Synthetic Dipeptide, *N*-Stearoyl-D-Ser–L-Pro–OEt, Induces Release of Tissue-Type Plasminogen Activator in Cultured Cells and in Experimental Animals[1)]

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The tissue-type plasminogen activator (t-PA)-releasing action of synthetic dipeptides containing Gly, Ser or Pro was investigated. Among 10 dipeptides, Boc–L-Ser–L-Pro–OH and H–L-Ser–L-Pro–OH induced t-PA release *in vitro*, but the others were inactive. Since Boc–L-Ser–L-Pro–OH was more effective than H–L-Ser–L-Pro–OH, 7 related dipeptides with *N*-acylation were synthesized. Five of them enhanced the release of t-PA; *N*-stearoyl-L-Ser–L-Pro–OH (FK-5) had the greatest effect. Four compounds were further examined for activity to enhance the release of t-PA in rats. FK-5 produced a two-fold increase in fibrinolytic activity, and *N*-palmitoyl-L-Ser–L-Pro–OH (FK-4) also markedly enhanced the release of t-PA. Since FK-5 caused severe hemolysis, 7 analogues of FK-5 were synthesized. All of them enhanced the release of t-PA from melanoma (Bowes) cells. In rats, FK-5, *N*-stearoyl-D-Ser–L-Pro–OH (FK-8) and *N*-stearoyl-D-Ser–L-Pro–OEt (FK-10) enhanced the fibrinolytic activity two-fold. FK-5 and FK-8 also exhibited strong hemolytic activity, but FK-10 did not induce hemolysis. Therefore, FK-10 was examined in rabbits. After the injection of this compound, the fibrinolytic activity in the euglobulin fraction was markedly enhanced without accompanying hemolysis. Thus, FK-10 potently enhances fibrinolytic activity both *in vitro* and *in vivo*.

Keywords tissue-type plasminogen activator release; *N*-acyl dipeptide; synthetic peptide; endothelial cell; melanoma (Bowes)

Plasminogen activator (PA) converts plasminogen to an active enzyme, plasmin, which digests both fibrin and fibrinogen, the precursor protein of fibrin. PA can be divided into two categories, direct-type PA and indirect-type PA. The former group includes tissue-type PA (t-PA), and urokinase-type PA (u-PA), which activate plasminogen directly without any other cofactor. The latter group includes streptokinase (SK) and staphylokinase (SAK), which are inactive by themselves, but form a complex with plasminogen, and this complex expresses the enzymatic activity of PA.[2)] PA has been used in thrombolytic therapy[3)] for the treatment of thromboembolic diseases such as acute myocardial infarction, deep vein thrombosis, pulmonary embolism, *etc.* First generation drugs for thrombolytic therapy, such as SK and u-PA, have no affinity for fibrin, and activate plasminogen in circulating plasma. They can easily induce a "lytic state," in which bleeding tends to occur.[4)] In contrast, t-PA has a high affinity for fibrin, and activates plasminogen on the fibrin surface,[5)] thereby inducing effective thrombolysis. Under physiological conditions, t-PA is produced by endothelial cells[6)] and secreted into the circulation as part of the antithrombotic activity of the vasculature.[7)] The release of t-PA is a major marker to assess the antithrombotic

