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Synthesis of Chromogenic Substrates Specific for Human Spleen Fibrinolytic Proteinase (SFP) and Human Leukocyte Elastase (LE)

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Various peptide anilides were synthesized by a conventional method with the object of obtaining specific substrates for human spleen fibrinolytic proteinase (SFP) and human leukocyte elastase (LE) in order to compare the substrate specificity of SFP with that of LE. It was found that the P_1 Val compound among succinyl tripeptide *p*-nitroanilides (Suc–Tyr–Leu–X–*p*NA) exhibited the highest k_{cat}/K_m values for hydrolysis by SFP and LE, however, the tetrapeptide Suc–Ala–Tyr–Leu–Val–*p*NA [k_{cat}/K_m values ($M^{-1}\,s^{-1}$) for hydrolysis by SFP and LE: 84000 and 48000, respectively] was the preferred chromogenic substrate for SFP and LE because of its high solubility in the buffer and its moderate k_{cat}/K_m values. The substrate specificity of SFP was found to be similar to that of LE.

Keywords——human spleen fibrinolytic proteinase (SFP); human leukocyte elastase (LE); substrate specificity; chromogenic substrates; chemical synthesis; Suc–Ala–Tyr–Leu–Val–*p*NA

Okamoto *et al.*[2] isolated and purified a neutral proteinase which degrades fibrin and fibrinogen from human spleen tissue (SFP) and found that most of its properties, such as molecular weight and mobility in electrophoresis, were similar to those of human leukocyte elastase (LE), which is responsible for the tissue destruction that occurs in disease such as emphysema and bronchitis;[3] however, SFP did not degrade elastin. A program has been initiated in our laboratories directed to the synthesis of chromogenic substrates and inhibitors specific for SFP and LE in order to facilitate further studies on the enzymatic properties. Recently, we reported that a newly synthesized chromogenic substrate, Suc–Tyr–Leu–Val–*p*NA, which is related to insulin B chain (16—18), was specific for SFP.[4,5] This report describes the synthesis of chromogenic substrates with substitution of Suc–Tyr–Leu–Val–*p*NA at the P_1, P_2 or P_3 position and elongation of the peptide chain, as well as the amidolytic activities of SFP towards those synthetic substrates in comparison with those of LE.

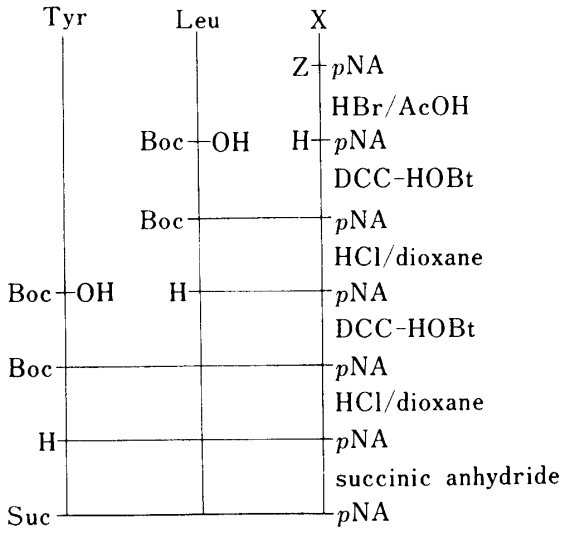

Fig. 1. Synthetic Route to Suc–Tyr–Leu–X–*p*NA

X: Val, Ile, Ala, Leu, Phe Met, Gly, Pro, Lys(Z).

The synthetic route to the tripeptide substrates is shown in Fig. 1. Z-amino acid-*p*NAs were synthesized by the phospho-azo method[6] and DCC–HOBt method.[7] The phospho-azo method generally gave better yields of anilides than the DCC–HOBt methods, as shown in Table I. After removal of the Z group from Z-amino acid-*p*NA by HBr/AcOH treatment, the resulting amine was coupled with Boc–Leu–OH by the DCC–HOBt method to afford Boc–Leu–X–*p*NA (X=amino acid). Boc–Leu–X–*p*NA was treated with HCl/dioxane to give

TABLE I. Yields of Benzyloxycarbonyl-amino Acid *p*-Nitroanilides (%)

Method	Amino acid									
	Gly	Ala	Val	Pro	Arg(NO_2)	Ile	Phe	Leu	Met[a]	Lys(Z)[b]
DCC-HOBt	9.1	8.7	trace	56.5	6.8	8.2	28.3	35.7	22.2	30.0
Phosphoazo	63.0	52.2	48.6	32.0	56.2	12.7	51.7	63.8	—	—

a) Boc-Met-OH was coupled with *p*NA by treatment with DCC alone. *b*) Boc-Lys(Z)-OH was coupled with *p*NA by treatment with DCC alone.

dipeptide amine hydrochloride, which was coupled with Boc-Tyr-OH by the DCC-HOBt method to yield Boc-Tyr-Leu-X-*p*NA. After removal of the Boc group with HCl/dioxane, the resulting amine was acylated with succinic anhydride[8] to increase the water solubility of the peptides. Suc-Tyr-Leu-Lys-*p*NA and Suc-Tyr-Leu-Arg-*p*NA were synthesized as follows. Suc-Tyr-Leu-Lys(Z)-*p*NA was treated with HBr/AcOH to remove the Z group on the ε-amino function of Lys residue to give the desired substrate. Prior to succinylation of P_1 Arg substrate, Boc-Tyr-Leu-Arg(NO_2)-*p*NA was treated with HF containing anisole[9] to afford H-Tyr-Leu-Arg-*p*NA hydrogen fluoride. This was converted to the corresponding acetate by treating it with Amberlite IRA 45 (acetate form). The acetate was further converted to the hydrochloride with 1 N HCl. This was succinylated with succinic anhydride in pyridine to afford Suc-Tyr-Leu-Arg-*p*NA. P_2-Substituted substrate, Suc-Tyr-Pro-Val-*p*NA and P_3-substituted substrates, Suc-Phe-Leu-Val-*p*NA and Suc-Ala-Leu-Val-*p*NA, were synthesized by the same route as described above. These peptide anilides obtained above were purified by recrystallization and/or silica gel column chromatography. Their homogeneity was assessed by thin-layer chromatography, elemental analysis and amino acid analysis of acid hydrolysates. Amidolytic activity was assayed by measuring the released *p*-nitroaniline (E_{410}). Kinetic parameters for the amidolysis of Suc-Tyr-Leu-X-*p*NA (X=amino acid) by SFP are presented in Table II. From the results, it can be seen that SFP exhibited a high

