Staplabin, a Novel Fungal Triprenyl Phenol which Stimulates the Binding of Plasminogen to Fibrin and U937 Cells

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A novel triprenyl phenol, designated staplabin, has been isolated from a culture of *Stachybotrys microspora* IFO 30018 by solvent extraction and successive chromatographic fractionation using silica gel, Sephadex LH-20 and silica ODS columns. By a combination of spectroscopic analyses, the structure of staplabin is proposed to be 5-(2-(5,7-dihydroxy-8-methyl-8-(4,8-dimethyl-3,7-nonadienyl)-3-oxo-7,8-dihydro-6*H*-pyrano[2,3-*e*][1,3]dihydroisoindolyl)pentanoic acid. Staplabin stimulated the binding of plasminogen, the zymogen of the fibrinolytic serine protease plasmin, to both fibrin and U937 cells. Binding was elevated 2-fold at a concentration of 0.3~0.5 mM.

The fibrinolytic system is involved in the dissolution of blood clots, which are formed in response to the injury and rupture of blood vessels. Plasmin, the primary fibrinolytic serine protease, is derived from the zymogen plasminogen by a limited proteolytic cleavage catalyzed by plasminogen activators[1]. The activation of plasminogen by tissue-type plasminogen activators is accelerated markedly on the surface of endothelial cells and fibrin[2]. Plasminogen has an ability to bind to fibrin and various cells, thereby localizing the fibrinolytic activity on cell surfaces and fibrin clots[3]. Elevating plasminogen binding to fibrin and cells thus would be expected to enhance local fibrinolytic activity. In a search for a microbial metabolite which enhances plasminogen binding to fibrin, we have found a fungal strain which produces a novel triprenyl phenol compound designated staplabin (Fig. 1). The present paper deals with the production, isolation, structure and biochemical activity of staplabin.

Materials and Methods

Materials

Human plasminogen (amino-terminal glutamic acid form) was purchased from Enzyme Research Laboratories, U.S.A. Human fibrinogen, human thrombin, bovine serum albumin and ε-aminocaproic acid were obtained from Sigma, U.S.A. Carrier-free $Na^{125}I$ was purchased from New England Nuclear/DuPont, U.S.A.

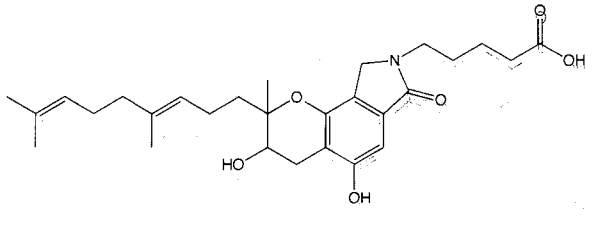
Fermentation Production of Staplabin

The producing strain *S. microspora* IFO 30018, which was obtained from the Institute for Fermentation, Osaka, was subcultured on potato glucose agar slants. For both seed and production cultures, 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. The pH of the medium before autoclaving was 5.8~6.0 (not adjusted). 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 9 days.

O-Methylation of Staplabin and Isolation of Trimethylstaplabin

Twenty mg of staplabin were dissolved in 1.2 ml of DMSO and 0.3 ml of DMSO carbanion[4]. The mixture was stirred under nitrogen gas for 4 hours at room temperature, following which 160 μl of CH_3I was added to initiate methylation. The reaction continued for 17 hours at room temperature with stirring. Subsequently, the mixture was extracted with $CHCl_3$ after adding 20 ml of water. The organic extract was subjected to HPLC on an Inertsil PREP-ODS column (6 mm × 250 mm). The column was developed with CH_3CN-water (7:1) at a rate of 1 ml/minute. The *O*-methyl derivative of staplabin (trimethylstaplabin) appeared as a peak at a retention

Fig. 1. Structure of staplabin.



time of 16 minutes. The fraction containing the derivative was lyophilized to give 7.5 mg of a residue.

Trimethylstaplabin: Light brown oil; FAB-MS, m/z 528 $(M+H)^+$; ^1H NMR (DMSO-d_6), δ 6.76 (1H, s), 5.10 (1H, t, $J=6.9$ Hz), 5.03 (1H, t, $J=6.9$ Hz), 4.23 (2H, s), 3.84 (3H, s), 3.56 (3H, s), 3.51 (1H, m), 3.49 (2H, m), 3.33 (3H, s), 2.91 (1H, dd, $J=5.0$ and 17.8 Hz), 2.59 (1H, dd, $J=6.3$ and 17.8 Hz), 2.34 (2H, t, $J=7.3$ Hz), 2.07 (2H, m), 1.98 (2H, m), 1.93 (2H, m), 1.60 (3H, s), ~1.6 (2H, m), ~1.6 (2H, m), 1.52 (3H, s), 1.51 (3H, s), 1.46 (2H, m) and 1.22 (3H, s).

