¹	TLC *Rf*²
Boc-L-Phe-BZA	40	156—157	+60.8° (*c* = 1.0, DMF)	$C_{27}H_{28}N_2O_4$	73.0 6.35 6.30 (72.9 6.27 6.14)	0.71	0.71
Boc-D-Phe-BZA	63	152—157	−62.4° (*c* = 1.0, DMF)	$C_{27}H_{28}N_2O_4$	73.0 6.35 6.30 (72.8 6.33 6.30)	0.71	0.71
Boc-L-Phe-ACA	59	158—159	+54.0° (*c* = 1.4, MeOH)	$C_{22}H_{26}N_2O_4$	69.1 6.85 7.33 (69.2 6.84 7.33)	0.71	0.58
Boc-D-Phe-ACA	68	155—157	−58.6° (*c* = 1.1, MeOH)	$C_{22}H_{26}N_2O_4$	69.1 6.85 7.33 (69.1 6.81 7.32)	0.70	0.58
Boc-L-Phe-BPP	72	Oil				0.78	0.85
Boc-D-Phe-BPP	77	Oil				0.78	0.85

TABLE IV. Yields, Melting Points, $[\alpha]_D$ Values, Elemental Analyses and *Rf* Values of Boc-L-Leu-L-Phe-R and Boc-D-Leu-D-Phe-R (R = BZA, ACA and BPP)

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$	Formula	Elemental analysis Calcd (Found) C H N	TLC *Rf*¹	TLC *Rf*²
Boc-L-Leu-L-Phe-BZA	53	100—105	+14.7° (*c* = 1.0, DMF)	$C_{33}H_{39}N_3O_5 \cdot 3/4\,H_2O$	69.4 7.14 7.35 (69.2 6.93 7.63)	0.60	0.64
Boc-D-Leu-D-Phe-BZA	91	95—100	−18.2° (*c* = 1.0, DMF)	$C_{33}H_{39}N_3O_5$	71.7 7.05 7.54 (70.9 7.24 7.38)	0.60	0.64
Boc-L-Leu-L-Phe-ACA	74	85—91	−11.7° (*c* = 1.0, MeOH)	$C_{28}H_{37}N_3O_5$	67.9 7.53 8.48 (68.0 7.75 8.61)	0.74	0.84
Boc-D-Leu-D-Phe-ACA	52	83—88	+11.8° (*c* = 1.0, MeOH)	$C_{28}H_{37}N_3O_5$	67.9 7.53 8.48 (67.7 7.66 8.76)	0.74	0.84
Boc-L-Leu-L-Phe-BPP	89	Oil	−24.5° (*c* = 0.7, MeOH)	$C_{32}H_{45}N_3O_4 \cdot 3/4\,H_2O$	70.0 8.47 7.70 (70.1 8.52 7.65)	0.84	0.81
Boc-D-Leu-D-Phe-BPP	80	Oil	—		—	0.84	0.81

by silica gel column chromatography, if necessary. Yield, melting point, $[\alpha]_D$ value, *Rf* values and the elemental analysis data are summarized in Table III.

General Procedure for the Preparation of Stereoisomeric Boc-Leu-Phe-R (R = BZA, ACA and BPP)—Boc-Leu-OH (0.69 g, 3.0 mmol), H-Phe-R [prepared from the corresponding Boc-Phe-R (3.0 mmol) and 3.5 N HCl/dioxane (4.2 ml, 15 mmol)] and HOBt (0.41 g, 3.0 mmol) were dissolved in DMF (20 ml) containing Et_3N (0.42 ml, 3.0 mmol) and cooled with ice-salt. DCC (0.68 g, 3.3 mmol) was added to the above solution and the reaction mixture was stirred at 4 °C overnight. After removal of 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 evaporated down. Petroleum ether was added to the residue to give the desired compound. Yield, melting point, $[\alpha]_D$ value, *Rf* values and analytical data are summarized in Table IV.