TABLE II. Kinetic Parameters for the Hydrolysis of Succinyl Tripeptide *p*-Nitroanilides by Human Spleen Fibrinolytic Proteinase (SFP)

Substrate P_3 P_2 P_1	K_m (mM)	k_{cat} (s^{-2})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Suc-Tyr-Leu-Val-*p*NA	0.17	3.85	22600
-Ile-	0.4	1.7	4250
-Ala-	0.8	1.2	1500
-Leu-	n.d.	n.d.	
-Phe-	n.d.	nid.	
-Gly-	n.d.	n.d.	
-Met-	n.d.	n.d.	
-Pro-	n.d.	n.d.	
-Lys-	n.d.	n.d.	
-Arg-	n.d.	n.d.	

k_{cat} was calculated on the assumption that all protein in the purified preparation was SFP.
n.d. = No hydrolysis was detectable.

degree of specificity with respect to the P_1 amino acid residue. The first three substrates were hydrolyzed by SFP to release *p*-nitroaniline with the following k_{cat}/K_m values ($M^{-1}\,s^{-1}$), Val: 22600; Ile: 4250; Ala: 1500. In contrast, peptides where X=Leu, Phe, Met, Gly, Pro, Lys, and Arg, were not cleaved by SFP to any measurable extent. From the finding that the P_1 Val compound exhibited the highest k_{cat}/K_m value for hydrolysis by SFP, it can be deduced that two methyl groups on the β-carbon atom of the P_1 amino acid are most favorable for

binding with the SFP active site whereas, an ethyl group or hydrogen atoms on the β-carbon atom of the P_1 amino acid are not so suitable for binding with the enzyme (Fig. 2). With regard to the P_2 or P_3 substitution, the replacement of P_2 Leu with Pro increased both k_{cat} and K_m values and resulted in a higher k_{cat}/K_m value than that of the parent molecule. P_3 Tyr compound exhibited a higher k_{cat}/K_m value than that of P_3 Phe or Ala compound, indicating that a Tyr residue at P_3 was favorable for binding with SFP. These results are summarized in Table III.

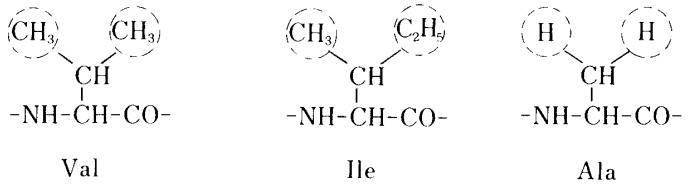

Fig. 2. Structure of P_1 Residues of Suc–Tyr–Leu–X–*p*NA

TABLE III. P_2 and P_3 Residue Substitution and SFP Amidolysis

Substrate P_3 P_2 P_1	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Suc–Tyr–Leu–Val–*p*NA	0.17	3.85	22600
–Pro–	0.21	7.7	36700
–Phe–	0.37	6.7	18100
–Ala–	0.68	9.6	14100
–Ala–Pro–	1.3	27.0	20770

TABLE IV. Comparative Activities of Human Spleen Fibrinolytic Proteinase (SFP) and Human Leucocyte Elastase (LE) on Various Synthetic Substrates

Substrate	SFP	LE
Suc–Tyr–Leu–Val–*p*NA	100%	100%
Suc–Tyr–Leu–Ile–*p*NA	33	23
Suc–Tyr–Leu–Ala–*p*NA	16	13
Suc–Tyr–Leu–Leu–*p*NA	0.2	0.2
Suc–Tyr–Leu–Phe–*p*NA	0	0
Suc–Tyr–Leu–Gly–*p*NA	0	0
Suc–Tyr–Leu–Met–*p*NA	0	0
Suc–Tyr–Leu–Pro–*p*NA	0	0
Suc–Tyr–Leu–Lys–*p*NA	0	0
Suc–Tyr–Leu–Arg–*p*NA	0	0

Table IV compares the amidolytic activity of SFP towards a series of compound, Suc–Tyr–Leu–X–*p*NA, with that of LE. From Table IV, it can be seen that substrate specificity of SFP is quite similar to that of LE. The finding of amidolytic activity of LE towards the compound containing the Val residue at the P_1 position is consistent with the results reported previously.[10–12]

Next, in order to determined the amidolytic activity of SFP and LE towards peptide anilides with various different chain lengths, Suc–Val–*p*NA, Suc–Leu–Val–*p*NA, Suc–Tyr–Leu–Val–*p*NA,[4,5] Suc–Ala–Tyr–Leu–Val–*p*NA, Suc–Ala–Ala–Tyr–Leu–Val–*p*NA, and Suc–

Ala–Ala–Ala–Tyr–Leu–Val–*p*NA were also synthesized by the stepwise elongation method (DCC–HOBt method) with succinylation by succinic anhydride in the same way as described above. Table V summarizes the kinetic parameters for hydrolysis of a series of substrates with different chain lengths containing Val at the P_1 position by SFP and LE. The k_{cat}/K_m value is increased by lengthening the peptide up to pentapeptide. Suc–Leu–Val–*p*NA was cleaved slightly by SFP, but Suc–Val–*p*NA and Suc–Ala–Ala–Ala–Tyr–Leu–Val–*p*NA were not hydrolyzed by the enzymes. Suc–Ala–Tyr–Leu–Val–*p*NA and Suc–Ala–Ala–Tyr–Leu–Val–*p*NA were hydrolyzed by both enzymes and exhibited much higher k_{cat}/K_m values than that for Suc–Tyr–Leu–Val–*p*NA. Among these substrates, Suc–Ala–Tyr–Leu–Val–*p*NA is the preferred chromogenic substrate for SFP and LE because of its high solubility in the buffer and its moderate k_{cat}/K_m value. The substrate specificities of SFP and LE were confirmed to be similar by the results obtained here. However, further work is necessary on the physiological roles of SFP as well as LE.

