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SMTP-4D, -5D, -6D, -7D and -8D, a New Series of the Non-lysine-analog Plasminogen Modulators with a D-Amino Acid Moiety

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Staplabin and SMTPs, triprenyl phenol metabolites of the fungus *Stachybotrys microspora*, are a family of non-lysine-analog plasminogen modulators that enhance both activation and fibrin binding of plasminogen by modulating plasminogen conformation. These compounds, including SMTP-4, -5, -6, -7 and -8, have an amino acid or an amino alcohol moiety in their structure, and precursor amine feeding greatly increases the biosynthesis of a metabolite of interest. In the present study, we have isolated five novel SMTPs (designated SMTP-4D, -5D, -6D, -7D and -8D) from precursor D-amino acid-fed cultures. Physico-chemical properties as well as chromatographic behavior were distinct from those of the corresponding L-amino acid analogs, which are selectively accumulated in L-amino acid-fed cultures and share common properties with corresponding natural products. The D-series SMTPs enhanced urokinase-catalyzed plasminogen activation by 10-fold at 80~180 μM.

The plasminogen/plasmin system is involved in a variety of 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 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 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 cellular receptors[11,12].

Staplabin, a metabolite of *Stachybotrys microspora*[13], is a first non-lysine-analog plasminogen modulator to be discovered that enhances both plasminogen-fibrin binding and activation of plasminogen by relaxing plasminogen conformation[14]. Conformational modulation of plasminogen is thus an attractive means to regulate localized plasminogen/plasmin system. *S. microspora* produces a variety of staplabin analogs designated SMTPs[15~17], which have an amino acid moiety as a structural constituent, and the feeding of the microbial cultures with a precursor amine selectively increases the production of a metabolite of interest[18]. To elucidate the stereochemistry and structure-activity relationships of SMTPs, we have isolated five novel congeners from D-amino acid-fed cultures.

Materials and Methods

Materials

Human native plasminogen (Glu^1-plasminogen) was isolated by lysine-Sepharose affinity chromatography as described[19]. H-Val-Leu-Lys-*p*-nitroanilide (VLK-*p*NA) was obtained from Bachem (Bubendorf, Switzerland) and urokinase (two-chain form; high molecular weight) from JCR Pharmaceuticals (Kobe, Japan). For use in experiments

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in vitro SMTPs were dissolved directly in buffer (TBS/T; 50 mM Tris-HCl, 100 mM NaCl and 0.01% Tween 80, pH 7.4).

Production of SMTPs

A loopful of a slant culture of *S. microspora* IFO 30018 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of medium consisting 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% $MgSO_4 \cdot 7H_2O$, 100 mg of D-amino acid (D-Phe, D-Leu, D-Trp, D-Orn or D-Lys) and 0.01% CB442 (pH 5.5), and the flask was incubated as above for 4~6 days.

Determination of Plasminogen Activation

Urokinase-catalyzed plasminogen activation was determined by measuring initial velocity for plasmin generation using VLK-*p*NA, a chromogenic substrate for plasmin. A reaction mixture consisted of 50 nM plasminogen, 50 U/ml urokinase and 0.1 mM VLK-*p*NA in 50 μl of TBS/T was incubated in a well of a 96-well microplate at 37°C for up to 40 minutes. Absorbance at 405 nm was measured with an interval of 1 to 2 minutes using a model 450 microplate reader (BioRad). From the slope of the plots of A_{405} nm *versus* t^2, the initial velocity of plasmin generation was calculated.

General Procedures

UV spectrum was measured in MeOH on a model 320 spectrometer (Hitachi, Tokyo, Japan) and IR spectrum on an IR-810 spectrometer (JASCO, Tokyo, Japan) with NaCl. FAB-MS spectrum was taken on an SX-102A spectrometer (JEOL, Tokyo, Japan) using glycerol as a matrix. NMR spectra were measured in DMSO-d_6 at 40°C on an EX-270 or an Alpha-600 spectrometer (JEOL). Optical rotation was measured on a model DIP-360 (JASCO) in MeOH.