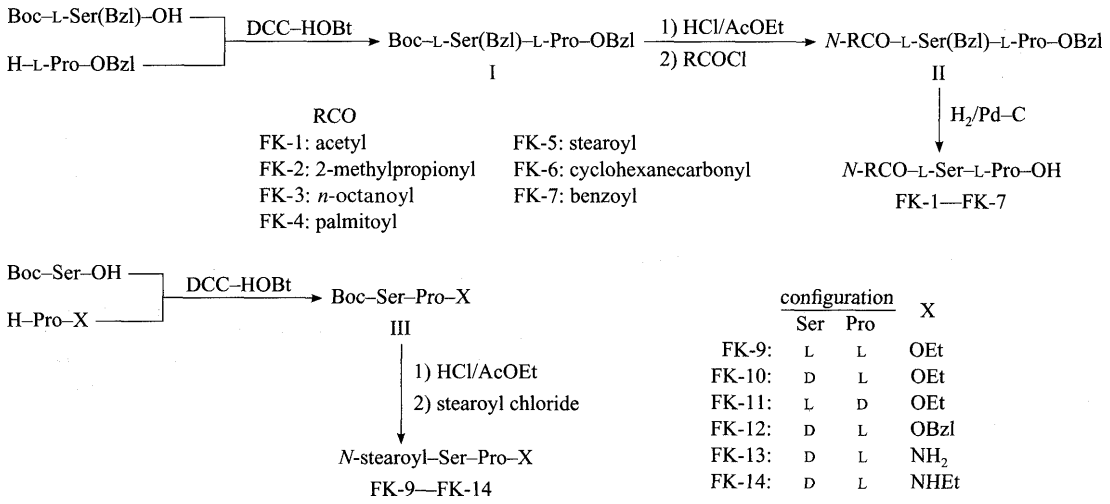

Chart 1. Synthetic Scheme for *N*-Acylated Dipeptides

function of endothelial cells in the clinical context (venous occlusion test).[8] Many attempts have been made to develop drugs that would induce the release of t-PA from endothelial cells, and reported active compounds include hypothalamic hormone,[9] steroid hormone,[10] and plant sterols.[11] However, these compounds have side-effects. In the present studies, new dipeptides were synthesized and examined for t-PA-releasing activity.

Synthesis The following dipeptides were synthesized by the solution method essentially as described elsewhere; Gly–Gly, Gly–L-Leu, L-Leu–Gly, Gly–L-Phe, Gly–L-Pro, L-Ser–Gly, L-Ser–L-Pro, L-Pro–L-Pro, Boc–L-Ser–L-Pro–OH and Boc–L-Pro–L-Pro–OH. *N*-Acylated dipeptides (FK-1—FK-14) were synthesized by the methods shown in Chart 1. Briefly, Boc–L-Ser(Bzl)-OH and H–L-Pro–OBzl were first condensed by the DCC/HOBt method.[12] After removal of the Boc group from Boc–L-Ser(Bzl)–L-Pro–OBzl (I) by treatment with HCl/AcOEt, we obtained *N*-acylated dipeptide esters (II) by acylation either by the acid chloride (RCOCl) method or by the DCC method with carboxylic acids (RCO_2H). The *O*-Bzl group and Bzl ester were removed from II by hydrogenolysis in the presence of Pd–C. Thus, the following *N*-acylated dipeptides were obtained: acetyl (FK-1), 2-methylpropionyl (FK-2), octanoyl (FK-3), palmitoyl (FK-4), stearoyl (FK-5), cyclohexanecarbonyl (FK-6) and benzoyl–L-Ser–L-Pro–OH (FK-7). *N*-Stearoyl-dipeptides (FK-9—FK-14) were prepared as follows; Boc–Ser–OH and H–Pro–X (X=OEt, OBzl, NH_2 or NHEt) were first condensed by the DCC/HOBt method to afford Boc–Ser–Pro–X (III). After removal of the Boc group from III by treatment with HCl/AcOEt, we obtained the desired compounds by *N*-acylation. *N*-Stearoyl-D-Ser–L-Pro–OH (FK-8) was obtained from benzylester (FK-12) by hydrogenolysis.

Results

The ability of the synthetic dipeptides to enhance the release of t-PA from melanoma (Bowes) cells was examined. As melanoma (Bowes) cells secrete t-PA, the effect of the synthetic dipeptides was assessed in terms of the increase in fibrinolytic activity. That is, a more than 10% increase in fibrinolytic activity was regarded as effective. First, single L-amino acids were examined for ability to enhance the release of t-PA from the cells; Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His. Among them, Gly, Ser and Pro exhibited weak fibrinolytic activity. These findings led us to synthesize 10 dipeptides containing these amino acids. Among them, only L-Ser–L-Pro and Boc–L-Ser–L-Pro–OH enhanced the release of t-PA from the melanoma (Bowes) cells. The other 8 dipeptides, Gly–Gly, Gly–L-Leu, L-Leu–Gly, Gly–L-Phe, Gly–L-Pro, L-Ser–Gly, L-Pro–L-Pro, and Boc–L-Pro–L-Pro–OH were ineffective.