Table V. Kinetic Parameters for the Amidolysis of Synthetic Substrates of Various Chain Lengths by SFP and LE

Substrate	Enzyme	K_m(mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Suc–Val–*p*NA	SFP	n. d.	n. d.	
	LE	—	—	
Suc–Leu–Val–*p*NA	SFP	7.0	2.52	360
	LE	—	—	
Suc–Tyr–Leu–Val–*p*NA	SFP	0.17	3.85	22600
	LE	0.21	3.7	17600
Suc–Ala–Tyr–Leu–Val–*p*NA	SFP	0.125	10.5	84000
	LE	0.13	6.3	48500
Suc–Ala–Ala–Tyr–Leu–Val–*p*NA	SFP	0.11	15.2	138000
	LE	0.125	17	136000
Suc–Ala–Ala–Ala–Tyr–Leu–Val–*p*NA	SFP	n. d.	n. d.	
	LE	—	—	

k_{cat} was calculated on the assumption that the protein in the purified preparation was only SFP or LE.
n.d. = No hydrolysis was detectable.

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 a JEOL JLC-6AH amino acid analyzer. For column chromatography, a Toyo SF-160K fraction collector was used. For thin-layer chromatography (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), *n*-BuOH, pyridine, AcOH and H_2O (4: 1: 1: 2), *n*-BuOH, AcOH and H_2O (4: 1: 5, upper phase), *n*-BuOH, pyridine, AcOH and H_2O (1: 1: 1: 2) and *n*-BuOH, pyridine, AcOH and H_2O (30: 20: 6: 24), respectively.

General Procedure for the Synthesis of Boc-Leu-X-*p*NA (X=Val, Ile, Ala, Leu, Phe, Met, Gly, Pro, Lys(Z) and Arg(NO_2))——H–X–*p*NA (10 mmol) [prepared from the corresponding Z–X–*p*NA by treatment with 25% HBr/AcOH or from Boc–X–*p*NA (X=Lys(Z) and Met) by treatmen with HCl/dioxane] and Boc–Leu–OH (10 mmol) were dissolved in DMF (20 ml) and the solution was cooled with ice-salt. DCC (2.3 g, 11 mmol) and HOBt (1.4 g, 10 mmol) were added to the above cold solution. The reaction mixture was stirred at room temperature overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford a crystalline material; the yield, mp, optical rotation, *Rf* values and analytical data are summarized in Table VI.

General Procedure for the Synthesis of Boc-Tyr-Leu-X-*p*NA (X=Val, Ile, Ala, Leu, Phe, Met, Gly, Pro, Lys(Z), and Arg(NO_2))——A solution of Boc–Leu–X–*p*NA (3 mmol) in 6.4 N HCl in dioxane (1.6 ml) was kept at room temperature for 10 min, then diluted with dioxane (1.6 ml) and further kept at room temperature for 30 min. Ether was added to the solution to give a white precipitate, which was collected by centrifugation,

washed with ether and dried over KOH pellets *in vacuo*. The resulting amine hydrochloride, Boc–Tyr–OH (0.84 g, 3 mmol) and HOBt (0.40 g, 3 mmol) were dissolved in DMF (20 ml) containing Et_3N (0.42 ml) and cooled with ice-salt. DCC (0.68 g, 3.3 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 dissolved in AcOEt. The organic layer was washed with 10% citric acid, 5% Na_2CO_3 and H_2O, dried over

Table VI. Yield, mp, Optical Rotation, *Rf* Values and Analytical Data of Protected Dipeptide Derivatives

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
Boc–Leu–Ile–*p*NA	49.5	169—171.5	−55.6 (c = 0.77)	$C_{23}H_{36}N_4O_6$	59.46 (59.18	7.81 7.80	12.06 12.06)	0.56	0.98
Boc–Leu–Ala–*p*NA	63.1	90—92	−67.7 (c = 1.10)	$C_{20}H_{30}N_4O_6$	56.86 (57.18	7.15 7.40	13.26 13.02)	0.55	0.87
Boc–Leu–Leu–*p*NA	72.5	109—113	−58.8 (c = 0.91)	$C_{23}H_{36}N_4O_6$	59.46 (59.18	7.81 7.72	12.06 11.97)	0.84	
Boc–Leu–Phe–*p*NA	16.0	98—100	− 8.7 (c = 1.1)	$C_{26}H_{34}N_4O_6$	62.63 (62.90	6.87 6.98	11.23 11.33)	0.69	
Boc–Leu–Met–*p*NA	67.4	84—87	−51.9 (c = 0.96)	$C_{22}H_{34}N_4O_6S$	54.75 (54.50	7.10 6.95	11.60 11.67)	0.64	0.76
Boc–Leu–Gly–*p*NA	42.8	168—170	+3.26 (c = 0.92)	$C_{19}H_{28}N_4O_6$	55.87 (55.55	6.90 6.78	13.71 13.61)	0.56	0.78
Boc–Leu–Lys(Z)–*p*NA	50.3	78—85	−34.7 (c = 0.68)	$C_{31}H_{43}N_5O_8$	60.67 (60.66	7.06 7.01	11.41 11.45)	0.69	
Boc–Leu–Arg(NO_2)–*p*NA	34.4	125—130	−39.7 (c = 0.77)	$C_{23}H_{36}N_8O_8$	49.99 (50.06	6.56 6.47	20.27 20.01)	0.27	0.67
Z–Leu–Pro–*p*NA	15.5	172—174	−132.0 (c = 0.75)	$C_{25}H_{30}N_4O_6$	62.22 (62.45	6.26 6.79	11.61 11.73)	0.86	0.87