Results and Discussion

Isolation of SMTP-4D, -5D and -7D

SMTP-4D, -5D and -7D were isolated from *S. microspora* cultures fed with D-Phe, D-Leu and D-Orn, respectively. The supernatant from each culture (0.6~0.9 liters) was extracted with 2-butanone (once with 1 volume and twice with 1/2 volume), and the combined organic extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The resulting oily residue (379, 236 and 165 mg from cultures fed with D-Phe, D-Leu and D-Orn, respectively) was dissolved in MeOH at approximately 100 mg/ml, passed through a LiChrolut® RP-18 solid phase extraction column and subjected to preparative HPLC on an Inertsil PREP-ODS column (30×250 mm; GL Sciences, Tokyo, Japan). The column was developed at a rate of 25 ml/minute at 40°C with 50 mM ammonium acetate in 70% (for the isolation of SMTP-4D and -5D) or 80% (for SMTP-7D) aqueous MeOH. SMTP-4D, -5D and -7D were eluted at a retention time of 46.5~50, 59~62 and 34~39 minutes, respectively. Fractions containing each compound were evaporated to remove MeOH and extracted with ethyl acetate, giving 42.4, 15.7 and 17.9 mg of purified SMTP-4D, -5D and -7D, respectively.

Isolation of SMTP-6D and -8D

SMTP-6D and -8D were isolated from *S. microspora* cultures fed with D-Trp and D-Lys, respectively. The supernatant (1.6~1.8 liters) was extracted with 2-butanone (once with 1 volume and twice with 1/2 volume), and the combined organic extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The resulting oily residue (789 and 752 mg from cultures fed with D-Trp and D-Orn, respectively) was applied to a silica gel column (30×240 mm), and the column was developed successively with 200 ml *n*-hexane, 300 ml of *n*-hexane - ethyl acetate (60 : 40 and 20 : 80) and 300 ml of ethyl acetate - MeOH (100 : 0, 98 : 2, 95 : 5 and 90 : 10). SMTP-6D was found in the *n*-hexane - ethyl acetate (20 : 80) fraction, which was concentrated to give 79.5 mg of purified SMTP-6. SMTP-8D was found in the ethyl acetate - MeOH (95 : 5 and 90 : 10) fractions, which were concentrated to give 163 mg of a residue. The residue was subjected to re-chromatography on a silica gel column (30×240 mm), which was developed successively with 100 ml hexane, 200 ml ethyl acetate and ethyl acetate - MeOH (98 : 2 and 95 : 5). The 95 : 5 fraction, which contained SMTP-8D, was concentrated to give 143 mg of a residue. The residue was further purified using preparative HPLC on an Inertsil PREP-ODS column (30×250 mm), which was developed at 40°C with 50 mM ammonium acetate in 80% aqueous MeOH at a rate of 25 ml/minute. The fraction containing SMTP-8D (retention time of 42~50 minutes) was evaporated to remove MeOH and extracted with ethyl acetate, giving 33.7 mg of purified SMTP-8D.

Table 1. Physico-chemical properties of SMTP-4D, -5D, -6D, -7D and -8D.

