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Activation of Fibrinolysis by SMTP-7 and -8, Novel Staplabin Analogs with a Pseudosymmetric Structure

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Two novel staplabin analogs, SMTP-7 and -8, have been isolated from cultures of *Stachybotrys microspora* IFO 30018. Spectroscopic analyses showed that the SMTP-7 molecule consisted of two identical staplabin core structures and ornithine which bridges the two partial structures. In the SMTP-8 molecule, the bridging unit was lysine. At concentrations of 80~150 μM, the two compounds caused 2- to 12-fold increase in urokinase-catalyzed plasminogen activation, fibrin binding of plasminogen, and urokinase- and plasminogen-mediated fibrinolysis. These activities of SMTP-7 and -8 were two to ten times higher than those of staplabin and previously isolated SMTPs, which exerted such effects at concentrations ranging from 150 to 800 μM.

The plasminogen/plasmin system is involved in many physiological and pathological conditions including fibrinolysis, inflammation, tissue remodeling, ovulation, tumor metastasis and tissue invasion of pathogens[1~4]. In this system, the zymogen, plasminogen, is proteolytically activated to plasmin by plasminogen activators, such as urokinase and tissue plasminogen activator. Circulating plasminogen with NH_2-terminal Glu (Glu^1-plasminogen; hereafter simply referred to as plasminogen) is a single-chain glycoprotein with multiple functional domains, which consist of an NH_2-terminal peptide, five homologous kringle domains and a trypsin-like serine protease domain[5]. The binding of plasminogen to fibrin and cell surfaces, which is mediated by lysine binding sites in some kringle domains, localizes fibrinolytic activity on these surfaces[6]. Plasminogen exhibits a tight, spiral structure due to an intramolecular interaction between a lysine residue(s) (Lys^{50} and/or Lys^{62}) in the NH_2-terminal peptide and the lysine binding site (or aminohexyl site) of kringle 5[7~10]. The tight conformation of plasminogen attenuates both its activation and binding to fibrin and to cellular receptors[11,12].

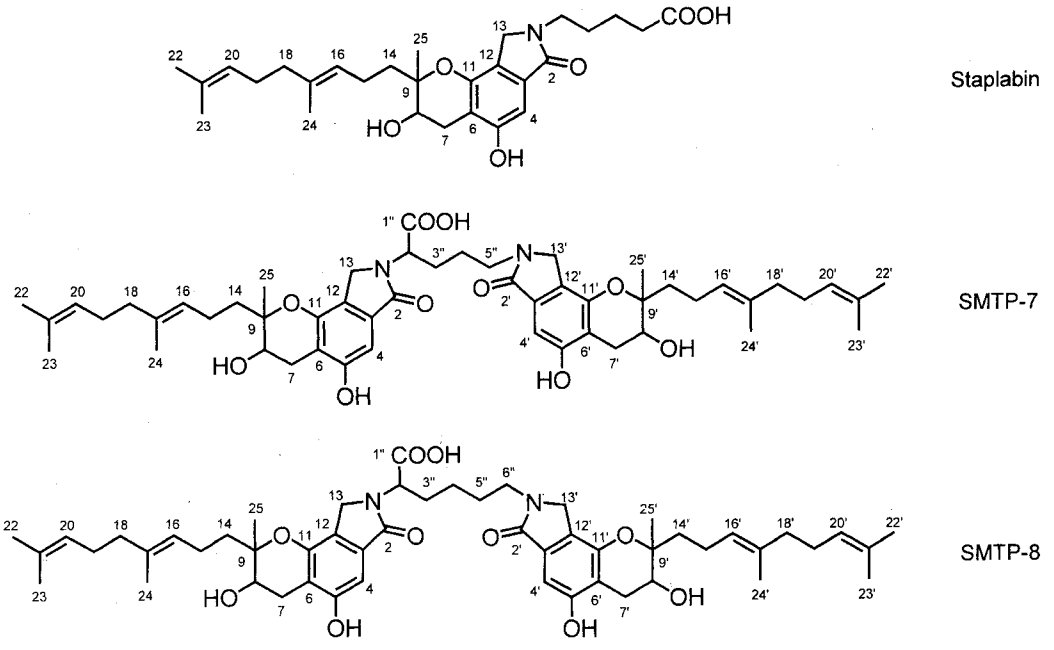
The triprenyl phenol staplabin[13] is the first low molecular weight compound that enhances both plasminogen-fibrin binding and activation of plasminogen by relaxing the plasminogen conformation to be discovered[14]. Conformational modulation of plasminogen is thus an attractive means to regulate the localized plasminogen/plasmin system. *Stachybotrys microspora* IFO 30018 produces a variety of staplabin analogs (SMTPs)[15,16], and some are found to be several times as active as staplabin[16]. In the present study, we have searched for more potent congeners and isolated two compounds, SMTP-7 and -8, which have distinct structural features (Fig. 1). The two analogs are 5- to 10-times more potent than staplabin in enhancing plasminogen-fibrin binding, urokinase-catalyzed activation of plasminogen, and urokinase- and plasminogen-mediated fibrin degradation.