Radioiodination of Plasminogen

Plasminogen was radioiodinated using Chloramine-T and $Na^{125}I$ as described by MILES and PLOW[3]. The specific radioactivity of the ^{125}I-plasminogen obtained was 1,500~3,000 cpm/ng. Over 97% of the radioactivity was precipitable upon treatment with 10% trichloroacetic acid.

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

A 100-μl solution of 0.2 mg/ml human fibrinogen in buffer A (20 mM sodium phosphate and 150 mM NaCl, pH 7.4) was dispensed into each well of a 96-well plastic tissue culture dish (Corning) and left 3~5 days at 37°C to dry. Subsequently, 75 μl of human thrombin (0.68 international unit/ml in buffer A) was added to each well. After incubation at 37°C for 3 hours, the fibrin clot was washed 3 times with 0.1 ml of buffer A. To each fibrin-coated well, 50 μl of buffer B (Hanks' balanced salt solution supplemented with 1 mg/ml bovine serum albumin and 50 mM Hepes, pH 7.4) containing 50 nM ^{125}I-plasminogen was added and the well was incubated at 37°C for 60 minutes in the absence or presence of 0.2 M ε-aminocaproic acid. The wells were washed successively with buffer A (twice with 0.2 ml and once with 0.1 ml). The ^{125}I-plasminogen bound to fibrin was dissolved in 50 μl of 0.2 M NaOH and 2% (wt/vol) SDS and incubated 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 ^{125}I-Plasminogen Binding to U937 Cells

Human monocytoid line U937 cells were obtained from the Japanese Cancer Research Resources Bank. The cells were grown at 37°C in suspension in a medium (RPMI-1640 medium containing 100 units/ml penicillin G and 100 μg/ml streptomycin) supplemented with 10% fetal bovine serum. Exponentially growing cells were harvested and washed twice with buffer B by centrifugation. U937 cells were incubated in 70 μl of buffer B containing the indicated concentration of ^{125}I-plasminogen at 37°C for 60 minutes in the absence or presence of ε-aminocaproic acid (0.2 M). Subsequently, a portion (50 μl) of the mixture was removed and layered onto 0.3 ml of 20% sucrose in buffer B in a conical 0.5-ml polypropylene tube[3]. After centrifugation at 4,900 × g for 2.5 minutes at 4°C, the cell pellet was removed by cutting the bottom of the tube off and counted for radioactivity using a γ-counter.

General Procedures

The UV spectrum was measured in MeOH on a model 320 spectrometer (Hitachi, Japan) and the IR spectrum on a IR-810 spectrometer (JASCO, Japan) as a pellet with KBr. The FAB mass spectrum was taken on a SX-102A spectrometer (JEOL, Japan). NMR spectra were measured on a GX-270 spectrometer (JEOL) at 270 MHz (for ^1H) and 67.9 MHz (for ^{13}C). The optical rotation was measured on a model DIP-360 (JASCO).

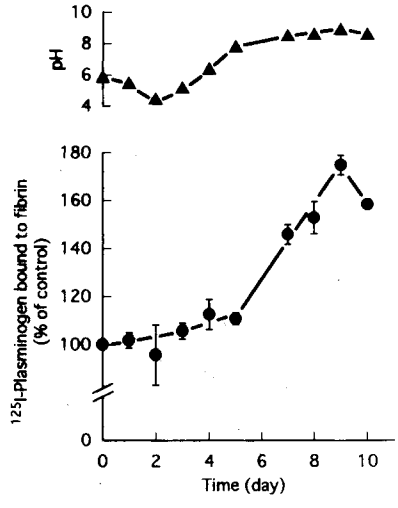
Results

Production and Isolation

A typical time course of the fermentation is presented in Fig. 2, which shows that activity to enhance ^{125}I-plasminogen binding to fibrin in the culture extracts increased after cultivation for 5 days and reached a maximum on day 9 of culture.

The combined supernatant (1 liter) obtained from 9-day cultures was extracted with 1-BuOH at pH 3 (once with 1 liter and twice with 0.5 liter). The organic layer was concentrated to give 4.8 g of an oily residue, which was then applied to a silica gel column (40 mm × 300 mm). After washing with 750 ml of $CHCl_3$-MeOH (19:1), the column was developed with 750 ml of $CHCl_3$-MeOH (9:1). The active fractions were com-

Fig. 2. Time course and pH profile of the production of activity enhancing ^{125}I-plasminogen binding to fibrin by *S. microspora* IFO 30018.