Table VII. Yield, mp, Optical Rotation, *Rf* Values and Analytical Data of Boc–Tyr–Leu–X–*p*NA

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
Boc–Tyr–Leu–Ile–*p*NA	52.6	226—230	−43.00 (c = 0.77)	$C_{32}H_{45}N_5O_8$	61.22 (60.99	7.07 7.24	11.15 11.02)	0.31	0.75
Boc–Tyr–Leu–Ala–*p*NA	42.5	164—167	−58.65 (c = 0.77)	$C_{29}H_{39}N_5O_8$	59.47 (59.72	6.71 6.98	11.96 11.98)	0.48	0.69
Boc–Tyr–Leu–Leu–*p*NA	36.6	210—212	−56.06 (c = 0.95)	$C_{32}H_{45}N_5O_8$	61.22 (61.36	7.07 7.44	11.15 11.13)	0.49	0.72
Boc–Tyr–Leu–Phe–*p*NA	75.6	120—132	− 8.96 (c = 0.78)	$C_{35}H_{43}N_5O_8$	63.52 (63.40	6.54 6.82	10.58 10.30)	0.46	0.81
Boc–Tyr–Leu–Met–*p*NA	77.4	110—115	−41.70 (c = 1.00)	$C_{31}H_{43}N_5O_8S$	57.74 (57.72	6.72 7.06	10.86 10.45)	0.64	0.67
Boc–Tyr–Leu–Gly–*p*NA	88.1	198—199	+ 8.23 (c = 0.99)	$C_{28}H_{37}N_5O_8$	58.83 (58.84	6.52 6.71	12.25 12.20)	0.37	0.75
Boc–Tyr–Leu–Pro–*p*NA	33.4	145—151	−122.89 (c = 0.83)	$C_{31}H_{41}N_5O_8$	60.87 (60.10	6.75 6.87	11.41 11.47)	0.25	0.81
Boc–Tyr–Leu–Lys(Z)–*p*NA	39.6	109—111	−35.79 (c = 0.69)	$C_{40}H_{52}N_6O_{10}$	61.84 (61.61	6.74 6.69	10.81 10.71)	0.26	0.80
Boc–Tyr–Leu–Arg(NO_2)–*p*NA	67.3	153—155	−31.25 (c = 0.48)	$C_{32}H_{45}N_9O_{10}\cdot H_2O$	52.34 (52.92	6.45 6.66	17.16 16.87)	0.16	0.70

Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to afford a crystalline material; the yield, mp, optical rotation, analytical data and *Rf* values are presented in Table VII.

Suc-Tyr-Leu-X-*p*NA (X=Val, Ile, Ala, Leu, Phe, Met, Gly, Pro, Lys(Z))——A solution of Boc-Tyr-Leu-X-*p*NA (0.64 mmol) in 6.4 N HCl (in dioxane (1.0 ml) was kept at room temperature for 10 min then diluted with dioxane (1.5 ml) and further kept at room temperature for 30 min. Ether was added to the solution to give a white precipitate, which was collected by filtration, washed with ether and dried over KOH pellets *in vacuo*. The resulting amine hydrochloride was dissolved in water (15 ml) and the pH of the solution was adjusted to 8 with Na_2CO_3. The oily precipitate was extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. Succinic anhydride (0.19 g, 1.9 mmol) was added to the above AcOEt solution containing Et_3N (0.09 ml) in five equal portions over a period of 1 h with ice cooling. During the reaction, the pH of the solution was maintained at 8—9 by adding Et_3N. The precipitate formed was collected by centrifugation. It was dissolved in AcOEt and 10% AcOH (20 ml+20 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 a white precipitate, which was collected by filtration; the yield, mp, optical rotation, *Rf* values and analytical data are presented in Table VIII.

Table VIII. Yield, mp, Optical Rotation, *Rf* Values and Analytical Data of Suc-Tyr-Leu-X-*p*NA

