STUDIES ON WB-3559 A, B, C AND D, NEW POTENT FIBRINOLYTIC AGENTS

I. DISCOVERY, IDENTIFICATION, ISOLATION AND CHARACTERIZATION

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WB-3559 A, B, C and D were produced by a bacterium which was classified as a member of the genus *Flavobacterium*. These compounds were purified by solvent extraction followed by chromatography on silica gel and then isolated by HPLC. The chemical structures were determined on the basis of chemical and spectroscopic evidence as in the succeeding papers. WB-3559 A, B, C and D stimulated rabbit plasma euglobulin clot lysis time. A chemically synthesized compound (WB-3559 D-syn) stimulated mouse plasma euglobulin clot lysis time when injected intravenously.

Thrombosis is classified into two categories according to its pathological characteristics. White thrombi mainly induced by platelet aggregation occur in arterial vasculature. Red thrombi mainly composed of fibrin clot occlude venous blood stream. Platelet aggregation inhibitors *i.e.* aspirin, dipyridamole and sulfinpyrazone have been used to prevent arterial platelet dependent thrombosis in clinical field. Recently more potent platelet aggregation inhibitor, ticlopidine became available to treat the patients of thrombosis in many countries. We have reported several platelet aggregation inhibitors isolated from microbial products.[1,2] On the other hand, thrombolytic therapy for the patients of pulmonary embolism, myocardiac infarction and cerebral embolus is important to relieve the symptoms. Urokinase and streptokinase have been used for the purpose of thrombolytic therapy.[3,4] Tissue plasminogen activator currently produced by advanced biotechnology will become available for fibrinolytic therapy. But all these fibrinolytic agents are high molecular weight and effective when injected intravenously. Some effort have been done to develop low-molecular-weight fibrinolytics,[5,6] but none is commercially available. Therefore, we undertook a screening program directed towards the isolation and evaluation of new compound with fibrinolytic activity. As a result, we discovered four new compounds designated as WB-3559 A, B, C and D which stimulated euglobulin clot lysis time of rabbit plasma *in vitro*. In this paper we will describe characterization of the producing bacterium, fermentation and isolation procedures, physico-chemical and biological properties of WB-3558 A, B, C and D, together with a result of *ex vivo* experiment on the chemically synthesized compound, WB-3559 D-syn (*vide infra*). The chemical structures were determined as in the succeeding papers.[7,8]

Methods

Fermentation

The growth of *Flavobacterium* sp. No. 3559 on slant culture was used to inoculate twenty 500-ml flasks containing 150 ml of sterile medium of Polypeptone 1%, meat extract 0.7% and NaCl 0.3%. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) for two days at 30°C. The content of the flasks was used to inoculate 150 liters of the same medium in a 200-liter volume of stainless steel fermentation tank. Fermentation was allowed to proceed for two days at the temperature of 30°C, at the air flow of 150 liters per minute and with agitation of 250 rpm.

Euglobulin Clot Lysis Time

The active compound present in the fermentation broth or in preparation obtained during purification process was detected by its stimulating activity to euglobulin clot lysis time of rabbit plasma. Euglobulin clot lysis time was measured according to the method reported by NILSSON *et al.*[9] Briefly, rabbit blood was obtained *via* polyethylene catheter from carotid artery of male Japanese white rabbit and mixed with 1 volume of sodium citrate (130 mM) to 9 volumes of blood. Citrated rabbit plasma was prepared by centrifugation of the blood at 3,000 rpm for twenty minutes at 4°C. Citrated plasma (450 μl) was mixed with test sample (50 μl) and incubated 15 minutes at 37°C and then diluted with 0.025% acetic acid solution (4 ml). The solution was kept on ice for 60 minutes and then centrifuged at 3,000 rpm for 10 minutes at 4°C to obtain the precipitate of euglobulin fraction. The supernatant was discarded and the precipitate was dissolved in veronal buffer saline (500 μl, 5 mM barbital, 0.803% NaCl, pH 7.3, ionic strength 0.145). Bovine thrombin (250 μl, 5 U/ml) was added to the euglobulin solution to get the fibrin clot. The fibrin clot was incubated at 37°C and the spontaneous lysis time of the clot was determined visually. Activity of inhibitor was expressed as ED_{50} value *i.e.* concentration required to stimulate the clot lysis time by 50%.

