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Isolation of SMTP-3, 4, 5 and -6, Novel Analogs of Staplabin, and Their Effects on Plasminogen Activation and Fibrinolysis

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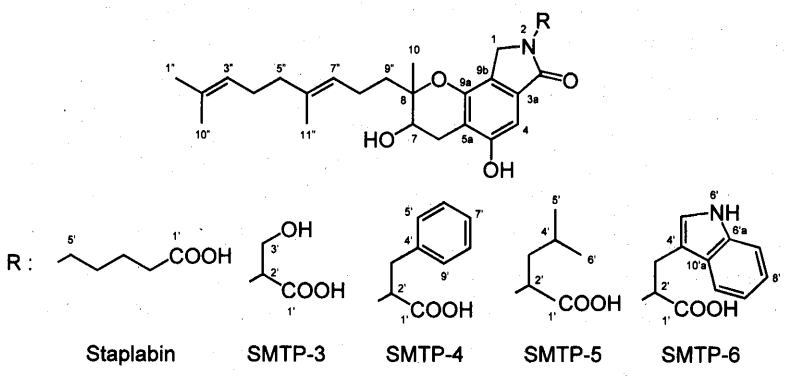
Four novel triprenyl phenol metabolites, designated SMTP-3, -4, -5, and -6, have been isolated from cultures of *Stachybotrys microspora* IFO 30018 by solvent extraction and successive chromatographic fractionation using silica gel and silica ODS columns. A combination of spectroscopic analyses showed that SMTP-3, -4, -5, and -6 are staplabin analogs, containing a serine, a phenylalanine, a leucine or a tryptophan moiety in respective molecules in place of the *N*-carboxybutyl portion of the staplabin molecule. SMTP-4, -5, and -6 were active at 0.15~0.3 mM in enhancing urokinase-catalyzed plasminogen activation and plasminogen binding to fibrin, as well as plasminogen- and urokinase-mediated fibrinolysis. On the other hand, the concentration of staplabin required to exert such effects was 0.4~0.6 mM, and SMTP-3 was inactive at concentrations up to 0.45 mM.

The plasminogen/plasmin system is involved in a variety of physiological and pathological processes requiring localized proteolysis, such as fibrinolysis, inflammation, tissue remodeling, ovulation, tumor metastasis and tissue invasion of pathogens[1~5]. Circulating, native plasminogen (Plg) with NH_2-terminal Glu^1 (Glu-Plg) is a single-chain glycoprotein with multiple functional domains, which consist of an NH_2-terminal peptide (NTP), five homologous kringle domains and a trypsin-like serine protease domain[6,7]. Glu-Plg exhibits a tight spiral structure because of an intramolecular interaction between a lysine residue in the NTP and the aminohexyl site of kringle 5[8,9], giving rise to resistance to activation by Plg activators and low affinity binding to fibrin and cellular receptors[10~12]. In a local site where Plg is activated, plasmin hydrolyzes Glu-Plg to yield a truncated form of Plg called Lys-Plg[13,14], which lacks NTP and adopts a relaxed conformation. Lys-Plg has a high susceptibility to activation and a high affinity to fibrin and cellular receptors[9,15]. Plg binding to fibrin and cell surface receptors localizes and facilitates the Plg/plasmin system[16].

We have recently isolated a fungal triprenyl phenol metabolite (designated staplabin; Fig. 1) from a culture

Fig. 1. Structures of staplabin and SMTP-3, -4, -5 and -6.



of *Stachybotrys microspora* as an activator of Glu-Plg binding to fibrin and monocytoid U937 cells[17]. Staplabin induces a conformational change in both Glu-Plg and Lys-Plg to allow them to bind fibrin and to be activated by Plg activators with higher efficiency[18]. Later, *S. microspora* was found to produce minor staplabin analogs, two of which were isolated and shown to be far less active than staplabin[19]. In the present study, four more analogs (Fig. 1) were isolated and their effects on Plg activation, fibrin binding of Plg and *in vitro* fibrinolysis was examined. The results indicated that the analogs having a phenylalanine, a leucine or a tryptophan moiety in place of the *N*-carboxybutyl portion of the staplabin molecule were several times more active compared to staplabin.

Materials and Methods

Materials

Human Glu-Plg and Lys-Plg were purchased from Enzyme Research Laboratories, USA. Human fibrinogen, human thrombin, bovine serum albumin and S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide) were obtained from Sigma, USA. Staplabin was isolated from a culture of *S. microspora* IFO30018 as described previously[17]. Urokinase-type Plg activator (two-chain form) was obtained from JCR Farmaceuticals, Japan, and aprotinin from CosmoBio, Japan. Carrier-free $Na^{125}I$ was purchased from Amersham. The compositions of buffers were: buffer A, 50 mM Tris-HCl, 100 mM NaCl and 0.01% (wt/vol) Tween 80, pH 7.4; buffer B, 20 mM sodium phosphate and 150 mM NaCl, pH 7.4; buffer C, Hanks' balanced salt solution containing 1 mg/ml bovine serum albumin and 50 mM HEPES, pH 7.4.