Since Boc–L-Ser–L-Pro–OH exhibited slightly higher t-PA releasing activity than L-Ser–L-Pro, 7 derivatives with *N*-acylation were synthesized. Table I shows the t-PA-releasing activity of these compounds. Five of the 7 compounds enhanced t-PA release from the melanoma (Bowes) cells. Among them, *N*-stearoyl-L-Ser–L-Pro–OH (FK-5) exhibited the strongest activity, followed by *N*-palmitoyl-L-Ser–L-Pro–OH (FK-4).

To confirm the t-PA-releasing activity *in vivo*, the synthesized compounds were injected intravenously into rats. Blood taken at 3 min after the injection was used for the measurement of fibrinolytic activity. Among 5 compounds which enhanced the t-PA-releasing activity *in vitro*, FK-5 exhibited the strongest fibrinolytic activity; almost a two-fold increase (Table I). FK-4 also exhibited stronger fibrinolytic activity. However, since hemolysis was encountered during the experiments, all the synthesized compounds were examined for hemolytic activity. FK-5 exhibited the strongest hemolytic activity and FK-4 had very weak hemolytic activity.

To examine whether the enhancement of fibrinolytic activity could be separated from the hemolytic effect, 7 analogues of FK-5 were synthesized. As shown in Table II, *N*-stearoyl-D-Ser–L-Pro–OH (FK-8) and *N*-stearoyl-D-Ser–L-Pro–OEt (FK-10) enhanced fibrinolytic activity almost two-fold, whereas *N*-stearoyl-L-Ser–D-Pro–OEt (FK-11) and *N*-stearoyl-D-Ser–L-Pro–NH_2 (FK-13) did not enhance the fibrinolytic activity. The other 3 compounds, *N*-stearoyl-L-Ser–L-Pro–OEt (FK-9), *N*-stearoyl-D-Ser–L-Pro–OBzl (FK-12) and *N*-stearoyl-D-Ser–L-Pro–NHEt (FK-14) also increased the fibrinolytic activity in rats. However, FK-8 induced marked hemolysis. FK-9, FK-10 and FK-11 induced hemolysis to a similar degree to saline. Therefore, FK-10 was chosen as a candidate for

TABLE I. Effect of *N*-RCO–L-Ser–L-Pro–OH on Fibrinolytic Activity *in Vitro* and *in Vivo* and Hemolysis in Rats

Compound	RCO	*In vitro*[a]	*In vivo*		
		Fibrinolytic activity	*n*	Fibrinolytic activity[b]	Hemolysis (A_{540})[d]
FK-1	Acetyl	+	3	127±15	– (0.060)
FK-2	2-Methylpropionyl	+	2	104	– (0.060)
FK-3	*n*-Octanoyl	+	2	88	ND
FK-4	Palmitoyl	++	2	142	– (0.089)
FK-5	Stearoyl	+++	4	217±64[c]	+ (0.353)
FK-6	Cyclohexanecarbonyl	–		ND	ND
FK-7	Benzoyl	–		ND	ND
Control	Saline		15	100±13	– (0.054)

a) The final concentration of compounds in the culture medium was 10^{-6} M. The t-PA-releasing activities were compared with the control and the results were expressed according to the following criteria: +++, more than 30% increase, ++, 30—20% increase, +, 20—10% increase; –, less than 10% increase. *b*) The fibrinolytic activity of the euglobulin fraction was measured and the relative activity of the compounds to that of saline was expressed as % increase (mean±SE). The significance of differences from the control value is indicated. *c*) $p<0.01$, *t*-test. *d*) A_{540} of diluted plasma (10 portions) was measured. ND: not determined.