Ex Vivo Experiment

A group of 5 to 10 male *dd*Y mouse was used to evaluate the effect of chemically synthesized WB-3559 D-syn (*vide infra*) to the fibrinolytic activity *in vivo*. WB-3559 D-syn was dissolved in 1% $NaHCO_3$ and injected intravenously (10 ml/kg). Thirty minutes later, blood was obtained by the cardiac puncture under light ether anesthesia into the disposable plastic syringe containing 0.1 ml of sodium citrate. After centrifuging the blood at 3,000 rpm for 10 minutes at 4°C, mouse plasma was used to measure the fibrinolytic activity according to the same method described above.

Results

Identification of Strain No. 3559

WB-3559 substances-producing strain is a bacterium isolated from a soil sample obtained at Iriomote-shima, Okinawa Prefecture. The identification of the strain was performed according to the procedures described in BERGEY's Manual of Determinative Bacteriology (8th Ed., 1974).

Strain No. 3559 was characterized by Gram-negative, non-sporulating, non-motile rod. Growth on solid media was pigmented yellow. Colonies of the strain showed typically translucent and smooth type. Morphological characteristics were summarized in Table 1.

Table 1. Morphological characteristics of strain No. 3559.

Spore formation	Negative
Motility	Negative
Gram staining	Negative
Shape	Rod with rounded ends
Size	0.5×1.5~2.5 μm

Oxidase and catalase reaction were positive. The growth-permissible temperature range was between 8°C and 33°C, with optimum temperature at 26°C. The guanine-plus-cytosine content of DNA in the strain was estimated to be between 34 and 37 mol %. Results were shown in Tables 2 and 3.

Table 2. Physiological characteristics of strain No. 3559.

Oxidase reaction	Positive
Catalase reaction	Positive
Carbohydrate metabolism (O/F medium)	Fermentative
Change of pH in milk	Slightly acidified
Milk peptonization	Slow peptonization
Milk coagulation	Negative
Gelatin liquefaction	Slow liquefaction
Voges-Proskauer reaction	Negative
Indole production in peptone broth	Negative
H_2S production	Negative
Nitrates reduced to nitrites	Negative
Temperature range for growth	8～33°C
Optimum temperature for growth	25～27°C
pH Range for growth	6～8
Optimum pH for growth	7
Energy metabolism	Aerobic
Growth in 2% NaCl	Negative
Growth on citrate medium	Negative
Growth on sodium acetate medium	Negative
Urease activity	Negative
Growth on ammonium ion and glucose as sole sources of nitrogen and carbon	Negative
Growth on arginine as a sole source of nitrogen and carbon	Negative
Growth on ornithine	Negative
Growth on lysine	Negative
G+C content of DNA	34～37 mol%

Table 3. Cleavage of carbohydrate by strain No. 3559.

Carbon source	Acid formation	Gas formation
D-Glucose	+	−
Maltose	±	−
Sucrose	−	−
Salicin	−	−
Sorbitol	−	−
Mannitol	−	−
Lactose	−	−
Inositol	−	−
D-Fructose	±	−
D-Xylose	−	−
L-Arabinose	−	−
Starch	−	−
Glycerol	−	−
D-Galactose	−	−
D-Mannose	−	−

Fig. 1. Extraction and purification of WB-3559.

Broth (150 liters)
↓ Centrifugation
Cells (2 kg)
↓ Me_2CO extract (10 liters)
Extract (aq soln 1 liter)
↓ EtOAc extraction (pH 2)
EtOAc fraction
↓ Silicic acid column chromatography
EtOAc eluate (oil 3 g)
↓ ODS column chromatography
MeOH eluate (WB-3559 A～D mixture 1.2 g)
↓
HPLC

Morphological and physiological characteristics of strain No. 3559 indicate that the strain belongs to the genus *Flavobacterium*. Therefore, it is identified as *Flavobacterium* sp.

Isolation of WB-3559 A, B, C and D

The isolation diagram is shown in Fig. 1. Fermentation broth (150 liters) was centrifuged con-

Table 4. Isolation of WB-3559 A, B, C and D by HPLC.

Apparatus	Model M-6000 A pump U 6K injector (Waters)
Stationary phase	μBondapack C_{18} (Waters)
Mobile phase	MeOH - H_2O - AcOH, 900: 100: 0.1
Flow rate	2 ml/minute
Detector	RI

Table 5. Retention time and recovery of WB-3559 A, B, C and D from 700 mg of mixture.