S. microspora IFO 30018 was grown aerobically in a 500-ml Erlenmeyer flask at 25°C. A one milliliter aliquot of the culture broth was lyophilized and extracted with 1 volume of 90% aq. methanol. The resulting organic extracts were dried and assayed for stimulation of ^{125}I-plasminogen binding to fibrin at a concentration of 5% (vol/vol).

bined and concentrated to dryness, giving 664 mg of a residue. The residue was subjected to fractionation on a Sephadex LH-20 column (20 mm × 300 mm). The column was developed with 180 ml of MeOH. The resulting active fraction was subjected to a second fractionation, which was performed using MeOH - water (3 : 1) as an eluting solvent. The active fraction (298 mg) was then subjected to HPLC on an Inertsil PREP-ODS column (30 mm × 250 mm, GL Sciences, Japan). The column was developed with CH_3CN - water (3 : 2) and the resulting active fraction was lyophilized, giving 24 mg of purified staplabin as brown oil.

Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of staplabin are summarized in Table 1. Its ^1H and ^{13}C NMR spectra are shown in Figs. 3 and 4, respectively. From the ^{13}C NMR and HRFAB-MS spectra, the molecular formula of staplabin was established to be $C_{28}H_{39}NO_6$. In the UV spectrum, staplabin showed absorption maxima at 216, 258 and 300 nm, the characteristic UV absorption by some triprenyl phenol compounds[5]. An intense absorption band at 1650 cm^{-1} in the IR spectrum suggested the presence of an amide carbonyl. The DEPT experiments showed that the staplabin molecule contained four methyl, ten methylene, four methine and ten quarternary (one sp^3 and nine sp^2) carbons (Table 2).

Table 1. Physico-chemical properties of staplabin.

Appearance	Light brown oil
Molecular formula	$C_{28}H_{39}NO_6$
HRFAB-MS (m/z)	
Found:	486.2862 $(M+H)^+$
Calcd:	486.2859 for $C_{28}H_{40}NO_6$
UV λ_{max}^{MeOH} nm (ε)	216 (33,500), 258 (7,300), 300 (2,500)
IR ν_{max} (KBr) cm^{-1}	3150, 2920, 1700, 1650, 1610, 1460, 1370, 1330, 1240, 1200, 1070, 1040, 900, 840, 770
Specific rotation $[\alpha]_D^{20}$	−11.0° (c 0.1, $CHCl_3$)

Fig. 3. ^1H NMR spectrum of staplabin (DMSO-d_6, 270 MHz).

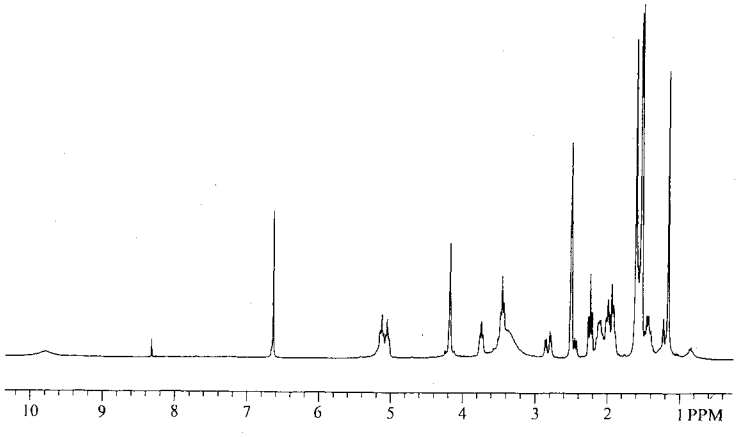
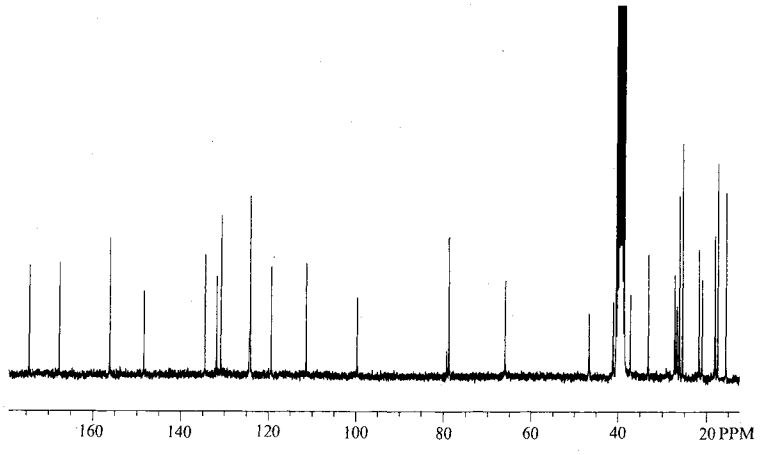


Fig. 4. ^{13}C NMR spectrum of staplabin (DMSO-d_6, 67.9 MHz).