TABLE II. Effect of *N*-Stearoyl-dipeptides, *N*-Stearoyl-Ser–Pro–X, on Fibrinolytic Activity *in Vitro* and *in Vivo* and Hemolysis in Rats

Compound	Configuration		X	*In vitro*[a]	*In vivo*		
	Ser	Pro		Fibrinolytic activity	*n*	Fibrinolytic activity[b]	Hemolysis (A_{540})[e]
KF-8	D	L	OH	+	3	205±53[d]	+ (0.291)
FK-9	L	L	OEt	+	4	150±24[c]	− (0.052)
FK-10	D	L	OEt	+	7	200±44[d]	− (0.035)
FK-11	L	D	OEt	+	2	106	− (0.041)
FK-12	D	L	OBzl	ND	11	158±21[c]	ND
FK-13	D	L	NH_2	ND	3	112±12	ND
FK-14	D	L	NHEt	ND	3	140±30	ND
Control		Saline			15	100±13	− (0.054)

a) The final concentration of compounds in a culture medium was 10^{-6} M. The t-PA-releasing activities were compared with control and the results were expressed as + for more than 10% increase. *b*) The fibrinolytic activity of euglobulin fraction was measured and the relative activities of the compounds to the saline were expressed as % increase. The significance of differences from the control value is indicated. *c*) $p<0.05$. *d*) $p<0.01$. *e*) A_{540} of diluted plasma (10 portions) was measured. ND: not determined.

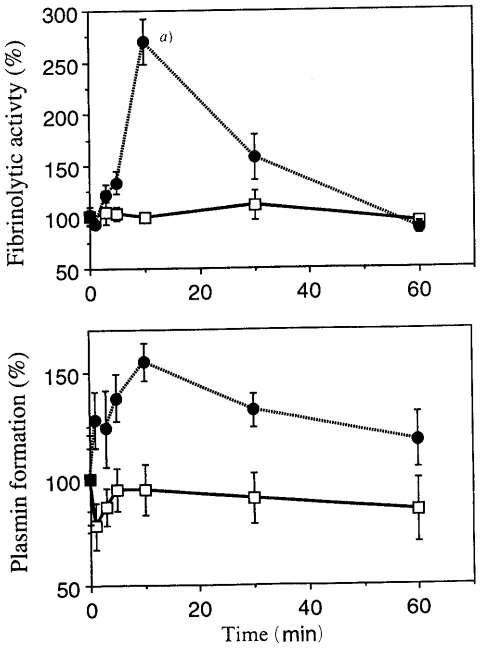

Fig. 1. Fibrinolytic Activity and Plasmin Formation after Intravenous Injection of *N*-Stearoyl-D-Ser–L-Pro–OEt (FK-10) into Rabbits

FK-10 (●, 10 mg/kg, $n=5$), control (□, $n=6$). The relative activity of the compound with respect to the control was expressed as % increase (mean ± S.E.). The significance of differences from the control value is indicated (*a*) $p<0.01$, *t*-test).

further experiments with rabbits.

Injection of FK-10 into rabbits enhanced the fibrinolytic activity. As shown in Fig. 1, the fibrinolytic activity in the euglobulin fraction after injection of the compound increased gradually from 3—5 min, showed the maximum increase at 10 min, decreased gradually and returned to the pre-injection level at 60 min. The degree of plasmin formation was measured by incubating the euglobulin fraction with S-2251, a synthetic chromogenic substrate. The plasmin formation increased gradually after the injection of the compounds and reached a maximum at 10 min. Then, it decreased gradually. In the control rabbits, neither enhancement of fibrinolytic activity nor plasmin formation was observed after the injection of saline. Thus, FK-10 is a potent enhancer of fibrinolytic activity both *in vitro* and *in vivo*.

TABLE III. Physicochemical and Analytical Data for RCO–L-Ser(Bzl)–L-Pro–OBzl

Compound	RCO	Yield[a] (%)	$[\alpha]_D^{25}$ (°)[b]	*Rf*[c]
IIb	2-Methylpropionyl	88	−52.0	0.37
IIc	Octanoyl	90	−53.7	0.50
IId	Palmitoyl	92	−46.2	0.60
IIe	Stearoyl	89	−44.3	0.60
IIf	Cyclohexanecarbonyl	85	−51.8	0.49
IIg	Benzoyl	95	−53.0	0.58

a) All compounds described above were isolated as oils. *b*) $c=1$, EtOH. *c*) Benzene : AcOEt = 2 : 1 (v/v).