Fermentation Production of SMTPs

The producing strain *S. microspora* IFO 30018 (obtained from the Institute for Fermentation, Osaka, Japan) was subcultured on potato glucose agar slants. For the production of SMTP-3, a medium containing the following was used: 30 g glucose, 10 g soybean meal, 3 g meat extract, 3 g peptone, 3 g yeast extract, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.1 g CB442 (an antifoam, Nippon Oil & Fat Co., Japan) in 1 liter of reverse osmotic water (pH 5.8~6.0). SMTP-4, -5 and -6 were produced in a medium containing 20 g glucose, 5 g peptone, 3 g yeast extract, 3 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$ and 0.1 g CB442 in 1 liter of reverse osmotic water (pH 5.5). A loopful of a slant culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of medium, which was then incubated at 25°C for 3 days on a rotary shaker at 180 rpm. A 1-ml portion of the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of medium and the flask was incubated as above for 12 days for production of SMTP-3 and for 11 days for other SMTPs.

Radioiodination of Plg and Fibrinogen

Both Glu-Plg and Lys-Plg were radioiodinated using Chloramine-T and $Na^{125}I$ as described by Miles and Plow[20] to a specific radioactivity of 1,500~3,000 cpm/ng. Over 97% of the radioactivity was precipitable upon treatment with 10% trichloroacetic acid. Fibrinogen was labeled by the iodine monochloride method[21] to a specific activity of 200~400 cpm/ng. Upon treatment with thrombin, approximately 80% of the radioactivity of ^{125}I-fibrinogen were incorporated into the clot.

Determination of Plasminogen Activation

Plg activation was assayed either by measuring initial velocity for plasmin generation using a chromogenic substrate for plasmin or by determining the rate for conversion of ^{125}I-Plg to ^{125}I-plasmin using SDS-polyacrylamide gel electrophoresis (SDS-PAGE)[18]. In the former assay, 50 nM Plg and 0.1 mM S-2251 were incubated in 60 μl of buffer A with urokinase (50 units/ml for Glu-Plg and 3 units/ml for Lys-Plg) at 37°C for up to 60 minutes. From the slope of the plots of A_{405} nm versus t^2, the velocity of plasmin generation was calculated[23]. In the latter assay, 50 nM ^{125}I-Plg was incubated with urokinase (30 units/ml for ^{125}I-Glu-Plg and 2 units/ml for ^{125}I-Lys-Plg) and aprotinin (1000 kallikrein inhibitor units/ml) in 15 μl of buffer A. Aprotinin was used to inhibit plasmin activity that forms Lys-Plg during incubation of Glu-Plg. After incubation at 37°C for 30 minutes, the mixture received 4.5 mg of solid urea and 5 μl of a solution containing 3.6% (wt/vol) SDS, 3.6% (wt/vol) dithiothreitol and 0.08% (wt/vol) bromophenol blue, then heated at 60°C for 15 minutes. A portion (5 μl) of the mixture was subjected to SDS-PAGE[24] on a 10% gel in triplicate. After fixing and drying, the gel was exposed to an X-ray film at −60°C for 2~5 days. Radioactive bands corresponding to Plg and the light and heavy chains of plasmin were excised from the gel and counted for radioactivity in a γ-counter.

Determination of ^{125}I-Plasminogen Binding to Fibrin

The assay was performed as described previously[17,22]. Briefly, a 100-μl solution of human fibrinogen (0.2 mg/ml)

in buffer B was dispensed into each well of a 96-well polyvinyl chloride plate. After drying the well by incubating at 37°C for 3~5 days, each well received 75 μl of human thrombin (0.68 international unit/ml in buffer B) and was incubated at 37°C for 3 hours. Fibrin clots formed in the wells were washed 3 times with 100 μl of buffer B, then incubated at 37°C for 60 minutes with 50 μl of buffer C containing 50 nM ^{125}I-Plg. After washing the wells with buffer B (twice with 200 μl, then once with 100 μl), ^{125}I-Plg bound was dissolved in 50 μl of 0.2 M NaOH and 2% (wt/vol) SDS at 37°C for 30 minutes. A portion (40 μl) of the lysate was removed and counted for radioactivity using a γ-counter.