General Procedure for Preparation of Stereoisomeric Boc-Tyr-Leu-Phe-R (R = BZA, ACA and BPP)—Boc-Tyr-OH (0.34 g, 1.3 mmol), H-Leu-Phe-R·HCl [prepared from the corresponding Boc-Leu-Phe-R (1.6 mmol) and 3.5 N HCl/dioxane (2.2 ml, 8.0 mmol)] and HOBt (0.18 g, 1.3 mmol) were dissolved in DMF (15 ml) containing Et_3N (0.18 ml, 1.3 mmol) and cooled with ice-salt. DCC (0.32 g, 1.6 mmol) was added to the above cold solution. The reaction mixture was stirred at 4 °C overnight. After removal of the dicyclohexylurea and 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 evaporated down. Petroleum ether was added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt or AcOEt and ether. Yield, melting point, $[\alpha]_D$ value, elemental analysis data

TABLE V. Yields, Melting Points, $[\alpha]_D$ Values, Elemental Analyses and *Rf* Values of Boc-L-Tyr-L-Leu-L-Phe-R and Boc-L-Tyr-D-Leu-D-Phe-R (R = BZA, ACA and BPP)

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (DMF)	Formula	Elemental analysis Calcd (Found) C H N	TLC Rf^1	TLC Rf^2
Boc-L-Tyr-L-Leu-L-Phe-BZA	74	166—168	+13.7° (c=1.0)	$C_{42}H_{48}N_4O_7 \cdot 1/2\,H_2O$	69.1 6.76 7.67 (69.1 6.80 7.56)	0.56	0.31
Boc-L-Tyr-D-Leu-D-Phe-BZA	36	226—228	+25.5° (c=1.0)	$C_{42}H_{48}N_4O_7$	70.0 6.71 7.77 (69.7 6.94 7.88)	0.56	0.21
Boc-L-Tyr-L-Leu-L-Phe-ACA	59	209—213	−93.7° (c=1.0)	$C_{37}H_{46}N_4O_7$	67.5 7.04 8.51 (67.5 7.09 8.41)	0.61	0.54
Boc-L-Tyr-D-Leu-D-Phe-ACA	85	237—239	+27.7° (c=1.0)	$C_{37}H_{46}N_4O_7$	67.5 7.04 8.51 (67.4 7.05 8.45)	0.61	0.54
Boc-L-Tyr-L-Leu-L-Phe-BPP	89	107—112	−10.0° (c=1.0)	$C_{41}H_{54}N_4O_6 \cdot 1/2\,H_2O$	69.6 7.78 7.91 (69.5 7.84 7.82)	0.65	0.28
Boc-L-Tyr-D-Leu-D-Phe-BPP	43	109—112	+7.5° (c=0.9)	$C_{41}H_{54}N_4O_6$	70.5 7.79 8.02 (70.4 7.89 7.80)	0.65	0.28

and *Rf* values are summarized in Table V.

Suc-Phe-BZA (8)——H-Phe-BZA·HCl [prepared from Boc-Phe-BZA (0.11 g, 0.25 mmol) and 3.5 N HCl/dioxane (0.72 ml, 2.5 mmol)] was dissolved in pyridine (6 ml) containing Et_3N (0.035 ml, 0.25 mmol). Succinic anhydride (0.075 g, 0.75 mmol) was added to the above solution in three equal portions over a period of 30 min. During the reaction, the pH of the solution was maintained at 8—9 by adding Et_3N. The reaction mixture was stirred at room temperature for 90 min. After removal of the solvent, AcOEt and 10% AcOH were added to the residue. The organic layer was washed with water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give crystals, which were collected by filtration. Yield 0.090 g (81%), mp 76—85 °C, $[\alpha]_D^{25}$ +19.2° (c=1.1, DMF), Rf^1 0.59. *Anal.* Calcd for $C_{26}H_{24}N_2O_5 \cdot 2.5H_2O$: C, 63.8; H, 5.93; N, 5.32. Found: C, 63.9; H, 5.52; N, 5.06.

Suc-Leu-Phe-BZA (9)——The title compound was prepared from H-Leu-Phe-BZA·HCl [prepared from Boc-Leu-Phe-BZA (0.36 g, 0.64 mmol) and 3.5 N HCl/dioxane (1.8 ml, 6.4 mmol)] and succinic anhydride (0.19 g, 1.9 mmol) in the same manner as described for the synthesis of **8**. Yield 0.18 g (50%), mp 213—215 °C, $[\alpha]_D^{25}$ +14.8° (c=0.9, DMF), Rf^1 0.82. *Anal.* Calcd for $C_{32}H_{35}N_3O_6 \cdot 0.25H_2O$: C, 68.4; H, 6.32; N, 7.48. Found: C, 68.4; H, 6.23; N, 7.29.