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC *Rf*[1]	*Rf*[2]
Suc-Tyr-Leu-Ile-*p*NA	67.1	232—232.5	−42.25 (c = 0.35)	$C_{31}H_{41}N_5O_9$	59.32 (58.87	6.58 6.98	11.16 11.11)	0.37	0.36
Suc-Tyr-Leu-Ala-*p*NA	16.8	141—144	−43.47 (c = 0.32)	$C_{28}H_{35}N_5O_9$	57.42 (57.16	6.02 5.89	11.95 11.83)		0.28
Suc-Tyr-Leu-Leu-*p*NA	30.0	125—130	−39.45 (c = 0.29)	$C_{31}H_{41}N_5O_9$	59.32 (58.89	6.58 6.87	11.16 10.76)		0.38
Suc-Tyr-Leu-Phe-*p*NA	64.5	149—154	− 1.93 (c = 0.29)	$C_{34}H_{39}N_5O_9\cdot H_2O$	60.01 (59.58	6.07 5.85	10.29 9.90)	0.37	0.36
Suc-Tyr-Leu-Met-*p*NA	34.1	113—117	−29.61 (c = 0.87)	$C_{30}H_{39}N_5O_9S$	55.80 (55.50	6.08 6.13	11.84 11.55)		0.45
Suc-Tyr-Leu-Gly-*p*NA	25.0	139—143	+ 5.18 (c = 0.77)	$C_{27}H_{33}N_5O_9$	56.37 (56.63	5.81 6.10	12.25 11.97)	0.22	
Suc-Tyr-Leu-Pro-*p*NA	66.9	140—148	−115.66 (c = 0.69)	$C_{30}H_{37}N_5O_9\cdot H_2O$	57.22 (57.54	6.24 6.21	11.12 10.62)	0.13	0.57
Suc-Tyr-Leu-Lys(Z)-*p*NA	42.6	130—135	—	$C_{39}H_{48}N_6O_{11}\cdot 1/2H_2O$	60.29 (59.58	6.28 6.06	10.69 10.48)	0.38	0.52
Suc-Tyr-Leu-Lys-*p*NA	46.2	166—170	−40.52 (c = 0.53)	$C_{31}H_{42}N_6O_9\cdot CH_3COOH\cdot H_2O$	54.96 (54.88	6.70 6.53	11.65 11.86)	0.72[a]	0.65[b]
Suc-Tyr-Leu-Arg-*p*NA	24.4	142—146	−31.57 (c = 0.19)	$C_{31}H_{42}N_8O_9\cdot HCl\cdot H_2O$	51.31 (51.83	6.25 6.41	15.46 14.62)	0.68[c]	0.81[d]

a) *Rf*[3], *b*) *Rf*[5], *c*) *Rf*[4], *d*) *Rf*[6].

Suc-Tyr-Leu-Lys-*p*NA——Suc-Tyr-Leu-Lys(Z)-*p*NA (400 mg) was dissolved in 25% HBr/AcOH (1 ml) containing anisole (0.05 ml). This solution was kept at room temperature for 1 h. Ether was added to the solution to give an oily precipitate, which was washed with ether by decantation, dried over KOH pellets *in vacuo* and dissolved in H_2O (10 ml). This solution was washed with ether and treated with Amberlite IRA-45 (acetate form) to convert hydrochloride to acetate. After removal of the resin and the solvent, the residue was dissolved in H_2O and lyophilized to afford an amorphous powder. This was purified by silica gel column chromatography; the yield, mp, optical rotation, *Rf* values and analytical data are shown in Table VIII.

H-Tyr-Leu-Arg-*p*NA·2HCl——A solution of Boc-Tyr-Leu-Arg(NO_2)-*p*NA (500 mg) in HF (10 ml) containing anisole (0.4 ml) was stirred at 0°C for 30 min. After removal of HF, the residue was dried over KOH pellets *in vacuo*. The oily material was dissolved in H_2O (60 ml). The solution was washed with ether and treated with Amberlite IRA-45 (acetate form). After removal of the resin by filtration, the filtrate was concentrated to a small volume and 1 N HCl (1.4 ml) was added. Lyophilization gave an amorphous powder, yield 206 mg (45.8%), *Rf*[4] 0.73, *Rf*[6] 0.38. *Anal.* Calcd for $C_{27}H_{38}N_8O_6\cdot 2HCl\cdot 3H_2O$: C, 46.5; H, 6.64; N, 16.1. Found: C, 46.7; H, 6.45; N, 16.0.

Suc-Tyr-Leu-Arg-*p*NA·HCl——H–Tyr–Leu–Arg–*p*NA·2HCl (100 mg) was dissolved in pyridine (5 ml) containing Et_3N (0.02 ml). Succinic anhydride (45 mg) was added to the above solution in three equal portions over a period of 1 h under cooling with ice. After removal of the solvent, the residue was dissolved in AcOEt and 10% AcOH (10 ml+10 ml). The organic layer was washed with H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a white precipitate, which was collected by filtration; the yield, mp, optical rotation, *Rf* values and analytical data are presented in Table VIII.

Boc-Pro-Val-*p*NA——H–Val–*p*NA·HBr (prepared from 3.7 g of Z–Val–*p*NA and 9.7 ml of 25% HBr/AcOH), Boc–Pro–OH (2.8 g), and HOBt (1.35 g) were dissolved in DMF (20 ml) containing Et_3N (1.4 ml) and cooled with ice-salt. DCC (3.1 g) was added to the above cold solution, and then reaction mixture was stirred for 15 h. After removal of the urea derivative and the solvent, the oily residue was dissolved in AcOEt. The AcOEt solution was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. The residue was crystallized from AcOEt and petroleum ether, yield 2.65 g (61%), mp 96—100°C, $[\alpha]_D^{25}$ −108.9° (c=0.9, MeOH), Rf^1 0.53, Rf^2 0.92. *Anal.* Calcd for $C_{21}H_{30}N_4O_6$: C, 58.05; H, 6.95; N, 12.89. Found: C, 58.23; H, 7.04; N, 12.63.

Boc-Tyr-Pro-Val-*p*NA——H–Pro–Val–*p*NA (prepared from 2.0 g of Boc–Pro–Val–*p*NA and 7.2 ml of 6.4 N HCl/dioxane), Boc–Tyr–OH (1.3 g) and HOBt (0.62 g) were dissolved in DMF (20 ml) containing Et_3N (0.64 ml) and cooled with ice-salt. DCC (1.1 g) was added to the above cold solution, and then 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 H_2O, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford crystals, which were collected by filtration, yield 1.65 g (60%), mp 96—100°C, $[\alpha]_D^{25}$ −87.0° (c=1.0, MeOH), Rf^1 0.68. *Anal.* Calcd for $C_{30}H_{39}N_5O_8\cdot 1/2H_2O$: C, 59.32; H, 6.63; N, 11.52. Found: C, 60.13; H, 6.74; N, 11.09.