Determination of Fibrinolytic Activity

^{125}I-Fibrinogen (diluted with unlabeled fibrinogen to a specific activity of ~2,500 cpm/μg, 20 μg in 0.1 ml of buffer B) was used to form fibrin clots in microplate wells. The procedure for the preparation of fibrin clot was identical to that described for the unlabeled material used in the Plg binding assay. The clot was washed twice with 0.1 ml of buffer B supplemented with 0.1% Tween 80 and once with 0.1 ml of buffer B, then incubated with 0.2 ml of buffer B containing 5 mg/ml of calf skin gelatin at 37°C for 60 minutes[25]. After removing the buffer, the clot was incubated at 37°C for 60 minutes with 60 μ of buffer B containing 2.5 mg/ml gelatin, 0.01% Tween 80, 0.3 unit/ml urokinase and 100 nM Glu-Plg. Subsequently, a portion (30 μl) of the mixture was removed to determine radioactivity released from the ^{125}I-fibrin clot.

General Procedures

The UV spectrum was measured in MeOH on a model 320 spectrometer (Hitachi, Japan) and the IR spectrum on an IR-810 spectrometer (JASCO, Japan) with NaCl. The FAB mass spectrum was taken on an SX-102A spectrometer (JEOL, Japan) using glycerol as a matrix. The NMR spectra were measured in DMSO-d_6 at 40°C on an EX-270 spectrometer (JEOL) at 270 MHz (for ^{1}H) and 68 MHz (for ^{13}C). The optical rotation was measured on a model DIP-360 (JASCO) in MeOH.

Results

Isolation of SMTP-3

SMTP-3 was produced as a minor metabolite along with staplabin and SMTP-1 and -2. The combined culture supernatant (4.9 liters) was applied to a Diaion HP-20 column (60 × 320 mm) at pH 7. After washing with 4.9 liters of water, the column was developed with 4.9 liters of 50% aqueous MeOH. The 50% MeOH eluate was concentrated to give 11.7 g of an oily residue. The residue was suspended with 1 liter of chloroform-MeOH (4:1), and the suspension was centrifuged to obtain supernatant, which was concentrated to give 6.4 g of a residue. This material was applied to a silica gel column (60 × 270 mm), and the column was successively developed with a mixture of chloroform and MeOH (19:1, 6.4 liters; 9:1, 6.4 liters). The chloroform-MeOH (9:1) fraction was dried *in vacuo*, giving 981 mg of a residue. The residue was dissolved in a small volume of MeOH and subjected in multiple batches to preparative HPLC on an Inertsil PREP-ODS column (30 × 250 mm, GL Sciences, Japan). The column was developed at 40°C with 50 mM ammonium acetate in 70% aqueous MeOH at a rate of 25 ml/minutes. The fractions eluting with a retention time of 12~13 minutes were pooled, evaporated to remove MeOH and extracted with ethyl acetate, giving 7.3 mg of purified SMTP-3.

Isolation of SMTP-4, -5 and -6

In the modified medium, several metabolites including SMTP-4, -5 and -6 were accumulated as well as staplabin. The combined culture supernatant (9.15 liters) was extracted with 2-butanone (once with 9 liters and twice with 4.5 liters). The organic layer was concentrated, giving 3.89 g of an oily residue. The residue was applied to a silica gel column (30 × 240 mm), and the column was developed successively with a mixture of dichloromethane and MeOH (98:2, 95:5 and then 90:10; 3.9 liters each). SMTP-4 and -5 were found in the dichloromethane-MeOH (95:5) fraction, which were concentrated to give 946 mg of a residue. The residue was subjected to preparative HPLC on an Inertsil PREP-SIL column (20 × 250 mm, GL Sciences), which was developed with *n*-hexane-EtOH (93:7) at a rate of 9.9 ml/minute. The fractions containing SMTP-4 (retention time: 20.5~22.0 minutes) and SMTP-5 (retention time: 18.0~19.5 minutes) were concentrated *in vacuo*, yielding 15.9 and 12.5 mg of respective purified compounds. SMTP-6 was found in the dichloromethane-MeOH (90:10) fraction in the silica gel column chromatography. The fraction was dried *in vacuo*, giving 845 mg of a residue. This material was subjected to preparative HPLC on an Inertsil PREP-ODS column (30 × 250 mm), which was developed at 40°C with 50 mM ammonium acetate in 68% aqueous MeOH at a rate of 25 ml/minute. The fractions containing SMTP-6 (re-

Table 1. Physico-chemical properties of SMTP-3, -4, -5 and -6.