	A	B	C	D
Retention time (minutes)	10.8	12.2	13.2	15.3
Recovery (mg)	10	110	15	150

tinuously to get bacterial cells (2 kg). The wet bacterial cells were extracted with acetone (10 liters). The extract was concentrated *in vacuo* and the aqueous solution was extracted twice with same volume of ethyl acetate (1 liter) after acidification to pH 2.0 with 6 N HCl. The ethyl acetate layer was evaporated *in vacuo* and the oily matter obtained was applied to silicic acid column chromatography. The column was washed with *n*-hexane and then the active ingredients were eluted with ethyl acetate. The fractions containing the active compounds were combined and concentrated to dryness. The residue was dissolved in a small volume of methanol and then applied to reverse phase ODS column chromatography, eluted with methanol to give a mixture of WB-3559 A, B, C and D which was designated as WB-3559. In order to purify each component, the mixture (WB-3559) was subjected to high performance liquid chromatography under the condition shown in Table 4. The HPLC showed the ratio of WB-3559 A, B, C and D in WB-3559 to be *ca.* 1: 4: 1: 4, even though the isolated ratio was 10: 110: 15: 150 as shown in Table 5.

Physico-chemical Properties of WB-3559 A, B, C and D

Each of the four components (A, B, C and D) of WB-3559 has been isolated as colorless wax. The individual components of WB-3559 showed color reactions similar to each other. Thus, they gave positive reactions to cerium sulfate reagent, but were all negative to ninhydrin, Dragendorff and Molish reactions. There is also no significant difference in their solubilities. The four components are soluble in chloroform, slightly soluble in benzene and hexane, and insoluble in water, although WB-3559 A and B are soluble while WB-3559 C and D sparingly soluble in methanol.

The other physico-chemical properties of WB-3559 A, B, C and D are summarized in Table 6. The chemical structures of these compounds (Fig. 2) will be described in the succeeding paper.[7]

Synthesis of WB-3559 D-syn

For evaluation of fibrinolytic activity in *ex vivo* model, a substantial amount of material was

Fig. 2. Structures of WB-3559 A, B, C and D.

	R_1	R_2
WB-3559 A	$(CH_3)_2CH(CH_2)_{10}$	$(CH_3)_2CH(CH_2)_7CH=CHCH_2CH_2$
WB-3559 B	$(CH_3)_2CH(CH_2)_{11}$	$(CH_3)_2CH(CH_2)_7CH=CHCH_2CH_2$
WB-3559 C	$(CH_3)_2CH(CH_2)_{10}$	$(CH_3)_2CH(CH_2)_{11}$
WB-3559 D	$(CH_3)_2CH(CH_2)_{11}$	$(CH_3)_2CH(CH_2)_{11}$

Table 6. Physico-chemical properties of WB-3559 A, B, C and D.

	WB-3559 A		WB-3559 B		WB-3559 C		WB-3559 D	
MP (°C)	144~145		126~127		154~155		137~138	
Optical rotation $[\alpha]_D^{25}$ (c in $CHCl_3$)	+15.3° (0.3)		+16.7° (0.33)		+15.9° (0.37)		+19.75° (0.8)	
UV λ_{max}^{MeOH} nm (ε)	207 (2,480)		207 (2,160)		206 (1,920)		206 (1,900)	
MW	638		652		640		654	
Anal	Found	Calcd for $C_{36}H_{66}N_2O_7$	Found	Calcd for $C_{37}H_{68}N_2O_7$	Found	Calcd for $C_{36}H_{68}N_2O_7$	Found	Calcd for $C_{37}H_{70}N_2O_7$
C	67.37	67.67	67.95	68.06	67.28	67.46	67.74	67.85
H	10.42	10.41	10.43	10.50	10.48	10.69	10.55	10.77
N	4.14	4.39	4.10	4.29	4.15	4.37	4.19	4.28
Color reaction								
Cerium sulfate	Positive		Positive		Positive		Positive	
Ninhydrin	Negative		Negative		Negative		Negative	
Dragendorff	Negative		Negative		Negative		Negative	
Molish	Negative		Negative		Negative		Negative	
IR $\nu_{max}^{CHCl_3}$ cm^{-1}	3350, 2950, 2860, 1725, 1660, 1525, 1475, 1385, 1370, 1230, 1080, 1065		3350, 2950, 2860, 1730, 1715, 1660, 1525, 1475, 1370, 1220, 1180, 1080		3350, 2950, 2860, 1725, 1660, 1525, 1475, 1392, 1370, 1230, 1080		3350, 2950, 2870, 1725, 1660, 1520, 1475, 1390, 1370, 1230, 1080	
NMR ($CDCl_3$ - CD_3OD, 1: 1) ppm	0.85 (12H, d, J=6 Hz), 1.1~1.5 (30H, m), 1.60 (4H, m), 2.04 (2H, m), 2.36 (4H, t, J=7 Hz), 2.55 (2H, d, J=6 Hz), 3.92 (4H, m), 4.53 (1H, m), 5.20 (1H, m), 5.35 (2H, m)		0.85 (12H, d, J=6 Hz), 1.1~1.5 (32H, m), 1.60 (4H, m), 2.04 (2H, m), 2.36 (4H, m), 2.55 (2H, d, J=6 Hz), 3.92 (4H, m), 4.53 (1H, m), 5.20 (1H, m), 5.35 (2H, m)		0.85 (12H, d, J=6 Hz), 1.1~1.5 (38H, m), 1.60 (4H, m), 2.32 (2H, t, J=7 Hz), 2.54 (2H, d, J=6 Hz), 3.92 (4H, m), 4.53 (1H, m), 5.20 (1H, m)		0.85 (12H, d, J=6 Hz), 1.1~1.5 (40H, m), 1.60 (4H, m), 2.32 (2H, t, J=7 Hz), 2.54 (2H, d, J=6 Hz), 3.92 (4H, m), 4.53 (1H, m), 5.20 (1H, m)	