	SMTP-4D	SMTP-5D	SMTP-6D	SMTP-7D	SMTP-8D
Appearance	Light brown oil	Light brown oil	Light brown oil	Light brown oil	Light brown oil
Molecular formula	$C_{32}H_{39}NO_6$	$C_{29}H_{41}NO_6$	$C_{34}H_{40}N_2O_6$	$C_{51}H_{68}N_2O_{10}$	$C_{52}H_{70}N_2O_{10}$
FAB-MS (*m/z*; M+H)$^+$	534	500	573.2881	869	883.5060
UV λ_{Max}^{MeOH} nm (ε)	214 (37,470), 259 (7,675), 302 (2,185)	213 (31,587), 259 (6,786), 300 (1,996)	214 (50,794), 258 (10,296), 282 (6,178, sh), 290 (6,349, sh), 308 (2,974, sh)	213 (68,832), 257 (15,103), 302 (4,687),	213 (63,769), 257 (14,200), 302 (4,234)
IR ν_{max} (KBr) cm^{-1}	3410, 2920, 2850, 1670, 1610, 1470, 1360, 1210, 1160, 1080	3410, 2960, 2930, 2870, 1710, 1670, 1610, 1470, 1360, 1210, 1170, 1080	3470, 2920, 1710, 1660, 1620, 1460, 1360, 1220, 1160, 1080	3370, 2920, 2850, 1670, 1610, 1470, 1350, 1210, 1170, 1080	3430, 2920, 1660, 1610, 1460, 1360, 1220, 1160, 1080
Specific rotation $[\alpha]_D^{20}$	51.4° (*c* 0.9, MeOH)	-9.8° (*c* 0.5,MeOH)	19.2° (*c* 0.5, MeOH)	-2.44° (*c* 0.8, MeOH)	-5.18° (*c* 0.8, MeOH)

Table 2. NMR spectral data for SMTP-4D, -5D and -6D.

No.	SMTP-4D		No.	SMTP-5D		SMTP-6D	
	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
2	168.10		2	168.17		168.16	
3	130.70		3	130.84		130.88	
4	99.52	6.58 (1H, s)	4	99.56	6.65 (1H, s)	99.54	6.59 (1H, s)
5	156.08		5	156.12		156.08	
6	111.66		6	111.62		111.68	
7	26.66	2.78 (1H, dd, *J* = 5.4, 17.6) 2.46 (1H, dd, *J* = 7.3, 17.8)	7	26.70	2.80 (1H, dd, *J* = 5.4, 17.6) 2.48 (1H, dd, *J* = 7.3, 17.3)	26.55	2.45 (1H, dd, *J* = 7.5, 17.5) 2.78 (1H, dd, *J* = 5.4, 17.5)
8	65.89	3.71 (1H, t, *J* = 6.1)	8	65.96	3.73 (1H, t, *J* = 6.0)	65.91	3.69 (1H, t, *J* = 6.4)
9	78.76		9	78.80		78.71	
11	148.24		11	148.32		148.28	
12	119.52		12	119.58		119.56	
13	44.55	4.20 (2H, d, *J* = 16.6) 4.10 (2H, d, *J* = 16.6)	13	43.88	4.07 (1H, d, *J* = 16.8) 4.29 (1H, d, *J* = 16.8)	44.04	4.14 (1H, d, *J* = 16.7) 4.26 (1H, d, *J* = 16.7)
14	37.05	1.57 (2H, m)	14	37.02	~1.56 (2H, m)	37.12	1.56 (2H, m)
15	21.06	2.08 (2H, m)	15	21.15	2.10 (2H, m)	20.93	2.06 (2H, m)
16	124.10	5.12 (1H, t, *J* = 6.8)	16	124.26	5.12 (1H, t, *J* = 6.8)	124.12	5.10 (1H, t, *J* = 7.3)
17	134.26		17	134.06		134.22	
18	39.21	2.00 (2H, m)	18	39.19	1.98 (2H, m)	~39.1	1.99 (2H, m)
19	26.18	1.92 (2H, m)	19	26.15	1.90 (2H, m)	26.08	1.90 (2H, m)
20	123.99	5.05 (1H, m)	20	123.95	5.02 (1H, t, *J* = 6.6)	124.01	5.04 (1H, tt, *J* = 1.2, 6.8)
21	130.60		21	130.57		130.54	
22	25.47	1.61 (3H, s)	22	25.44	1.59 (3H, s)	25.03	1.61 (3H, s)
23	17.53	1.51 (3H, s)	23	17.51	1.50 (3H, s)	17.38	1.47 (3H, s)
24	15.59	1.53 (3H, s)	24	15.55	1.51 (3H, s)	15.41	1.53 (3H, s)
25	18.27	1.16 (3H, s)	25	18.24	1.17 (3H, s)	17.97	1.15 (3H, s)
26	172.00		26	172.97		172.15	
27	54.54	5.07 (1H, m)	27	51.66	4.76 (1H, dd, *J* = 4.15, 11.7)	53.87	5.14 (1H, dd, *J* = 5.0, 10.6)
28	34.57	3.25 (1H, dd, *J* = 11.2, 14.6) 3.20 (1H, dd, *J* = 4.9, 14.6)	28	37.69	1.85 (1H, m) 1.69 (1H, m)	25.30	3.41 (1H, dd, *J* = 5.0, 15.5) 3.32 (1H, dd, *J* = 10.7, 15.5)
29	137.57		29	24.65	1.33 (1H, m)	109.86	
30, 34	128.30	7.20 (2H, s)	30	22.93	0.87 (3H, d, *J* = 7.1)	122.51	7.01 (1H, d, *J* = 2.3)
31, 33	128.20	7.21 (2H, s)	31	20.86	0.85 (3H, d, *J* = 7.1)		10.70 (1H, d, *J* = 1.9)
32	126.27	7.13 (1H, m)	32			136.00	
			33			111.32	7.28 (1H, d, *J* = 8.0)
			34			120.09	7.04 (1H, ddd, *J* = 1.1, 6.8, 8.1)
			35			118.29	6.96 (1H, ddd, *J* = 0.8, 6.8, 8.1)
			36			117.84	7.56 (1H, d, *J* = 7.8)
			37			126.88	