	SMTP-3	SMTP-4	SMTP-5	SMTP-6
Appearance	Light brown oil	Light brown oil	Light brown oil	Light brown oil
Molecular formula	$C_{26}H_{35}NO_7$	$C_{32}H_{39}NO_6$	$C_{29}H_{41}NO_6$	$C_{34}H_{40}N_2O_6$
HRFAB-MS (*m/z*)				
Found $(M+H)^+$:	474.2464	534.2842	500.2980	573.2938
Calculated:	474.2492 for $C_{26}H_{36}NO_7$	534.2856 for $C_{32}H_{40}NO_6$	500.3012 for $C_{29}H_{42}NO_6$	573.2965 for $C_{34}H_{41}N_2O_6$
UV λ_{Max}^{MeOH} nm (ε)	214 (43,000) 256 (9,500) 298 (3,400)	214 (40,700) 259 (8,400) 302 (2,500)	215 (36,700) 259 (7,600) 300 (2,300)	216 (50,300) 260 (10,100) 282 (6,200, sh) 290 (5,400, sh) 308 (2,500, sh)
IR ν_{max} (KBr) cm^{-1}	3400, 2920, 2860, 1660, 1600, 1460, 1380, 1160, 1080	3392, 2076, 1668, 1469, 1369, 1241, 1073, 674	3546, 2075, 1677, 1465, 1365, 1234, 1079, 773, 647	3397, 2073, 1666, 1463, 1365, 1234, 1083, 593
Specific rotation $[\alpha]_D^{20}$	19.0° (*c* 0.32, acetone)	-79.0° (*c* 0.9, MeOH)	-12.3° (*c* 0.5,MeOH)	-44.6° (*c* 0.5, MeOH)

tention time: 31.5~33.5 minutes) were evaporated to remove MeOH and extracted with ethyl acetate, giving 23.2 mg of purified compound.

Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of SMTPs are summarized in Table 1. The four SMTPs were soluble in chloroform, MeOH and DMSO. The molecular formulae were established from the NMR and HRFAB-MS spectra. The UV and IR spectra of the SMTPs were similar to those of staplabin[17]. In ^1H and ^{13}C NMR spectra of the four SMTPs (Tables 2, 3), signals corresponding to the ring structure and the 4,8-dimethyl-3,7-nonadienyl side chain of staplabin were found. The presence of such structures was further confirmed by the ^1H-^1H COSY, ^{13}C-^1H COSY and HMBC spectra. The structural difference between staplabin and the four SMTPs was found in the substituent at position 2 of the lactam ring. In the NMR spectra, the four analogs were shown to lack carboxybutyl signals found in staplabin but had the following signals: a carbonyl, a nitrogen-bearing methine and an oxygen-bearing methylene for SMTP-3; a carbonyl, a nitrogen-bearing methine, an aliphatic methylene, an olefinic quaternary carbon and five olefinic methines for SMTP-4; a carbonyl, a nitrogen-bearing methine, an aliphatic methylene, an aliphatic methine and two methyls for SMTP-5; a carbonyl, a nitrogen-bearing methine, an aliphatic methylene, three olefinic quaternary carbons and five olefinic methines for SMTP-6. The ^1H and ^{13}C chemical shift values for the side chains of SMTP-3, -4, -5 and -6 were comparable to those for the amino acids serine, phenylalanine, leucine and tryptophan, respectively. The side chain structures of the analogs were further confirmed by the ^1H-^1H COSY and HMBC spectra, and the results are summarized in Fig. 2. From these results, the structures of the four analogs were determined as shown in Fig. 1.

Effects on Plg Activation

The effects of SMTPs on Plg activation were first examined by determining the conversion of ^{125}I-Plg to ^{125}I-plasmin. As shown in Fig. 3A, the rate of urokinase-catalyzed conversion of ^{125}I-Glu-Plg to ^{125}I-plasmin was markedly elevated by SMTP-4, -5 and -6 at concentrations ranging from 0.15 to 0.4 mM, while the effect of SMTP-3 was not significant at concentrations up to 0.45 mM. Lys-Plg, which adopts a relaxed conformation, is more sensitive to activation than is Glu-Plg, and significant conversion to plasmin occurred at a lower concentration of urokinase (see Materials and Methods). SMTP-4, -5 and -6, but not SMTP-3, were also active in enhancing the conversion of ^{125}I-Lys-Plg to ^{125}I-plasmin at concentrations of 0.15~0.45 mM (Fig. 3B). However, the magnitude of the enhancement was significantly lower than that observed with Glu-Plg

Table 2. NMR spectral data for SMTP-3 and -4.