Fig. 3. Synthetic route of WB-3559 D-syn.

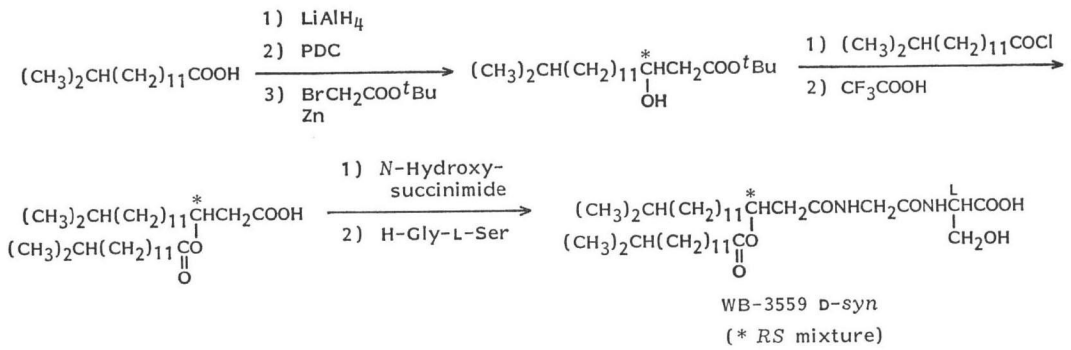

Table 7. Fibrinolytic activity of WB-3559 A, B, C and D measured with euglobulin clot lysis time.

(ED_{50}; μg/ml)

Urokinase (Fujisawa)	A	B	C	D
21 units/ml	100	52	32	40

Table 8. Fibrinolytic activity of intravenously injected synthetic WB-3559 D-syn in mice.

	Euglobulin clot lysis time decreased (%)		
	30 mg/kg	10 mg/kg	3 mg/kg
Expt 1	26	22	11
Expt 2	35	11	24
Expt 3	29	31	9
Expt 4	26*	23	5
x±SE	29±2	22±4	12±4

ddY, N=5~10.
*; $P < 0.05$.

needed. Then, the diastereomeric mixture of WB-3559 D, which was designated as WB-3559 D-syn, was synthesized starting from 13-methyltetradecanoic acid.[7] The synthetic route was shown in Fig. 3 (experimental data not shown). The fibrinolytic activity of the synthetic compound, WB-3559 D-syn, *in vitro* was same as that of the natural WB-3559 D.

Biological Properties of WB-3559 A, B, C, D and WB-3559 D-syn

WB-3559 A, B, C and D showed no antimicrobial activity at the concentration of 100 μg/ml by agar dilution method against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Saccharomyces cerevisiae*. The effect of WB-3559 A, B, C and D on the euglobulin clot lysis time of rabbit plasma was evaluated *in vitro* and ED_{50} is shown in Table 7. WB-3559 C was most potent and its ED_{50} was 32 μg/ml. ED_{50} of urokinase was 21 units/ml. Chemically synthesized WB-3559 D-syn was tested to evaluate the effect in *ex vivo* model using *dd*Y mouse. The result is shown in Table 8. WB-3559 D-syn stimulated euglobulin clot lysis time of mouse plasma in this model in the dose dependent manner. WB-3559 A, B, C and D showed no hemolytic activity at the concentration of 100 μg/ml against SRBC.