Materials and Methods

Materials

Proteins and chemicals were from the following sources: human Glu^1-plasminogen from Enzyme Research Laboratories (South Bend, IN, USA); human fibrinogen, human thrombin, bovine serum albumin and S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide) from Sigma (St. Louis, MO, USA); urokinase (two-chain form; high molecular weight) from JCR Pharmaceuticals (Kobe, Japan); carrier-free $Na^{125}I$ from Amersham. Plasminogen and fibrinogen were radioiodinated using the chloramine-T and the iodine

Fig. 1. Structures of staplabin and SMTP-7 and -8.



monochloride methods, respectively[16]. 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.

Production of SMTP-7 and -8

A loopful of a slant culture of *S. microspora* IFO 30018[13] was inoculated into a 500-ml Erlenmeyer flask containing100 ml of medium consisted of 3% glucose, 1% soybean meal, 0.3% peptone, 0.3% meat extract, 0.3% yeast extract, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% CB442 (an antifoam, Nippon Oil & Fat Co., Japan). The flask was 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 consisted of 2% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.01% CB442 (pH 5.5), and the flask was incubated as above for 11 days.

Determination of Plasminogen Activation

Urokinase-catalyzed plasminogen activation was determined by measuring the initial velocity for plasmin generation using S-2251, a chromogenic substrate for plasmin. A reaction mixture (50 nM plasminogen, 50 units/ml urokinase and 0.1 mM S-2251 in 60 μl of buffer A) was incubated in a well of a 96-well microplate at 37°C for up to 60 minutes. Absorbance at 405 nm was measured with an interval of 2 minutes using a model 450 microplate reader (BioRad). From the slope of the plot of A_{405} nm versus t^2, the velocity of plasmin generation was calculated[17].

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

Fibrin clots formed in a well of a 96-well tissue culture plate[16,18] were washed three times with 100 μl of buffer B (the last wash was left in wells at 37°C for 60 minutes). The fibrin clot was then incubated at 37°C for 60 minutes with 50 μl of buffer C containing 50 nM ^{125}I-plasminogen. Where indicated, 20 mM ε-aminocaproic acid was included in the reaction mixture to obtain nonspecific binding values. Subsequently, the wells were washed with buffer B (twice with 200 μl, then once with 100 μl), and ^{125}I-plasminogen 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

Fibrin clots were prepared as described above using ^{125}I-

Table 1. Physico-chemical properties of SMTP-7 and -8.

	SMTP-7	SMTP-8
Appearance	Light brown oil	Light brown oil
Molecular formula	$C_{51}H_{68}N_2O_{10}$	$C_{52}H_{70}N_2O_{10}$
HRFAB-MS (*m/z*)		
Found $(M+H)^+$:	869.4934	883.5060
Calculated:	869.4952 for $C_{51}H_{69}N_2O_{10}$	883.5109 for $C_{52}H_{71}N_2O_{10}$
UV λ_{Max}^{MeOH} nm (ε)	213 (82,500) 257 (18,800) 302 (5,820)	213 (79,500) 257 (18,200) 302 (5,600)
IR ν_{max} (KBr) cm^{-1}	3320, 2923, 1722, 1664, 1469, 1419, 1382, 1349, 1211 1079, 1049, 771	3322, 2931, 1708, 1673, 1469, 1376, 1382, 1351, 1213, 1078, 1049, 773
Specific rotation $[\alpha]_D^{20}$	-35.87° (*c* 0.85, MeOH)	-30.03° (*c* 1.0, MeOH)

fibrinogen (~2,500 cpm/μg). The clot was washed twice with 100 μl of buffer B containing 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° for 60 minutes[19]. After removing buffer, the clot was incubated at 37°C for 60 minutes with 60 μl of buffer B containing 2.5 mg/ml gelatin, 0.01% Tween 80, 0.3 unit/ml urokinase and 100 nM plasminogen. 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, Tokyo, Japan) and the IR spectrum on an IR-810 spectrometer (JASCO, Tokyo, Japan) with NaCl. The FAB-MS spectrum was taken on an SX-102A spectrometer (JEOL, Tokyo, 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-7 and -8