Position	Staplabin		SMTP-3		SMTP-4	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	46.6	4.17 (2H, s)	45.1	4.32 (2H, s)	44.6	4.15 (2H, s)
3	167.6		168.5		168.1	
3a	131.7		131.1		130.7	
4	99.6	6.63 (1H, s)	99.5	6.65 (1H, s)	99.5	6.59 (1H, s)
5	156.2		156.1		156.1	
5a	111.3		111.5		111.6	
6	26.7	2.47 (1H, dd, J=7.3, 17.5) 2.82 (1H, dd, J=5.3, 17.5)	26.7	2.47 (1H, dd, J=7.4, 17.5) 2.83 (1H , dd , J=5.3, 17.2)	26.5	2.42 (1H, dd, J=6.9, 14.2) 2.80 (1H, dd, J=5.3, 17.8)
7	66.0	3.73 (1H, dd, J=5.3, 7.3)	65.9	3.75 (1H, m)	65.7	3.73 (1H, dd, J=5.6, 5.9)
8	78.8		78.7		78.3	
9a	148.4		148.4		148.3	
9b	119.3		120.2		119.5	
10	18.2	1.16 (3H, s)	18.2	1.17 (3H, s)	18.4	1.18 (3H, s)
1″	25.5	1.61 (3H, s)	25.5	1.61 (3H, s)	25.3	1.62 (3H, s)
2″	130.7		130.7		130.5	
3″	124.1	5.04 (1H, t, J=6.6)	124.1	5.05 (1H, t, J=6.2)	124.0	5.05 (1H, t, J=5.3)
4″	26.2	1.99 (2H, m)	26.2	1.99 (2H, m)	26.1	2.03 (2H, m)
5″	39.8	1.94 (2H, m)	38.5	1.94 (2H, m)	39.0	2.00 (2H, m)
6″	134.4		134.4		134.3	
7″	124.2	5.12 (1H, t, J=6.6)	124.2	5.14 (1H, m)	124.1	5.12 (1H, t, J=6.6)
8″	21.1	2.11 (2H, m)	21.0	2.11 (2H, m)	21.0	2.07 (2H, m)
9″	37.2	~1.6 (2H, m)	37.2	~1.6 (2H, m)	36.9	1.55 (2H, m)
10″	17.5	1.53 (3H, s)	17.5	1.53 (3H, s)	17.4	1.53 (3H, s)
11″	15.6	1.54 (3H, s)	15.7	1.53 (3H, s)	15.5	1.53 (3H, s)
1′	174.4		171.2		171.9	
2′	33.1	2.23 (2H, t, J=7.3)	56.3	4.75 (1H, dd, J=4.2, 7.3)	54.7	5.07 (1H, m)
3′	21.8	1.44 (2H, m)	60.0	3.88 (2H, m)	34.5	3.27 (2H, m)
4′	27.2	~1.6 (2H, m)			137.6	
5′	41.1	3.45 (2H, t, J=6.7)				
7′					126.2	7.15 (1H, m)
5′, 9′					128.3	7.22 (2H, s)
6′, 8′					128.2	7.24 (2H, s)

The data for staplabin are from ref. 17. The chemical shift is relative to DMSO-d_6 (δ_C 39.5 ppm; δ_H 2.49 ppm). The coupling constant (J) is given in Hz.

activation. In control incubations in the absence of urokinase, SMTPs showed no effect on the conversion of both Glu-Plg and Lys-Plg. Since SMTP-3 was inactive in enhancing Plg activation, its activity was not evaluated further.

Next, urokinase-catalyzed Plg activation was determined by measuring the generation of plasmin activity using a chromogenic plasmin substrate, S-2251. In the activation of Glu-Plg, SMTP-4, -5 and -6 were shown to enhance 2- to 12-fold the generation of plasmin activity at concentrations ranging from 0.1 to 0.25 mM (Fig. 4A). As the concentration of staplabin required for elevating 2-fold Glu-Plg activation was 0.4 mM, these analogs are at least three times as active as staplabin. The activation of Lys-Plg was also significantly elevated by the three SMTPs at 0.1 ~ 0.25 mM, while the effects of SMTP-4 and -5 were rather moderate (Fig. 4B). These results were similar to the results obtained with the assay determining the conversion of Plg to plasmin, indicating that plasmin formed in the presence of SMTPs was functionally active. In the absence of urokinase, SMTPs showed no effect on the hydrolysis S-2251 by Plg.

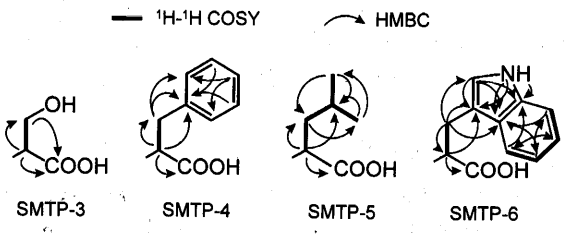
Effects on Plg Binding to Fibrin

The fibrin binding of ^{125}I-Plg was assayed in the presence of SMTPs at a concentration of 0.3 mM. The

Table 3. NMR spectral data for SMTP-5 and -6.