Suc-Phe-BPP (14)——The title compound was prepared from H-Phe-BPP·HCl [prepared from Boc-Phe-BPP (0.23 g, 0.56 mmol) and 3.5 N HCl/dioxane (1.6 ml, 5.6 mmol)] and succinic anhydride (0.17 g, 1.7 mmol) in the same manner as described for the synthesis of **8**. Yield 0.20 g (86%), amorphous, $[\alpha]_D^{25}$ −30° (c=0.1, MeOH), Rf^1 0.61. *Anal.* Calcd for $C_{25}H_{30}N_2O_4 \cdot 3.25H_2O$: C, 62.5; H, 7.11; N, 4.86. Found: C, 62.4; H, 6.89; N, 4.62.

Suc-Leu-Phe-BPP (15)——The title compound was prepared from H-Leu-Phe-BPP·HCl [prepared from Boc-Leu-Phe-BPP (0.19 g, 0.36 mmol) and 3.5 N HCl/dioxane (1.2 ml, 4.2 mmol)] and succinic anhydride (0.12 g, 1.3 mmol) in the same manner as described for the synthesis of **8**. Yield 0.11 g (61%), mp 85—99 °C, $[\alpha]_D^{25}$ −10.6° (c=0.9, MeOH), Rf^1 0.60. *Anal.* Calcd for $C_{31}H_{41}N_3O_5 \cdot 1.25H_2O$: C, 66.8; H, 7.80; N, 7.53. Found: C, 66.6; H, 7.56; N, 7.43.

General Procedure for Preparation of Stereoisomeric Suc-Tyr-Leu-Phe-R [R = BZA (10, 11), R = ACA (12, 13), and R = BPP (16, 17)]——Succinylation was performed with H-Tyr-Leu-Phe-R [prepared from the corresponding Boc-Tyr-Leu-Phe-R (0.80 mmol) and 3.5 N HCl/dioxane (2.2 ml, 8.0 mmol)] and succinic anhydride (0.21 g, 2.1 mmol) in the same manner as described for the synthesis of **8**. The crude product was dissolved in MeOH (30 ml) containing 1 N NaOH (1.2 ml). After 90 min at room temperature, the solution was neutralized with AcOH and 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 evaporated down. Ether was added to the residue to give crystals, which were collected by filtration. Yield, melting point, $[\alpha]_D$ value, elemental analysis data and *Rf* value are summarized in Table VI.

Z-Phe-CH_2Cl——Diazomethane [prepared from nitrosomethylurea (6.1 g, 60 mmol)] was added to a mixed anhydride [prepared from Z-Phe-OH (9.0 g, 30 mmol), ethyl chloroformate (2.8 ml, 30 mmol) and Et_3N (4.2 ml, 30 mmol)] in THF (100 ml) at −15 °C and the reaction mixture was stirred at 4 °C for 15 h. After addition of 6 N HCl/dioxane (15 ml, 90 mmol) at −15 °C, the reaction mixture was stirred at −15 °C for 3 h. After neutralization of the solution with Et_3N and removal of the solvent, the residue was dissolved in AcOEt. This solution was washed with

TABLE VI. Yields, Melting Points, $[\alpha]_D$ Values, Elemental Analyses and *Rf* Values of Suc-L-Tyr-L-Leu-L-Phe-R and Suc-L-Tyr-D-Leu-D-Phe-R (R = BZA, ACA and BPP)