Suc-Tyr-Pro-Val-*p*NA——A solution of Boc–Tyr–Pro–Val–*p*NA (1.0 g) in 2.6 ml of 6.4 N HCl in dioxane was stirred at room temperature for 10 min. The solution was diluted with dioxane (2.6 ml) and further stirred for 50 min. Ether and petroleum ether were added to the solution and the precipitate formed was collected by filtration, washed with ether and dried over KOH pellets *in vacuo*. It was dissolved in H_2O, and the pH of the solution was adjusted to 8 with Na_2CO_3. The oily precipitate was extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. Succinic anhydride (0.5 g) was added to the above solution containing Et_3N (0.23 ml) in four equal portions over a period of 40 min. The reaction mixture was further stirred at 0°C for 1 h. 10% AcOH (20 ml) was added to the solution. The organic layer was washed with water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to give crystals, which were collected by filtration. The crude product (0.6 g) was dissolved in MeOH (10 ml) containing 1 N NaOH (2 ml). The solution was stirred at room temperature for 2 h, then neutralized with AcOH. The solvent was removed by evaporation, and the residue was extracted with AcOEt. The extract was washed with 1 N HCl and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to afford crystals, which were collected by filtration, yield 0.54 g (54.1%), mp 145—160°C, $[\alpha]_D^{25}$ −80.4° (c=1.0, MeOH), Rf^2 0.36. *Anal.* Calcd for $C_{29}H_{35}N_5O_9\cdot H_2O$: C, 56.50; H, 6.04; N, 11.36. Found: C, 56.86; H, 5.94; N, 11.00.

Boc-Phe-Leu-Val-*p*NA——H–Leu–Val–*p*NA·HCl (prepared from 1.3 g of Boc–Leu–Val–*p*NA and 2.0 ml of 7.5 N HCl/dioxane as usual), Boc–Phe–OH (0.80 g) and HOBt (0.4 g) were dissolved in DMF (15 ml) containing Et_3N (0.42 ml) and cooled with ice-salt. DCC (0.68 g) was added to the solution and the reaction mixture was stirred at room temperature for 48 h. After removal of the urea derivative and the solvent, the oily residue was dissolved in AcOEt. The organic layer was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and concentrated to a small volume to give crystals, which were collected by filtration and dried, yield 1.16 g (67.4%), mp 214—216°C, $[\alpha]_D^{25}$ −53.4° (c=1.1, MeOH), Rf^1 0.72, Rf^2 0.71. *Anal.* Calcd for $C_{31}H_{43}N_5O_7$: C, 62.29; H, 7.25; N, 11.71. Found: C, 62.55; H, 7.40; N, 11.81.

Suc-Phe-Leu-Val-*p*NA——H–Phe–Leu–Val–*p*NA·HCl (prepared from 0.90 g of Boc–Phe–Leu–Val–*p*NA and 2.0 ml of 7.5 N HCl/dioxane as usual) was dissolved in H_2O (5 ml), and the pH of the solution was adjusted to 9 with Na_2CO_3. The oily precipitate was extracted with AcOEt. The extract was washed with H_2O and dried over Na_2SO_4. Succinic anhydride (450 mg) was added to the above solution containing Et_3N (0.21 ml) in five equal portions over a period of 1 h and the solution was stirred at 0°C for 3 h. The precipitate formed was collected by centrifugation. It was dissolved in AcOEt and 10% AcOH (20 ml+20 ml). The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated to a small volume to afford crystals, which were collected by filtration, yield 0.53 g (59.2%), mp 230—232°C, $[\alpha]_D^{25}$ −40.9° (c=1.0, MeOH), Rf^1 0.35, Rf^2 0.66. *Anal.* Calcd for $C_{30}H_{39}N_5O_8$: C, 60.29; H, 6.57; N, 11.72. Found: C, 60.56; H, 6.71; N, 11.80.

Boc-Ala-Leu-Val-*p*NA——H–Leu–Val–*p*NA (prepared from 1.3 g of Boc–Leu–Val–*p*NA and 2.0 ml of 7.5 N HCl/dioxane as usual), Boc–Ala–OH (0.57 g) and HOBt (0.4 g) were dissolved in DMF (10 ml) containing Et_3N (0.42 ml) and cooled with ice-salt. DCC (0.68 g) was added to the cold solution, and the reaction mixture was stirred at room temperature for 18 h. After removal of the urea derivative 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 to give crystals, which were collected by filtration, yield 1.31 g (83.8%), mp 199—202°C, $[\alpha]_D^{25}$ −81.0° (c=1.1, MeOH), Rf^1 0.74. *Anal.* Calcd for $C_{25}H_{39}N_5O_7$: C, 57.56; H,

7.53; N, 13.42. Found: C, 57.77; H, 7.60; N, 13.37.

Suc-Ala-Leu-Val-*p*NA——H–Ala–Leu–Val–*p*NA·HCl (prepared from 0.78 g of Boc–Ala–Leu–Val–*p*NA and 2 ml of 7.5 N HCl/dioxane as usual) was dissolved in H_2O, and the pH of the solution was adjusted to 9 with Na_2CO_3. The resulting precipitate was extracted with AcOEt and the extract was washed with H_2O and dried over Na_2SO_4. Succinic anhydride (0.45 g) was added to the above AcOEt solution containing Et_3N (0.21 ml) in five equal portions over a period of 1 h. The reaction mixture was stirred at 0°C for 3 h. The precipitate formed was collected by centrifugation and dissolved in AcOEt and 10% AcOH (20 ml+20 ml). The organic layer was washed with H_2O, 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, yield 0.4 g (51.2%), mp 233—240°C, $[\alpha]_D^{25}$ −87.3° (c=1.0, MeOH), Rf^1 0.24, Rf^2 0.58. *Anal.* Calcd for $C_{24}H_{35}N_5O_8$: C, 55.26; H, 6.76; N, 13.42. Found: C, 55.49; H, 6.94; N, 13.48.