Position	SMTP-5		SMTP-6	
	δ_C	δ_H	δ_C	δ_H
1	43.8	4.11 (1H, d, J=16.8) 4.31 (1H, d, J=16.8)	44.0	4.11 (1H, d, J=16.8) 4.36 (1H, d, J=16.8)
3	168.1		168.0	
3a	131.0		131.4	
4	99.6	6.66 (1H, s)	99.5	6.60 (1H, s)
5	156.1		156.0	
5a	111.5		111.3	
6	26.5	2.50 (1H, dd, J=7.1, 17.6) ~2.80 (1H, dd, J=5.8, 17.5)	26.6	2.45 (1H, dd, J=7.2, 10.6) 2.81 (1H, dd, J=5.6, 12.2)
7	65.8	3.75 (1H, dd, J=5.9, 6.6)	65.9	3.72 (1H, t, J=6.3)
8	78.7		78.6	
9a	148.3		148.2	
9b	119.6		119.6	
10	18.3	1.19 (3H, s)	18.1	1.13 (3H, s)
1″	25.3	1.61 (3H, s)	25.3	1.62 (3H, s)
2″	130.5		130.5	
3″	124.0	5.05 (1H, t, J=6.6)	124.0	5.08 (1H, m)
4″	26.0	2.02 (2H, m)	26.1	2.01 (2H, m)
5″	39.0	1.90 (2H, m)	39.1	1.95 (2H, m)
6″	134.2		134.3	
7″	124.1	5.13 (1H, t, J=6.6)	124.1	5.12 (1H, m)
8″	21.0	2.12 (2H, m)	21.0	2.06 (2H, m)
9″	37.0	~1.6 (2H, m)	37.1	~1.6 (2H, m)
10″	17.3	1.54 (3H, s)	17.4	1.55 (3H, s)
11″	15.5	1.54 (3H, s)	15.5	1.55 (3H, s)
1′	172.9		172.7	
2′	51.8	4.76 (1H, dd, J=4.6, 11.2)	54.5	5.10 (1H, m)
3′	37.7	~1.6 (2H, m)	25.5	3.36 (2H, m)
4′	24.5	1.38 (1H, m)	110.7	
5′	22.8	0.90 (3H, d, J=6.3)	122.3	7.00 (1H, s)
6′	20.8	0.88 (3H, d, J=6.3)		10.66 (1H, s)
6′a			136.0	
7′			111.2	7.29 (1H, d, J=8.3)
8′			120.8	7.04 (1H, t)
9′			118.1	6.96 (1H, t)
10′			118.0	7.57 (1H, d, J=7.9)
10′a			127.0	

Fig. 2. Spin coupling and long-range coupling observed in the ^{1}H-^{1}H COSY and HMBC spectra.

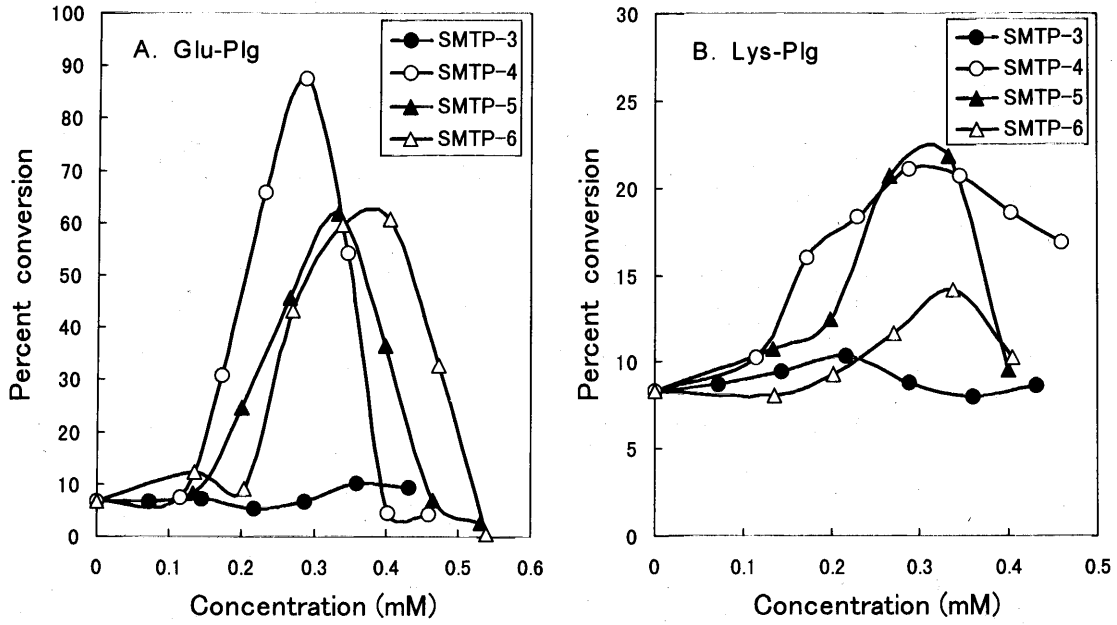


binding of ^{125}I-GluPlg was enhanced 3.5- to 4.6-fold by SMTP-4, -5 and -6 (Fig. 5A). Similarly, the binding of ^{125}I-Lys-Plg was also elevated by these analogs, while the effects were less prominent than the effects on ^{125}I-GluPlg binding (Fig. 5B). Although staplabin has been shown to be active in elevating binding of both Glu-Plg and Lys-Plg at higher concentrations (0.4~0.6 mM)[17,18], it was inactive at 0.3 mM.