Discussion

The release of t-PA from endothelial cells is induced by various compounds, of which some activate the fibrinolytic system in human beings; for example, 1-desamino-8-D-arginine vasopressin (DDAVP) increases t-PA release, but its side-effect include tachycardia, hypertension and skin rash.[13] Other active compounds, such as extracts of the hypothalamus[14] and plant sterols have not been examined in human beings. We have been searching for a new low-molecular-weight compound which can induce the release of t-PA from cells. Since melanoma (Bowes) cells secrete t-PA into medium,[15] this cell line was used for assay of t-PA-releasing activity.

Among 10 dipeptides, two were active, and Boc–L-Ser–L-Pro–OH showed greater t-PA-releasing activity than L-Ser–L-Pro. Therefore, substitution at the *N*-terminus was performed with various acyl groups (Table I). The compounds with alicyclic or aromatic groups showed little activity. Among the compounds with an aliphatic acyl group, ones with a short chain showed weak activity, and the longer the chain, the stronger the t-PA-releasing activity; *N*-stearoyl-L-Ser–L-Pro–OH (FK-5) had the strongest effect. This compound also showed strong fibrinolytic activity in rats. However, it had severe side effects, so we sought an analogue of FK-5 possessing t-PA-releasing activity without hemolytic activity.

FK-8, with the substitution of L-Ser in FK-5 to D-Ser, retained almost the same fibrinolytic activity both *in vitro* and *in vivo*. As the hemolytic activity had not disappeared, the carboxyl group in L-Pro was esterified. The esterifica-

TABLE IV. Physicochemical and Analytical Data for *N*-RCO–L-Ser–L-Pro–OH

Compound	RCO	Yield (%)	mp (°C) Recryst. solv.[a]	$[\alpha]_D^{25}$ (°)[b]	*Rf*[c]	Formula	Analysis Calcd (Found)		
							C	H	N
FK-2	2-Methylpropionyl	85	154—158 M–E	−40.8	0.25	$C_{12}H_{20}N_2O_5$	52.93 (53.12	7.40 7.20	10.29 10.14)
FK-3	Octanoyl	92	Oil	−74.3	0.43	$C_{16}H_{28}N_2O_5$	58.52 (58.39	8.59 8.76	8.53 8.32)
FK-4	Palmitoyl	89	96—104 EA–H	−51.4	0.49	$C_{24}H_{44}N_2O_5$	65.42 (65.28	10.07 10.11	6.36 6.09)
FK-5	Stearoyl	93	57—70 EA	−45.0	0.49	$C_{26}H_{48}N_2O_5$	66.63 (66.52	10.32 10.08	5.98 5.99)
FK-6	Cyclohexanecarbonyl	90	146—148 M–E	−38.1	0.33	$C_{15}H_{24}N_2O_5$	57.68 (57.81	7.74 7.56	8.97 8.83)
FK-7	Benzoyl	91	197—199 M–E	−29.4	0.29	$C_{15}H_{18}N_2O_5$	58.82 (58.67	5.92 5.99	9.15 9.03)

a) Recrystallization solvent: E = ether, EA = ethyl acetate, H = hexane, M = methanol. *b*) $c = 1$, EtOH. *c*) $CHCl_3$: MeOH : AcOH = 80 : 10 : 5 (v/v).

tion of the original compound decreased the *in vivo* fibrinolytic activity slightly, but the hemolytic action was completely lost. In particular, *N*-stearoyl-D-Ser–L-Pro–OEt (FK-10) had strong fibrinolytic activity and no hemolytic activity. Since the enantiomer of FK-10, *N*-stearoyl-L-Ser–D-Pro–OEt (FK-11), had no activity, the specific three-dimensional structure was required to induce the enhanced fibrinolytic activity. Further modification of the carboxyl group of L-Pro in FK-10 with benzyl ester, amide or ethylamide did not bring about any further increase in fibrinolytic activity. In the rabbit experiments, FK-10 induced marked fibrinolytic activity after intravenous injection, and showed no hemolytic activity. Thus, FK-10 was selected as the best candidate for further development as a thrombolytic drug.