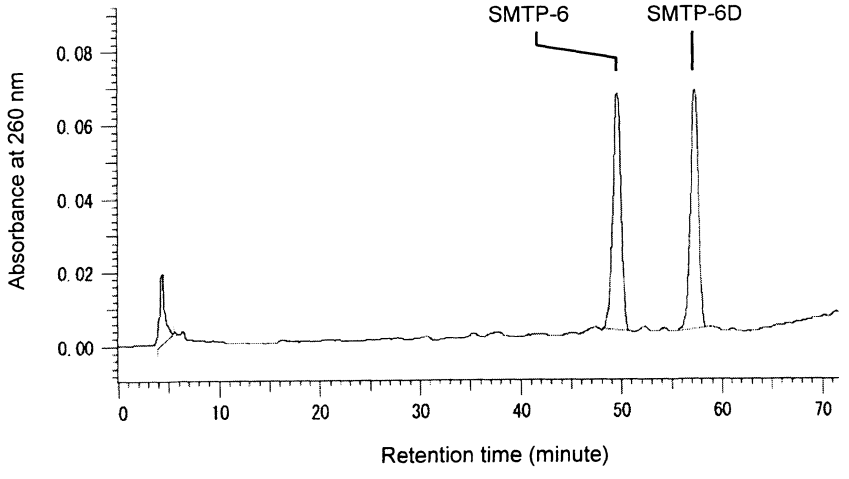
The chemical shift is relative to DMSO-d_6 (δ_C 39.5 ppm; δ_H 2.49 ppm). The coupling constant (*J*) is given in Hz.

Table 3. NMR spectral data SMTP-7D and -8D.