Effects on Fibrinolysis

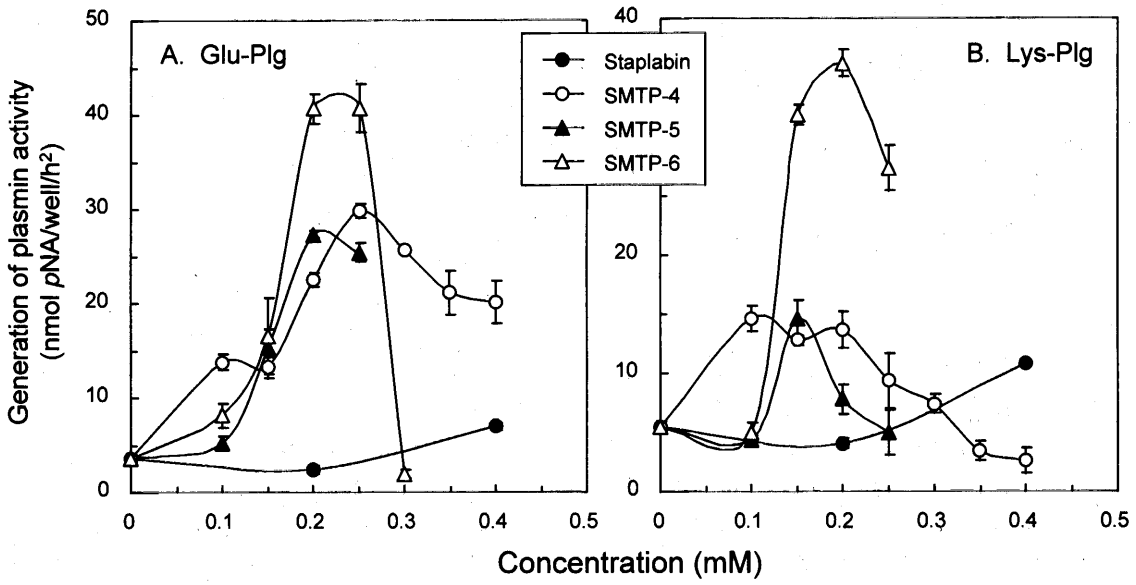
Fibrinolytic activity was determined by incubating

Fig. 3. Effects of SMTP-3, -4, -5 and -6 on urokinase-catalyzed conversion of ^{125}I-Plg to ^{125}I-plasmin.



The urokinase-catalyzed conversion of ^{125}I-Glu-Plg (A) and ^{125}I-Lys-Plg (B) to ^{125}I-plasmin was determined in the presence of the indicated concentrations of SMTPs. The specific activities of Plg used were 2.67×10^4 cpm/pmol for ^{125}I-Glu-Plg and 5.33×10^4 cpm/pmol for ^{125}I-Lys-Plg. The rate of conversion was calculated by dividing radioactivity of plasmin (heavy chain plus light chain) by radioactivity of Plg plus plasmin. Each value represents the mean from triplicate determinations.

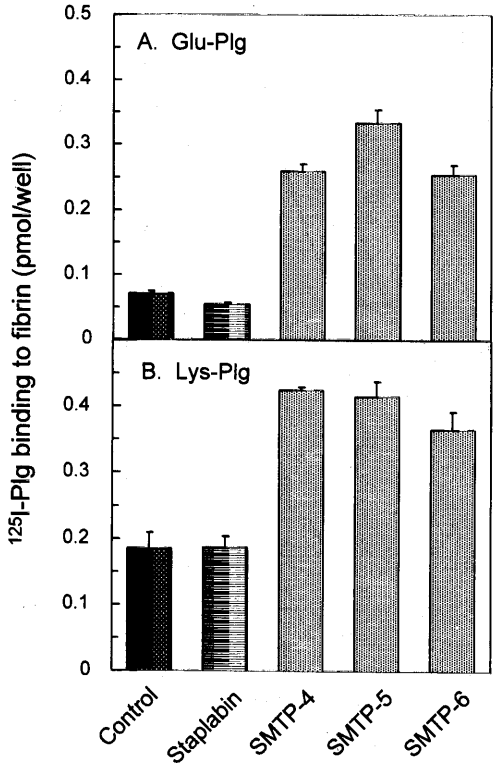
Fig. 4. Effects of SMTP-4, -5 and -6 on urokinase-catalyzed generation of plasmin activity from Plg.



The activation of Glu-Plg (A) and Lys-Plg (B) was determined by measuring the generation of plasmin activity in the presence of the indicated concentration of SMTPs and staplabin. Each value represents the mean ± S.D. from triplicate determinations.