SMTP-7 and -8 were produced as minor metabolites along with staplabin and SMTP-4, -5 and -6. 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 eluted successively with a mixture of dichloromethane and MeOH (98 : 2, 95 : 5, 90 : 10 and then 80 : 20; 3.9 liters each). SMTP-7 and -8 were found in the 80 : 20 fraction, which were concentrated to give 143 mg of a residue. This material was subjected to preparative HPLC on an Inertsil PREP-ODS column (30×250 mm; GL Sciences, Tokyo, Japan), which was developed at 40°C with 50 mM ammonium acetate in 80% aqueous MeOH at a rate of 25 ml/minute. The fractions containing SMTP-7 (retention time, 22~24.5 minutes) and SMTP-8 (retention time, 27.5~30.5 minutes) were evaporated to remove MeOH and extracted with ethyl acetate, giving 8.0 and 3.2 mg of purified SMTP-7 and -8, respectively.

Table 2. NMR spectral data for staplabin SMTP-7 and -8.

No.	Staplabin		SMTP - 7			SMTP -8		
	δ_C	δ_H	δ_C		δ_H	δ_C		δ_H
2, 2'	167.60		168.12	167.37		168.12	167.22	
3, 3'	131.70		131.43	130.74		131.49	130.72	
4, 4'	99.60	6.63 (1H, s)	99.46	99.44	6.65 (1H, s) 6.62 (1H, s)	99.49	99.46	6.65 (1H, s) 6.61 (1H, s)
5, 5'	156.20		155.90	155.79		155.91	155.78	
6, 6'	111.30		111.51	111.10		111.53	111.07	
7, 7'	26.70	2.82 (1H, dd, J=5.3, 17.5)	26.62	26.62	2.83 (2H, m)	26.62	26.62	2.83 (2H, m)
		2.47 (1H, dd, J=7.3, 17.5)			~2.5 (2H, m)			~2.5 (2H, m)
8, 8'	66.00	3.73 (1H, dd, J=5.3, 7.3)	65.93	65.80	3.73 (2H, dd, J=5.4, 11.6)	65.94	65.81	3.73 (2H, dd, J=6.0, 10.5)
9, 9'	78.80		78.65	78.51		78.66	78.52	
11, 11'	148.40		148.17	148.05		148.18	148.05	
12, 12'	119.30		119.55	119.14		119.53	119.09	
13, 13'	46.60	4.17 (2H, s)	46.76	44.15	4.16 (2H, s) 4.24 (1H, d, J=16.5)	45.54	43.96	4.17 (2H, d, J=1.6) 4.25 (1H, d, J=17)
					4.15 (1H, d, J=16.5)			4.15 (1H, d, J=17)
14, 14'	37.20	~1.6 (2H, m)	37.17	37.11	~1.6 (4H, m)	37.16	37.08	~1.6 (4H, m)
15, 15'	21.10	2.11 (2H, m)	21.01	20.97	~2.1 (4H, m)	21.04	21.00	~2.1 (4H, m)
16, 16'	124.20	5.12 (1H, t, J=6.6)	123.97	123.95	5.11 (2H, t, J=6.8)	124.01	123.97	5.12 (2H, t, J=6.8)
17, 17'	134.40		134.04	134.02		134.05	134.03	
18, 18'	39.80	1.94 (2H, m)	39.03	39.03	~1.9 (4H, m)	39.06	39.06	~1.9 (4H, m)
19, 19'	26.20	1.99 (2H, m)	26.08	26.08	~2.0 (4H, m)	26.11	26.11	~2.0 (4H, m)
20, 20'	124.10	5.04 (1H, t, J=6.6)	123.83	123.83	5.03 (2H, t, J=6.8)	123.84	123.84	5.04 (2H, t, J=6.8)
21, 21'	130.70		130.33	130.33		130.34	130.34	
22, 22'	25.50	1.61 (3H, s)	25.30	25.30	1.60 (6H, s)	25.31	25.31	1.60 (6H, s)
23, 23'	17.50	1.53 (3H, s)	17.39	17.39	1.52 (6H, s)	17.40	17.40	1.52 (6H, s)
24, 24'	15.60	1.54 (3H, s)	15.53	15.53	1.52 (6H, s)	15.57	15.54	1.52 (6H, s)
25, 25'	18.20	1.16 (3H, s)	18.19	17.98	1.18 (3H, s) 1.15 (3H, s)	18.19	17.99	1.17 (3H, s) 1.15 (3H, s)
1"	174.40		172.12			172.33		
2"	33.10	2.23 (2H, t, J=7.3)	53.47		4.71 (1H, dd, J=5.4, 10.0)	53.41		4.66 (1H, dd, J=5.3, 10.5)
3"	21.80	1.44 (2H, m)	26.30		~1.9 (2H, m)	28.52		~2.0 (2H, m)
4"	27.20	~1.6 (2H, m)	24.95		~1.6 (2H, m)	23.30		1.22 (2H, m)
5"	41.10	3.45 (2H, t, J=6.7)	41.12		3.48 (2H, t, J=6.6)	27.13		~1.6 (2H, m)
6"						41.26		3.41 (2H, m)

The data for staplabin are from ref. 13. 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.

Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of SMTP-7 and -8 are summarized in Table 1. The two compounds 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 two compounds were similar to those of staplabin[13]. In the ^{13}C NMR spectrum of each compound, two sets of signals corresponding to the ring structure and the 4,8-dimethyl-3,7-nonadienyl side chain of staplabin were found (Table 2). In the ^1H NMR spectrum, most of the signals for this moiety, except for signals for 4-H, 13-H_2 and 25-H_3, did not appear as doublets (Table 2). These observations suggested that the two compounds had a pseudosymmetric structure. The ^1H-^1H COSY, ^{13}C-^1H COSY and HMBC spectra were consistent with the notion that SMTP-7 and -8 had two

Fig. 2. Spin coupling and long-range coupling observed for the bridging units of SMTP-7 and -8.

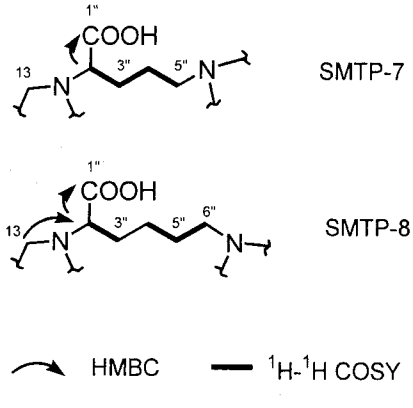
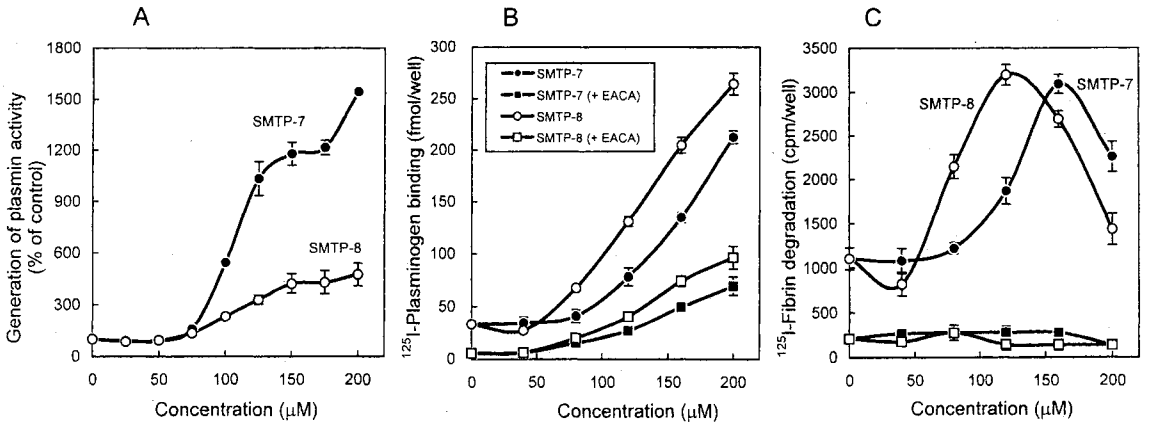


Fig. 3. Enhancement of plasminogen activation, fibrin binding of ^{125}I-plasminogen, and fibrinolysis by SMTP-7 and -8.



(A) The activation of plasminogen by urokinase was determined by measuring the generation of plasmin activity in the presence of the indicated concentration of SMTP-7 and -8. (B) The binding of ^{125}I-plasminogen to fibrin was determined in the presence of the indicated concentrations of SMTP-7 and -8. Where indicated, 20 mM ε-aminocaproic acid (EACA) was added to the reaction mixture. (C) Urokinase- and plasminogen-mediated ^{125}I-fibrin degradation was assayed in the presence of the indicated concentrations of SMTP-7 (closed symbols) and -8 (open symbols). Where indicated, urokinase was not included in the reaction mixture (squares). Each value represents the mean±S.D. from triplicate determinations.