Suc-Val-*p*NA——H–Val–*p*NA (prepared from 740 mg of Z–Val–*p*NA and 1.9 ml of 25% HBr/AcOH) was dissolved in H_2O (10 ml). The pH of the solution was adjusted to 9 with Na_2CO_3 and the precipitate formed was extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. Succinic anhydride (600 mg) was added to the above AcOEt solution containing Et_3N (0.28 ml) under cooling with ice. The reaction mixture was stirred at 0°C for 3 h, then 10% AcOH (20 ml) was added to the solution. The organic layer was washed with water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a white crystalline material, which was collected by filtration, yield 350 mg (73.5%), mp 172—174°C, $[\alpha]_D^{25}$ −78.3° (c=1.0, MeOH), Rf^1 0.40, Rf^2 0.46. *Anal.* Calcd for $C_{15}H_{19}N_3O_6$: C, 53.40; H, 5.68; N, 12.45. Found: C, 53.33; H, 5.67; N, 12.00.

Suc-Leu-Val-*p*NA——A solution of H–Leu–Val–*p*NA (prepared from 1.0 g of Boc–Leu–Val–*p*NA as usual) in AcOEt (40 ml) containing Et_3N (0.32 ml) was cooled with ice. Succinic anhydride (690 mg) was added to the above solution in five equal portions over a period of 1 h. The reaction mixture was stirred at 0°C for 5 h, during which the pH of the solution was maintained at 9 with Et_3N. The reaction mixture was washed with 10% AcOH and water, dried over Na_2SO_4 and evaporated down. Ether and petroleum ether were added to the residue to give a solid, which was collected by filtration, yield 820 mg (78.4%), mp 165—167°C, $[\alpha]_D^{25}$ −51.0° (c=1.0, MeOH), Rf^1 0.40, Rf^2 0.70. *Anal.* Calcd for $C_{21}H_{30}N_4O_7 \cdot 1/2H_2O$: C, 54.89; H, 6.80; N, 12.19. Found: C, 54.56; H, 6.62; N, 11.26.

Boc-Ala-Tyr-Leu-Val-*p*NA——H–Tyr–Leu–Val–*p*NA·HCl (prepared from 1.2 g of Boc–Tyr–Leu–Val–*p*NA[4,5] with 1.8 ml of 7.5 N HCl/dioxane as usual), Boc–Ala–OH (0.38 g) and HOBt (0.27 g) were dissolved in DMF (30 ml) containing Et_3N (0.28 ml) and cooled with ice-salt. DCC (0.45 g) was added to the above cold solution, and the reaction mixture was stirred at room temperature overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to afford a precipitate, which was collected by filtration. The crude material in $CHCl_3$ (5 ml) was applied to a silica gel column (2×26 cm) equilibrated and eluted with $CHCl_3$. The $CHCl_3$ eluate (500—900 ml) provided the purified material, yield 0.54 g (39.4%), mp 153—155°C, $[\alpha]_D^{25}$ −65.9° (c=1.4, MeOH), Rf^1 0.38, Rf^2 0.84. *Anal.* Calcd for $C_{34}H_{48}N_6O_9$: C, 59.64; H, 7.06; N, 12.27. Found: C, 59.44; H, 7.01; N, 12.23.

Suc-Ala-Tyr-Leu-Val-*p*NA——H–Ala–Tyr–Leu–Val–*p*NA (prepared from 340 mg of Boc–Ala–Tyr–Leu–Val–VNA with 1.2 ml of 7.5 N HCl/dioxane) was dissolved in H_2O (10 ml), and the pH of the solution was adjusted to 9 with Na_2CO_3. AcOEt (10 ml) was added to the solution to provide crystals, which were collected by filtration (yield 220 mg, mp 231—235°C). It was dissolved in pyridine (10 ml) containing Et_3N (0.07 ml) and cooled in an ice-bath. Succinic anhydride (150 mg) was added to the above solution in five equal portions over a period of 1 h. The reaction mixture was stirred at 0°C for 3 h. After removal of the solvent, the residue was dissolved in AcOEt (10 ml) and 10% AcOH (10 ml). The organic layer washed with water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to afford a white precipitate (Rf^2 0.52 (major spot), 0.40 (minor spot)). The crude product (240 mg) was dissolved in MeOH (15 ml) containing 1 N NaOH (0.70 ml) and the solution was stirred at room temperature for 40 min. After neutralization of the solution with AcOH, the solvent was removed by evaporation. The residue was extracted with AcOEt. The extract was washed with 10% AcOEt 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 180 mg (52.6%), mp 230—234°C, $[\alpha]_D^{25}$ −28.0° (c=1.0, MeOH), Rf^1 0.09, Rf^2 0.66. *Anal.* Calcd for $C_{33}H_{44}N_6O_{10}$: C, 57.88; H, 6.47; N, 12.27. Found: C, 57.71; H, 6.77; N, 11.97. Amino acid ratios in an acid hydrolysate: Ala 1.0; Val 1.0; Leu 1.0; Tyr 0.95 (average recovery 93.9%).