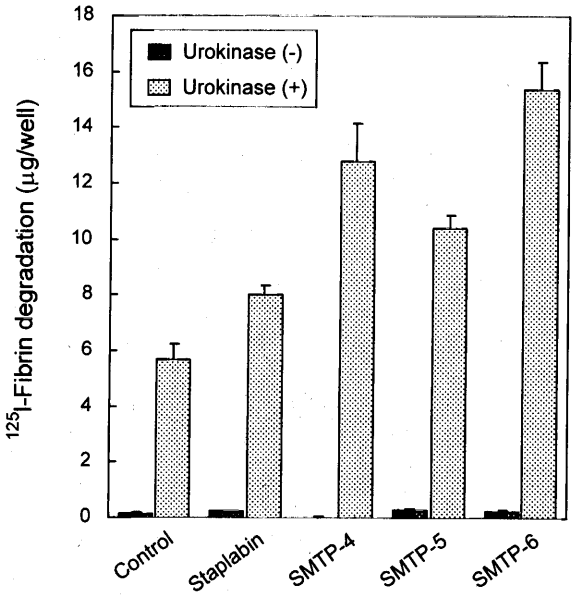
^{125}I-fibrin with urokinase and Glu-Plg in the presence of SMTPs at a concentration 0.25 mM (Fig. 6). The level of fibrinolysis was significantly elevated by SMTP-4, -5 and -6 (2.3-fold, 1.9-fold and 2.7-fold, respectively). Under these conditions, staplabin caused a slight activation of fibrinolysis. In the absence of urokinase,

Fig. 5. Effects of SMTP-4, -5 and -6 on fibrin binding of ^{125}I-Plg.



The binding of ^{125}I-Glu-Plg (A) and ^{125}I-Lys-Plg (B) to fibrin was determined in the presence of the indicated agent at a concentration of 0.3 mM. The specific activities of both Glu-Plg and Lys-Plg used were 2.00×10^4 cpm/pmol. Each value represents the mean ± S.D. from triplicate determinations.

Fig. 6. Effects of SMTP-4, -5 and -6 on fibrinolysis in the presence of urokinase and Glu-Plg.



^{125}I-Fibrin degradation was assayed in the presence of the indicated compounds at a concentration of 0.25 mM. Where indicated, urokinase was not included in the reaction mixture. Each value represents the mean ± S.D. from triplicate determinations.

the level of fibrinolysis was not elevated by these agents, indicating that these effects were mediated by Plg activation.

Discussion

In the present study, we have isolated four novel staplabin analogs, designated SMTP-3, -4, -5, and -6. The structural difference between these compounds lies on a substituent at the nitrogen atom of the chroman-lactam ring. SMTP-4, -5 and -6, which contain a phenylalanine, a leucine or a tryptophan moiety in respective molecules, are three times or more active in enhancing Plg activation than staplabin, which has a carboxybutyl substituent. On the other hand, SMTP-3, which has a serine moiety, was inactive even at higher concentrations. From these observations, it is suggested that staplabin analogs with a nonpolar amino acid moiety at this position are more active than staplabin.

Staplabin enhances both activation and fibrin binding of Plg. These effects are attributable to a conformational change of Plg induced by staplabin[18]. Like the effect of staplabin, the effects of SMTP-4, -5 and -6 on these activities are more prominent with Glu-Plg, which has a tight structure, than with Lys-Plg, which adopts a more relaxed conformation. Therefore, the enhancement of Plg activation and fibrin binding of Plg by these analogs may be related to a conformational change in the Plg molecule. As shown in the previous studies, staplabin action quenches at higher concentrations[18]. Similar results are observed with the analogs. These results can be partly explained if, at higher concentrations of SMTPs, Plg may undergo an excessive conformational change that is not favorable to activation and fibrin binding. Lysine analogs, such as 6-aminohexanoic acid, bind to the lysine binding sites and/or aminohexyl site in the kringle domain of Plg and induce a conformational change in Plg[9,26,27]. The change facilitates the Plg activator-catalyzed activation of Plg. With respect to such effects, SMTPs are similar to lysine analogs. However, lysine analogs inhibit fibrinolysis by abolishing Plg-fibrin binding, which is mainly mediated by an interaction

between the lysine binding sites (and/or aminohexyl site) of Plg and lysine residues of fibrin. Thus, SMTPs are unique in that they are small molecules that enhance both activation and fibrin binding of Plg.

SMTP-4, -5 and -6 are also active in enhancing fibrinolysis, which is mediated by urokinase and Glu-Plg. This observation provides support for the idea that compounds that induce a conformational change of Plg can activate fibrinolysis. Although the concentration of SMTPs required for exerting this activity is still high, the present results would be helpful to structure–activity relationship studies for designing a conformational modulator of Plg with therapeutic applicability.

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