From the ^1H-^1H COSY and ^1H-^{13}C COSY spectra, the presence of the following four spin systems was shown: $=CH-CH_2-CH_2-$, $=CH-CH_2-CH_2-$, $-CH(O-)-CH_2-$ and $(>N)-CH_2-CH_2-CH_2-CH_2-$. From the long-range couplings observed in the heteronuclear multiple-bond connectivity (HMBC) spectrum, seven sp^2 carbons and one methylene carbon were assigned to form a 1,3-dihydroisoindole-1-one moiety of the molecule (Fig. 5). In addition, the four spin systems were combined as shown in Fig. 5. An *E* configuration could be assigned to the $\Delta^{6''}$ double bond according to the chemical shift of the methyl carbon at position 11″ appearing at δ 15.6 (rather than at δ 22~25 for the *Z* configuration)[6,7]. Similarly, two methyl carbons at δ 25.5 and 17.5 (C-1″ and C-10″, respectively) were assigned as shown in Fig. 5. When the ^{13}C NMR spectrum of staplabin was measured in DMSO-d_6 after the addition of a drop of D_2O, the peak heights of signals at δ_C 156.2 (C-5) and 66.0 (C-7) were reduced by 60 and 36%, respectively, while the peak heights of signals at δ_C 78.7 (C-8) and 148.4 (C-9a) were not changed. These results suggested that the hydroxy groups of the staplabin molecule are attached to C-5 and C-7. To confirm this hypothesis, staplabin was methylated to afford a tri-*O*-methylated derivative. The HMBC spectrum of the derivative clearly showed the coupling of *O*-methyl protons at δ_H 3.84, 3.33 and 3.56 with C-5, C-7 and C-1′, respectively. The chemical shift value of C-1′ indicated that C-1′ represents a carboxylic acid, thus the third *O*-methylation involves the terminal carboxyl group. The structure of staplabin was proposed as shown in Fig. 1. The final assignments of ^1H and ^{13}C NMR data are shown in Table 2.

Table 2. Assignment of ^1H and ^{13}C NMR data for staplabin.

Position	δ_C (67.9 MHz)	δ_H (270 MHz)
1	46.6	4.17 (2H, s)
3	167.6	
3a	131.7	
4	99.6	6.63 (1H, s)
5	156.2	
5a	111.3	
6	26.7	2.47 (1H, dd, J=7.3, 17.5) 2.82 (1H, dd, J=5.3, 17.5)
7	66.0	3.73 (1H, dd, J=5.3, 7.3)
8	78.8	
9a	148.4	
9b	119.3	
10	18.2	1.16 (3H, s)
1′	174.4	
2′	33.1	2.23 (2H, t, J=7.3)
3′	21.8	1.44 (2H, m)
4′	27.2	~1.6 (2H, m)
5′	41.1	3.45 (2H, t, J=6.6)
1″	25.5	1.61 (3H, s)
2″	130.7	
3″	124.1	5.04 (1H, t, J=6.6)
4″	26.2	1.99 (2H, m)
5″	39.8	1.94 (2H, m)
6″	134.4	
7″	124.2	5.12 (1H, t, J=6.7)
8″	21.1	2.11 (2H, m)
9″	37.2	~1.6 (2H, m)
10″	17.5	1.53 (3H, s)
11″	15.6	1.54 (3H, s)

The spectra were measured in DMSO-d_6 at 20°C using TMS as an internal standard (δ 0). J=Hz.

Stimulation of Plasminogen Binding to Fibrin and U937 Cells

When ^{125}I-plasminogen (50 nM) was incubated at 37°C for 60 minutes with 20 μg of fibrin that had been formed in a well of a plastic tissue culture plate, it was found that 0.17 pmol of ^{125}I-plasminogen bound to the fibrin. In the presence of staplabin at a concentration of 0.3~0.6 mM, the binding of ^{125}I-plasminogen was increased to 110~270% of the control level (Fig. 6A). When ε-aminocaproic acid (0.2 M), a lysine analogue inhibiting specific binding of plasminogen[8], was included in the reaction mixture, the binding of ^{125}I-plasminogen was reduced to 0.02 pmol both in the absence and presence of staplabin.