identical staplabin core structure in each molecule. In addition to these signals, a carbonyl, a methine, and four methylene signals were observed in the ^{13}C NMR spectrum of SMTP-8. These signals were assigned to the partial structure shown in Fig. 2 according to the ^{1}H-^{1}H COSY and HMBC spectra. The chemical shift values for this moiety were similar to those of lysine. Furthermore, the NMR signals for this partial structure and lactam moiety of the SMTP-8 molecule were quite analogous to the signals for corresponding moiety of stachybocins[20] and spirodihydrobenzofuranlactam VI[21], related compounds with a pseudosymmetrical structure having lysine as a bridging unit. From these observations, 2″-CH (δ_C 53.41, δ_H 4.66) and 6″-CH_2 (δ_C 41.26, δ_H 3.41) were formulated as connected to the nitrogen atoms of the two staplabin core structures. Similar results were observed for SMTP-7. The difference between the two compounds was that the number of methylene signals in the bridging unit was three in the SMTP-7 molecule (Table 2 and Fig. 2). From these results, the structures of SMTP-7 and -8 were proposed as shown in Fig. 1.

Enhancement of Plasminogen Activation, Fibrin Binding of Plasminogen and Fibrinolysis

The effects of SMTP-7 and -8 on plasminogen activation were examined by determining the generation of plasmin activity using a chromogenic plasmin substrate, S-2251. As shown in Fig. 3A, the rate of urokinase-catalyzed generation of plasmin activity was markedly elevated by SMTP-7 and -8 at concentrations higher than 75 μM. The enhancement by SMTP-7 was 2-fold at approximately 80 μM and 12-fold at 150 μM. SMTP-8 was less active than SMTP-7, and gave 2- and 4-fold increases at 80 and 140 μM, respectively. In control incubations in the absence of urokinase, the two compounds showed no effect on the plasminogen activation (data not shown).

Next, fibrin binding of ^{125}I-plasminogen was assayed in the presence of SMTPs. The binding of ^{125}I-plasminogen was enhanced 2- to 8-fold by SMTP-7 and -8 at concentrations of 80~200 μM (Fig. 3B). The concentrations required for 2-fold enhancement were approximately 110 μM for SMTP-7 and 80 μM for SMTP-8. The binding was inhibited by the lysine analog, ε-aminocaproic acid: the inhibition was ~17% in the absence of SMTPs and 18~37% in their presence. These results suggested that the elevation of the plasminogen-fibrin binding in the presence

of SMTPs were mainly mediated through the lysine binding sites of plasminogen.

Fibrinolytic activity was determined by incubating ^{125}I-fibrin with urokinase and plasminogen in the presence of the two compounds (Fig. 3C). In this system, SMTP-7 and -8 elevated 3-fold the ^{125}I-fibrin degradation at 150 and 120 μM, respectively. In the absence of urokinase, fibrinolysis was not elevated by the two agents, indicating that these effects were mediated by plasminogen activation.

Discussion

Two new staplabin analogs, SMTP-7, and -8 have been isolated in this study. SMTP-7 has a structure that two identical units (chromanlactam with 4,8-dimethyl-3,7-nonadienyl side chain) are bridged by ornithine. In the SMTP-8 molecule, the bridging moiety is lysine. In the previous study, we have isolated four SMTPs with an amino acid side chain attached to the nitrogen atom of the chromanlactam moiety[16]. These metabolites are assumed to be formed by incorporating amino acids. Thus, the two amino groups of ornithine and lysine are likely to be similarly attacked to form two lactam ring structures of the SMTP-7 and -8 molecules. Supporting this hypothesis is the observation that the feeding *S. microspora* IFO 30018 with ornithine and lysine results in marked increases in the production of SMTP-7 and -8, respectively (manuscript in preparation).

As mentioned in the introduction, agents that relax plasminogen conformation and, hence, increase its activation and fibrin binding may be beneficial to stimulate localyzed endogenous fibrinolytic activity. The first such candidate agent with a low molecular weight is staplabin. However, staplabin is less potent and exerts effects at concentrations of 400~800 μM[13,14]. On the other hand, SMTP-7 and -8 enhance several fold the urokinase-catalyzed plasminogen activation and plasminogen-fibrin binding as well as urokinase- and plasminogen-mediated fibrinolysis at concentrations of 80~150 μM. Thus, the two analogs are 5~10 times more potent than staplabin. It is likely that these effects involve conformational relaxation of plasminogen, since, like staplabin, SMTP-7 and -8 cause direct activation of neither plasmin nor urokinase. SMTP-7 and -8 should provide useful tools for studies of the conformational regulation of plasminogen activation as well as for the development of anti-thrombotic agents.

Acknowledgments

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