No.	SMTP-7D						SMTP-8D					
	δ_C		δ_H				δ_C		δ_H			
2, 2'	168.36	167.53					168.26	167.46				
3, 3'	131.55	130.84					131.68	131.25				
4, 4'	99.56	99.49	6.65	(1H, s)	6.61	(1H, s)	99.63	99.61	6.65	(1H, s)	6.61	(1H, s)
5, 5'	156.11	155.99					156.04	156.04				
6, 6'	111.66	111.18					111.49	111.23				
7, 7'	26.70	26.70	~2.5	(2H, m)			26.63	26.63	2.46	(2H, m)		
			2.80	(2H, m)					2.82	(2H, m)		
8, 8'	65.97	65.93	3.71	(2H, dd, J = 5.1, 12.9)			66.07	66.00	3.73	(2H, dd, J = 6.5, 10.1)		
9, 9'	78.77	78.62					78.69	78.60				
10, 10'	148.31	148.21					148.30	148.25				
11, 11'	119.65	119.25					119.73	119.20				
12, 12'	46.75	44.10	4.23	(1H, d, J = 16.8)	4.18	(1H, d, J = 17.8)	46.60	43.86	4.13	(2H, s)	4.33	(1H, d, J = 16.8)
			4.13	(1H, d, J = 16.8)	4.09	(1H, d, J = 17.1)					4.08	(1H, d, J = 16.8)
14, 14'	37.13	37.13	~1.6	(4H, m)			37.15	37.15	~1.6	(4H, m)		
15, 15'	21.12	21.06	2.08	(4H, m)			21.03	20.98	2.10	(4H, m)		
16, 16'	124.15	124.09	5.10	(2H, t, J = 7.8)			124.27	124.18	5.11	(2H, s)		
17, 17'	134.15	134.15					134.18	134.08				
18, 18'	39.18	39.15	1.87	(4H, m)			~39.1	~39.1	1.98	(4H, m)		
19, 19'	26.14	26.14	1.95	(4H, m)			26.09	26.09	2.10	(4H, m)		
20, 20'	123.98	123.98	5.01	(2H, t, J = 7.1)			124.00	123.99	5.02	(2H, s)		
21, 21'	130.55	130.55					130.53	130.52				
22, 22'	25.45	25.45	1.58	(6H, s)			25.29	25.27	1.59	(6H, s)		
23, 23'	17.49	17.49	1.48	(3H, s)	1.49	(3H, s)	17.36	17.34	1.50	(3H, s)	1.52	(3H, s)
24, 24'	15.58	15.58	1.49	(6H, s)			15.52	15.45	1.52	(6H, s)		
25, 25'	18.15	18.12	1.16	(3H, s)	1.14	(3H, s)	17.95	17.95	1.16	(3H, s)	1.14	(3H, s)
26	172.41						172.85					
27	53.37		4.72	(1H, dd, J = 4.9, 10.6)			54.11		4.59	(1H, dd, J = 4.0, 10.5)		
28	26.29		~1.9	(2H, m)			28.96		~1.9	(2H, m)		
29	24.96		~1.6	(2H, m)			23.49		~1.2	(2H, m)		
30	41.08		3.45	(2H, t, J = 7.1)			27.23		~1.6	(2H, m)		
31							41.39		3.39	(2H, m)		

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.

Fig. 1. Chromatographic elution profiles of SMTP-6 and SMTP-6D.



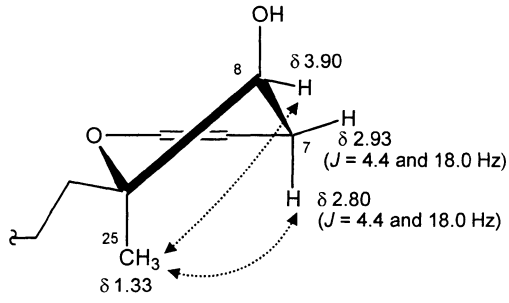
SMTP-6 and SMTP-6D (5 μg each) was applied to an Inertsil-PREP-ODS column (6×250 mm; GL Sciences, Tokyo, Japan) that was developed at a rate of 1 ml/minute at 40°C with a gradient of MeOH in 50 mM ammonium acetate, aq as follows: MeOH concentration was kept at 60% for initial 55 minutes, followed by linearly increasing MeOH concentration to 88% for next 15 minutes. The elution was monitored by absorption at 260 nm.