The binding of ^{125}I-plasminogen to U937 cells was 0.067 pmol/10^6 cells when the cells were incubated with

Fig. 5. Long-range coupling observed in the HMBC spectrum of staplabin.

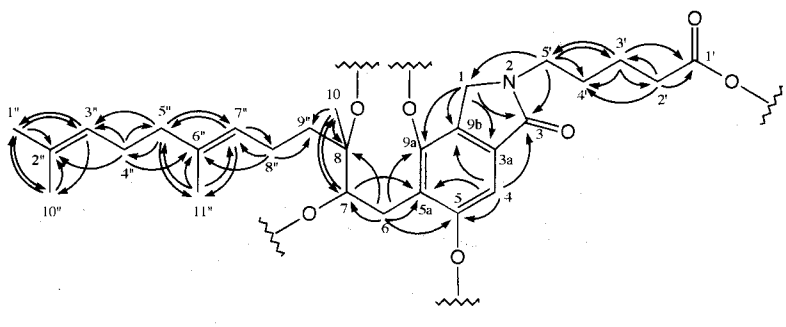
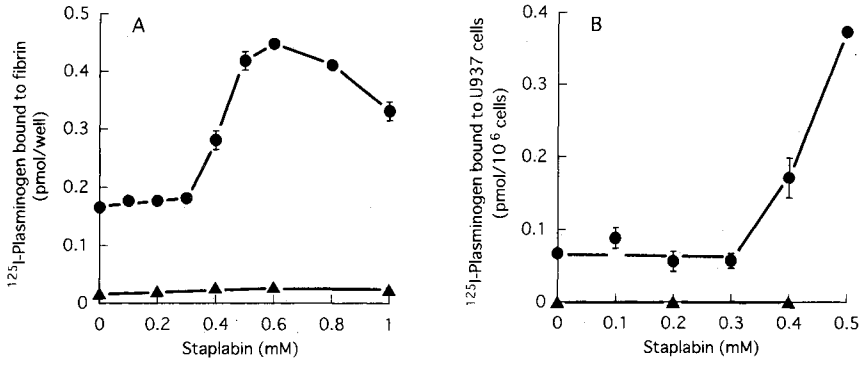


Fig. 6. Stimulation of ^{125}I-plasminogen binding to fibrin (A) and U937 cells (B) by staplabin.



Either fibrin-coated well (A) or U937 cells (B) received ^{125}I-plasminogen (50 nM for the fibrin-coated well and 12.3 nM for U937 cells) and the indicated concentrations of staplabin. After incubation at 37°C for 60 minutes in the absence (●) or presence (▲) of 0.2 M ε-aminocaproic acid, the amount of ^{125}I-plasminogen bound was determined. Each value represents the mean ± S.D. from triplicate determinations.

12.3 nM ^{125}I-plasminogen at 37°C for 60 minutes. Staplabin stimulated ^{125}I-plasminogen binding 2-fold at a concentration of 0.37 mM (Fig. 6B). Again, ε-aminocaproic acid inhibited the binding both in the absence and presence of staplabin.

Discussion

In the present study, we have isolated novel triprenyl phenol metabolite designated staplabin from a culture of *S. microspora*. A series of structurally related triprenyl phenols were reported to be produced by several fungal species belonging to the genus *Stachybotrys*. These include K-76[9], stachybotridial[10], stachybotramide[10,11], Mer-NF5003[12], eight compounds of the F-1839 series[5] and the stachybocins[13]. All of these compounds are characterized as having a phenylspirodrimane moiety. Unlike these *Stachybotrys* triprenyl phenols, the staplabin molecule has a linear prenyl group and a chroman skeleton. It is intriguing to study the biosynthetic relationships between staplabin and other triprenyl phenols.

Staplabin enhanced the binding of plasminogen to fibrin and U937 cells at concentrations in the order of 10^{-4} M. Recently, we have found that complestatin, a peptide-like metabolite of *Streptomyces*, has an activity similar to staplabin[14]. Complestatin exerts it effect at low micromolar concentrations. However, like staplabin's stimulation of plasminogen binding, plasminogen binding in the presence of complestatin is inhibited by a lysine analogue, suggesting these two agents promote plasminogen binding with by a similar mechanism. Details of the mechanism of action of staplabin will be reported elsewhere.

Acknowledgments

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