Boc-Ala-Ala-Tyr-Leu-Val-*p*NA——H–Ala–Tyr–Leu–Val–*p*NA·HCl (prepared from 490 mg of Boc–Ala–Tyr–Leu–Val–*p*NA with 0.28 ml of 7.5 N HCl/dioxane as usual) was dissolved in H_2O (10 ml), and the pH of the solution was adjusted to 8 with Na_2CO_3 to provide a solid precipitate, which was collected by filtration (mp 240—241°C, Rf^1 0.04, Rf^2 0.33, 440 mg). This product, Boc–Ala–OH (140 mg) and HOBt (100 mg) were dissolved in DMF (20 ml) and cooled with ice-salt. DCC (160 mg) was added to the cold solution. The reaction mixture was stirred at room temperature for 18 h. After removal of the urea derivative and the solvent, AcOEt was added to the residue to afford a precipitate (Rf^1 0.28 (main spot), 0.09, 0.62 (minor spots)). A solution of the crude product in $CHCl_3$, MeOH and H_2O (16: 3: 1, lower phase, 30 ml) was applied

to a silica gel column (2.3×24 cm) equilibrated and eluted with the same solvent. Individual fractions (10 g each) were collected. The solvent of the desired effluent fractions (Nos. 6—13) was removed by evaporation. Ether was added to the residue to provide a white precipitate, yield 370 mg (68.0%), mp 238—241°C, $[\alpha]_D^{25}$ −15.2° (c=0.48, DMF), Rf^1 0.40, Rf^2 0.81. *Anal.* Calcd for $C_{37}H_{53}N_7O_{10}$: C, 58.79; H, 7.06; N, 12.97. Found: C, 58.66; H, 7.21; N, 12.98.

Suc-Ala-Ala-Tyr-Leu-Val-*p*NA——H–Ala–Ala–Tyr–Leu–Val–*p*NA·HCl (prepared from 270 mg of Boc–Ala–Ala–Tyr–Leu–Val–*p*NA with 2.45 ml of 7.5 N HCl/dioxane) was dissolved in pyridine (20 ml) containing Et_3N (0.05 ml) and cooled with ice. Succinic anhydride (100 mg) was added to the solution in five equal portions over a period of 1 h. The reaction mixture was stirred at 0°C for 2 h. After removal of the solvent, the residue was dissolved in AcOEt and 10% AcOH (10 ml+10 ml). The organic layer was washed with water. A gelatinous product was collected by filtration and dried, yield 220 mg (81.6%), mp 233—235°C, $[\alpha]_D^{25}$ −42.1° (c=1.0, MeOH), Rf^1 0.16, Rf^2 0.58. *Anal.* Calcd for $C_{36}H_{49}N_7O_{11}\cdot 1/2H_2O$: C, 56.53; H, 6.53; N, 12.81. Found: C, 56.35; H, 6.56; N, 12.73. Amino acid ratios in an acid hydrolysate: Ala 1.96; Val 1.00; Leu 1.05; Tyr 1.05 (average recovery 79.3%).

Boc-Ala-Ala-Ala-Tyr-Leu-Val-*p*NA——H–Ala–Ala–Tyr–Leu–Val–*p*NA·HCl (prepared from 370 mg of Boc–Ala–Ala–Tyr–Leu–Val–*p*NA and 0.4 ml of 6.4 N HCl/dioxane as usual), Boc–Ala–OH (90 mg) and HOBt (70 mg) were dissolved in DMF (15 ml) containing Et_3N (0.07 ml) and cooled with ice-salt. DCC (100 mg) was added to the solution, and the reaction mixture was stirred at room temperature overnight. After removal of the urea derivative and the solvent, AcOEt was added to the residue to provide a precipitate, which was collected by filtration, washed with 5% Na_2CO_3 and water and treated with hot MeOH, yield 290 mg (71.9%), mp 242—244°C, $[\alpha]_D^{25}$ −11.8° (c=0.4, DMF), Rf^1 0.07, Rf^2 0.66. *Anal.* Calcd for $C_{40}H_{58}N_8O_{11}\cdot H_2O$: C, 56.86; H, 7.15; N, 13.26. Found: C, 56.65; H, 7.11; N, 12.96.

Suc-Ala-Ala-Ala-Tyr-Leu-Val-*p*NA——H–Ala–Ala–Ala–Tyr–Leu–Val–*p*NA·HCl (prepared from 210 mg of Boc–Ala–Ala–Ala–Tyr–Leu–Val–*p*NA with 0.39 ml of 6.4 N HCl/dioxane as usual) was dissolved in pyridine (8 ml) containing Et_3N (0.035 ml) and cooled in an ice bath. Succinic anhydride (75 mg) was added to the cold solution in three equal portions over a period of 30 min. The reaction mixture was stirred at 0°C for 40 min. After removal of the solvent, the residue was dissolved in AcOEt and 10% AcOH (20 ml+20 ml). The organic layer was washed with water and concentrated to a small volume. EtOH was added to the residue to afford a crystalline material (240 mg). The crude product was dissolved in MeOH (20 ml) containing 1 N NaOH (0.6 ml), and the reaction mixture was stirred at room temperature for 1 h. After neutralization with AcOH, the solvent was removed by evaporation. The residue was dissolved in AcOEt and 10% AcOH (20 ml+20 ml). The AcOEt layer was washed with water and concentrated to a small volume. EtOH and ether were added to the residue to afford crystals, yield 60 mg (29.1%), mp 239—242°C, $[\alpha]_D^{25}$ −16.7° (c=0.1, MeOH), Rf^2 0.22. *Anal.* Calcd for $C_{39}H_{54}N_8O_{12}\cdot 1.5H_2O$: C, 54.85; H, 6.72; N, 13.12. Found: C, 54.74; H, 6.47; N, 12.82. Amino acid ratios in an acid hydrolysate: Ala 3.44; Tyr 0.85; Leu 1.01; Val 1.00 (average recovery 78.4%).

Assay——SFP and LE were purified by gel-filtration[2] and affinity chromatography.[13] Amidolytic activity was assayed by measuring the released *p*NA (E_{410}). Michaelis constants were calculated from Lineweaver–Burk plots at a substrate concentration of 0.05—3.0 mM.

References and Notes

1) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration except in the case of glycine. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are Z=benzyloxycarbonyl, Boc=*tert*-butyloxycarbonyl, DCC=dicyclohexylcarbodiimide, Suc=succinyl, *p*NA=*p*-nitroanilide, HOBt=1-hydroxybenzotriazole, DMF=*N*,*N*-dimethylformamide.
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