Experimental

Melting points were determined on a Yanagimoto melting point apparatus without correction. Optical rotations were determined with a JASCO digital polarimeter. Fuji Davison silica gel BW-200 was used for column chromatography. The purity of all new compounds was monitored by analytical TLC on Merck silica gel plates in the following solvent systems: *Rf*[1], benzene : AcOEt (2 : 1, v/v); *Rf*[2], $CHCl_3$: MeOH : AcOH (80 : 10 : 5, v/v); *Rf*[3], $CHCl_3$: MeOH : AcOH (95 : 5 : 3, v/v).

General Procedure for the DCC/HOBt Method DCC solution in CH_2Cl_2 was added dropwise at −15 °C to a mixture of HOBt and paired amino acid derivatives to be coupled and the reaction mixture was stirred overnight at 5 °C. After removal of dicyclohexylurea by filtration, the filtrate was evaporated *in vacuo* at 30—40 °C. The product was extracted with AcOEt, followed by washing with 1 M HCl, H_2O, 10% Na_2CO_3 and finally H_2O, then dried over $MgSO_4$ and evaporated *in vacuo*. The residue was chromatographed on silica gel and/or recrystallized from appropriate solvents.

General Procedure for HCl/AcOEt Treatment *N*-Boc dipeptide was dissolved in 4 M HCl/AcOEt at room temperature for 1 h and the solution was concentrated *in vacuo*. The residue was solidified with ether, collected by filtration, and dried in a vacuum desiccator with NaOH. This product, without further purification, was dissolved in DMF and neutralized with Et_3N. The mixture was used for *N*-acylation as described below.

General Procedure for *N*-Acylation An acid chloride solution in CH_2Cl_2 and equimolar Et_3N were alternately added dropwise to a solution of an amino component [prepared from its *N*-Boc derivative by HCl/AcOEt treatment, followed by Et_3N treatment] in DMF at −10 °C, and the reaction mixture was stirred at the same temperature for 1 h, then poured into ice-cold 1 M HCl. The organic layer was extracted with CH_2Cl_2. The extract was washed with H_2O, 10% Na_2CO_3 and finally H_2O, then dried over $MgSO_4$ and evaporated *in vacuo*. The residue was chromatographed on silica gel and/or recrystallized from appropriate solvents.

TABLE V. Physicochemical and Analytical Data for Boc–Ser–Pro–X

Compound	Configuration Ser	Configuration Pro	X	Yield (%)	mp (°C) Recryst. solv.[a]	$[\alpha]_D^{25}$ (°)[b]	*Rf*[c]
IIIb	D	L	OEt	82	Oil	−33.6	0.64
IIIc	L	D	OEt	85	Oil	+29.4	0.64
IIId	D	L	OBzl	92	Oil	−32.6	0.64
IIIe	D	L	NH_2	76	132—136 EA–H	−37.4	0.27
IIIf	D	L	NHEt	85	Oil	−43.0	0.49

a) EA = ethyl acetate, H = hexane. *b*) $c = 1$, EtOH. *c*) $CHCl_3$: MeOH : AcOH = 95 : 5 : 3 (v/v).

General Procedure for Hydrogenolysis A mixture of an *O*-Bzl-protected dipeptide and 5% Pd–C in a mixture of MeOH and AcOH was vigorously stirred for several hours at room temperature under a hydrogen flow. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was recrystallized from appropriate solvents.