Physico-chemical Properties and Structure Elucidation

Except the optical rotation, the physico-chemical properties, including NMR, UV and IR spectra, of each of the five "D-series" SMTPs (Tables 1 and 2) are very similar to the properties of respective naturally occurring counterparts. ^{1}H and ^{13}C NMR data for the D-series SMTPs were reasonably assigned as shown in Tables 2 and 3 by comparing with those for the natural metabolites. These results suggest that SMTP-4D, -5D, -6D, -7D and -8D have a planar structural formula identical with SMTP-4, -5, -6, -7 and -8, respectively. As described previously, the physico-chemical properties as well as chromatographic behavior of SMTP-4, -5, -6, -7 and -8 that had been selectively accumulated in the L-amino acid-fed cultures were identical with those of respective natural products[18]. On the other hand, the chromatographic behavior and the optical rotation of the D-series SMTPs were apparently different from that of the naturally occurring counterparts. As shown in Fig. 1, for example, the retention time of SMTP-6D on analytical HPLC using a silica-C18 column was significantly longer than that of SMTP-6. Similarly, all of the remaining D-series SMTPs were eluted slower than respective natural counterparts. Since the precursor amino acid-feeding to *S. microspora* culture results in rapid and selective accumulation of a large amount of SMTP that has the fed amino acid as a structural constituent, all of the above observations strongly suggest that the configuration of the α-carbon in the amino acid moiety of SMTPs is the same as the amino acid fed to the culture for production of SMTP: *R* configuration for the D-series SMTPs and *S* for the natural counterparts.

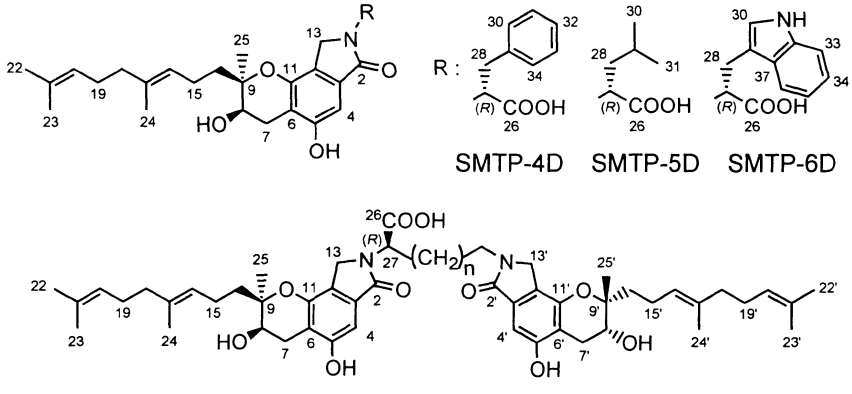
The stereochemistry of the dihydropyran moiety that is common to all SMTPs and staplabin[13,15~17] was also addressed using staplabin as a model compound. The NOE between 25-H_3 at δ 1.33 and H-7 at δ 2.80 indicated that the 25-H_3 is in an axial orientation. The NOE between 25-H_3 and 8-H at δ 3.90 demonstrated that 8-H is equatorially oriented and that 8-OH is trans to 25-H_3 (Fig. 2). These assignments were consistent with the chemical shift and coupling constant data: J=4.4 Hz for both axial 7-H at δ

Fig. 2. Relative configuration of the dihydropyran moiety.



The NMR spectra for the dihydropyran moiety of staplabin and SMTPs were very similar to each other, and staplabin was used as a model compound. The chemical shift and coupling constant data were from measurement in $CDCl_3$. Dashed arrows represent key NOE correlations.

Fig. 3. Proposed structures of SMTP-4D, -5D, -6D, -7D and -8D.



The configurations at the dihydropyran ring and the amino acid moiety are relative and absolute ones, respectively.

2.80 and equatorial 7-H at δ 2.93. Taken together, the structures of the five D-series SMTPs were elucidated as shown in Fig. 3.

Enhancement of Plasminogen Activation

The activity of the D-series SMTPs was determined as the activity to enhance urokinase-catalyzed plasminogen activation. The concentration of SMTPs to cause tenfold enhancement was: 160~180 μM for SMTP-4D and -5D, 150 μM for SMTP-6D, 80 μM for SMTP-7D, and 110 μM for SMTP-8D. Details of structure-activity relationships and *in vivo* efficacy (blood clot dissolution) of SMTP will be described elsewhere.

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