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^3
Suc-L-Tyr-L-Leu-L-Phe-BZA	86	116—117	−2.8° (*c*=0.9, MeOH)	$C_{41}H_{44}N_4O_8 \cdot 1/2\,H_2O$	67.5 (67.3)	6.21 (6.26)	7.67 (7.32)	0.25	0.71
Suc-L-Tyr-D-Leu-D-Phe-BZA	61	195—198	+122.9° (*c*=0.9, MeOH)	$C_{41}H_{44}N_4O_8 \cdot 1/2\,H_2O$	67.5 (67.2)	6.21 (6.15)	7.67 (7.69)	0.48	0.55
Suc-L-Tyr-L-Leu-L-Phe-ACA	83	165—175	−28.3° (*c*=1.0, DMF)	$C_{36}H_{42}N_4O_8 \cdot H_2O$	63.9 (63.9)	6.55 (6.44)	8.23 (8.23)	0.40	0.90
Suc-L-Tyr-D-Leu-D-Phe-ACA	90	198—202	+122.8° (*c*=1.0, MeOH)	$C_{36}H_{42}N_4O_8 \cdot 1/4\,H_2O$	65.2 (65.2)	6.42 (6.41)	8.45 (8.46)		0.70
Suc-L-Tyr-L-Leu-L-Phe-BPP	80	105—110	−18.1° (*c*=1.1, MeOH)	$C_{40}H_{54}N_4O_6 \cdot H_2O$	67.0 (67.1)	7.31 (7.23)	7.82 (7.67)	0.47	0.78
Suc-L-Tyr-D-Leu-D-Phe-BPP	64	110—115	+35.9° (*c*=1.0, MeOH)	$C_{40}H_{54}N_4O_6 \cdot 1/2\,H_2O$	67.9 (68.0)	7.26 (7.24)	7.92 (7.93)	0.47	0.84

0.1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give crystals, which were recrystallized from EtOH. Yield 7.4 g (75%), mp 88—91 °C, $[\alpha]_D^{25}$ −41.9° (*c*=0.9, MeOH), Rf^1 0.82. *Anal.* Calcd for $C_{18}H_{18}ClNO_3$: C, 65.2; H, 5.46; N, 4.22. Found: C, 65.4; H, 5.44; N, 4.25.

Boc-Tyr-Leu-Phe-CH_2Cl——Boc-Tyr-Leu-OH[13] (0.95 g, 2.4 mmol), HOBt (0.32 g, 3.0 mmol) and H-Phe-CH_2Cl·HBr [prepared from Z-Phe-CH_2Cl (1.0 g, 3.0 mmol) and 25% HBr/AcOH (2.0 ml, 6.0 mmol)] were dissolved in DMF containing Et_3N (0.34 ml, 2.4 mmol). DCC (0.62 g, 3.0 mmol) was added to the above cold solution and the reaction mixture was stirred at 4 °C 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 give a precipitate. The crude material in $CHCl_3$ (3 ml) was applied to a silica gel column (2 × 33.5 cm) equilibrated and eluted with $CHCl_3$, MeOH and water (95 : 4.6 : 0.4). Individual fractions (5 g each) were collected and the eluate (tube Nos. 24—60) was concentrated to a small volume. Ether and petroleum ether were added to the residue to give a white precipitate, which was collected by filtration. Yield 110 mg (8.1%), mp 155—159 °C, $[\alpha]_D^{25}$ −36.4° (*c*=0.9, MeOH), Rf^1 0.57, Rf^3 0.81. *Anal.* Calcd for $C_{30}H_{40}ClN_3O_6$: C, 62.7; 7.02; N, 7.31. Found: C, 62.4; H, 6.68; N, 7.20.

Kinetic Measurements——HLE[14] and cathepsin G[15] were isolated in our laboratory according to the procedures described previously. α-Chymotrypsin was purchased from Miles Co., Ltd., Elhardt. The cathepsin G preparation contains leukocyte elastase as an enzymatically active protein. The amidolytic activities of HLE and cathepsin G were assayed by measuring the *p*-nitroaniline (410 nm) released from the specific substrates Suc-Ala-Tyr-Leu-Val-*p*NA[5] and Suc-Val-Pro-Phe-*p*NA,[3] respectively. The enzyme reaction was carried out at 37 °C in Tris-HCl buffer (0.1 M, pH 8.0 for HLE, 0.1 M, pH 7.5 for cathepsin G and 0.1 M, pH 7.5 for α-chymotrypsin). Kinetic constants were estimated from the initial rates of amidolysis of substrates by the enzymes according to Lineweaver and Burk.[16] The K_i values were determined in the same manner after adding the peptide to be examined (synthetic peptides were dissolved in DMSO-containing buffer).

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