Boc–L-Ser(Bzl)–L-Pro–OBzl (I) Prepared from Boc–L-Ser(Bzl)–OH (29.5 g, 100 mmol), L-Pro–OBzl·TosOH (41.5 g, 110 mmol), Et_3N (15.4 ml, 110 mmol), HOBt (14.2 g, 105 mmol) and DCC (26.8 g, 130 mmol) by the DCC/HOBt method in DMF in the usual manner. The product was purified by silica gel column chromatography using $CHCl_3$–MeOH (50 : 1, v/v). Yield 45.4 g (94%), oil, *Rf*[1] 0.77, $[\alpha]_D^{23}$ −48.9° ($c = 1$, EtOH).

***N*-Acetyl-L-Ser(Bzl)–L-Pro–OBzl (IIa)** H–L-Ser(Bzl)–L-Pro–OBzl [prepared from its *N*-Boc-derivative (2.41 g, 5.00 mmol) by HCl/AcOEt treatment followed by Et_3N treatment] was *N*-acylated with acetyl chloride (471 mg, 6.00 mmol) and Et_3N (1.54 ml, 11.0 mmol) in DMF in the usual manner. The product was purified by silica gel column chromatography using $CHCl_3$–MeOH (50 : 1, v/v). Yield 1.87 g (88%), oil, *Rf*[1] 0.06, $[\alpha]_D^{23}$ −56.2° ($c = 1$, EtOH). Other protected dipeptides IIb—g were prepared as described for IIa and the results are shown in Table III.

***N*-Acetyl-L-Ser–L-Pro–OH (FK-1)** The above protected dipeptide (1.76 g, 4.15 mmol) was hydrogenated for 2 h in the usual manner. The product was reprecipitated from MeOH–ether. Yield 831 mg (82%), mp 75—78 °C, *Rf*[2] 0.14, $[\alpha]_D^{23}$ −44.8° ($c = 1$, EtOH). *Anal.* Calcd for $C_{10}H_{16}N_2O_5$: C, 49.18; H, 6.60; N, 11.47. Found: C, 49.25; H, 6.56; N, 11.39. Compounds FK-1—FK-7 were prepared in the same manner from IIb—g, respectively, and the results are shown in Table IV.

***N*-Stearoyl-D-Ser–L-Pro–OH (FK-8)** *N*-Stearoyl-D-Ser–L-Pro–OBzl (FK-12) (2.12 g, 3.79 mmol) was hydrogenated for 1.5 h in the usual manner. The product was reprecipitated from MeOH–ether. Yield 1.63 g (92%), mp 57—60 °C, *Rf*[3] 0.29, $[\alpha]_D^{23}$ −7.00° ($c = 1$, EtOH). *Anal.* Calcd for $C_{26}H_{48}N_2O_5$: C, 66.63; H, 10.32; N, 5.98. Found: C, 66.82; H, 10.55;

TABLE VI. Physicochemical and Analytical Data for *N*-Stearoyl-Ser–Pro–X

Compound	Configuration		X	Yield (%)	mp (°C) Recryst. solv.[a]	$[\alpha]_D^{25}$ (°)[b]	*Rf*[c]	Formula	Analysis Calcd (Found)		
	Ser	Pro							C	H	N
FK-10	D	L	OEt	83	74—75 EA–H	−8.3	0.62	$C_{28}H_{52}N_2O_5$	67.70 (67.87	10.55 10.80	5.64 5.58)
FK-11	L	D	OEt	88	74—75 EA–H	+9.0	0.62	$C_{28}H_{52}N_2O_5$	67.70 (67.56	10.55 10.58	5.64 5.61)
FK-12	D	L	OBzl	92	58—59 EA–H	−13.3	0.65	$C_{33}H_{54}N_2O_5$	70.93 (70.99	9.74 9.72	5.01 5.08)
FK-13	D	L	NH_2	82	93—97 M–E	−22.7	0.35	$C_{26}H_{49}N_3O_4$	66.77 (66.48	10.56 10.58	8.98 8.91)
FK-14	D	L	NHEt	87	Oil	−34.6	0.52	$C_{28}H_{53}N_3O_4$	67.84 (67.95	10.78 10.67	8.48 8.33)

a) Recrystallization solvent: E=ether, EA=ethyl acetate, H=hexane, M=methanol. *b*) $c=1$, EtOH. *c*) $CHCl_3$:MeOH:AcOH=95:5:3 (v/v).

N, 5.76.

Boc-L-Ser-L-Pro-OEt (IIIa) Prepared from Boc-L-Ser-OH (4.76 g, 23.2 mmol), H-L-Pro-OEt·HCl (5.00 g, 27.8 mmol), Et_3N (3.88 ml, 27.8 mmol), HOBt (3.45 g, 25.5 mmol) and DCC (6.22 g, 30.2 mmol) by the DCC/HOBt method in DMF in the usual manner. The product was recrystallized from EtOAc–hexane. Yield 6.43 g (84%), mp 108—109 °C, Rf^3 0.78, $[\alpha]_D^{23}$ −64.4° ($c=1$, MeOH). *Anal.* Calcd for $C_{15}H_{26}N_2O_6$: C, 54.53; H, 7.93; N, 8.48. Found: C, 54.62; H, 8.01; N, 8.22. Other *N*-Boc-dipeptides IIIb—f were prepared as described for IIIa and the results are shown in Table V.

***N*-Stearoyl-L-Ser-L-Pro-OEt (FK-9)** H-L-Ser-L-Pro-OEt [prepared from its *N*-Boc-derivative (5.09 g, 15.5 mmol) by HCl/AcOEt treatment followed by Et_3N treatment] was *N*-acylated with stearoyl chloride (4.90 g, 16.2 mmol) and Et_3N (2.27 ml, 16.2 mmol) in DMF in the usual manner. The product was reprecipitated from EtOAc–hexane. Yield 7.10 g (92%), mp 74—76 °C, Rf^3 0.78, $[\alpha]_D^{23}$ −64.4° ($c=1$, MeOH). *Anal.* Calcd for $C_{28}H_{52}N_2O_5$: C, 67.70; H, 10.55; N, 5.64. Found: C, 67.88; H, 10.61; N, 5.48. Other *N*-stearoyl-dipeptides FK-10—FK-14 were prepared as described for FK-9 and the results are shown in Table VI.

t-PA Release from Melanoma Cells (Bowes) The release of t-PA into the conditioned medium from melanoma cells (Bowes)[16] was examined by incubating the cells with synthetic compounds in calf serum-free medium. The conditioned medium was collected at intervals and used for the measurement of PA activity on the fibrin plate.[17] Briefly, the fibrin plate was prepared with plasminogen-rich bovine fibrinogen and human thrombin dissolved in 80 mM $CaCl_2$.

t-PA Release in Rats and Rabbis Male Wistar rats (190—260 g) (Sankyo Lab.) were anesthetized with urethane (1.25 g/kg, i.p.). The jugular vein was exposed for blood collection. With a plastic syringe containing 1/10 volume of sodium citrate (3.8%), 0.5 ml of blood was withdrawn at 10 min before the injection, and at 3 min after the injection of test compounds into the femoral vein, and used for the measurement of fibrinolytic activity. Male Japanese white domestic rabbits each weighing 2.8—4.0 kg (Sankyo Lab.) were also used to examine the induction of fibrinolytic activity by the test compounds. A compound was injected into the marginal vein, and blood collection (at 10 min before the injection, and at 1, 3, 5, 10, 30 and 60 min after the injection) was performed from the ear artery vein. As the control, a saline solution was injected.

The fibrinolytic activity was measured by the fibrin plate method in the experiments using both rats and rabbits, and by the chromogenic substrate method in the experiments using rabbits. The euglobulin fraction was obtained from plasma as described elsewhere. Plasmin activity was measured using the synthetic chromogenic substrate, S-2251 (H-D-Val–Leu–Lys–pNA).

Assay of Hemolysis The degree of hemolysis was measured by a spectrophotometer at 540 nm after 10-fold dilution of plasma.

References and Notes

1) Standard abbreviations for amino acids, protecting groups and peptides are used [*Eur. J. Biochem.*, **138**, 9 (1984)]. Boc=*tert*-butyloxycarbonyl. Other abbreviations include: DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, HOBt=1-hydroxybenzotriazole.
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