Discussion

Fibrinolytic therapy is important to treat the thromboembolic disease. Urokinase and streptokinase have been used for these patients. Anabolic steroid, stanozolol[10] and vasopressin analogue, DDAVP[11] are tested to stimulate the fibrinolytic activity in clinical field, but their efficacy and therapeutic benefit are still under controversy. Recently, a unique compound CP 2129 AL was reported to have fibrinolytic activity not only in animal model but also in human.[12] Low-molecular-weight

fibrinolytic agent is beneficial for the treatment of thromboembolism and might be advantageous for the prophylaxis of thrombosis. A peptidelipid, a strong surfactant named surfactin produced by *Bacillus subtilis* was reported to have plasmin stimulating effect on fibrin plate.[13] Chemical structure and biological properties of surfactin is quite different from those of WB-3559 A, B, C and D. Recently, IWAKI *et al.* reported the discovery of α_2-macroglobulin inhibitors, rishirilides A and B produced by a *Streptomyces*.[14] They might be potential fibrinolytic agents which inhibited the function of α_2-macroglobulin. Mode of action of WB-3559 A, B, C and D is not clear and further investigation will be needed.

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References

1) UMEHARA, K.; K. YOSHIDA, M. OKAMOTO, M. IWAMI, H. TANAKA, M. KOHSAKA & H. IMANAKA: Studies on WF-5239, a new potent platelet aggregation inhibitor. J. Antibiotics 37: 469~474, 1984
2) UMEHARA, K.; K. YOSHIDA, M. OKAMOTO, M. IWAMI, H. TANAKA, M. KOHSAKA & H. IMANAKA: Studies on new antiplatelet agents, WS-30581 A and B. J. Antibiotics 37: 1153~1160, 1984
3) BELL, W. R.: Streptokinase and urokinase in the treatment of pulmonary thromboemboli. Thromb. Haemostasis 35: 57~69, 1976
4) BELL, E. R. & A. G. MEEK: Guidelines for the use of thrombolytic agents. N. Engl. J. Med. 301: 1266~1270, 1979
5) VON KAULLA, K. N.: Synthetic fibrinolytic agents. Induction of fibrinolytic activity *in vitro*. Handb. Exp. Pharmacol. 46: 239~272, 1978
6) NANNINGA, L. B.: The binding of diiodosalicylate and flufenamate to the plasma antiactivator. Analysis of chemical fibrinolysis. Thromb. Haemostasis 41: 357~364, 1979
7) UCHIDA, I.; K. YOSHIDA, Y. KAWAI, S. TAKASE, Y. ITOH, H. TANAKA, M. KOHSAKA & H. IMANAKA: Studies on WB-3559 A, B, C and D, new potent fibrinolytic agents. II. Structure elucidation and synthesis. J. Antibiotics 38: 1476~1486, 1985
8) UCHIDA, I.; K. YOSHIDA, Y. KAWAI, S. TAKASE, Y. ITOH, H. TANAKA, M. KOHSAKA & H. IMANAKA: Structure and synthesis of WB-3559 A, B, C and D, new fibrinolytic agents isolated from *Flavobacterium* sp. Chem. Pharm. Bull. 33: 424~427, 1985
9) NILSSON, I. M.; U. HEDNER & M. PANDOLFI: The measurement of fibrinolytic activities. Handb. Exp. Pharmacol. 46: 107~134, 1978
10) JARRETT, P.; M. MORLAND & N. L. BROWSE: Treatment of Raynaud's phenomenon by fibrinolytic enhancement. Br. Med. J. 19: 523~525, 1978
11) JUHAN-VAGUE, I.; B. CONTE-DEVOLX, M. F. AILLAUD, C. MENDEZ, C. OLIVER & D. COLLEN: Effects of DDAVP and venous occlusion on the release of tissue-type plasminogen activator and Von Willebrand factor in patients with panhypopituitarism. Thromb. Res. 33: 653~659, 1984
12) ROBA, J.; F. DE PAERMENTIER, G. DEFREYN, F. DESSY, P. NIEBES, M. SNYERS & D. COLLEN: CP 2129 AL, a new fibrinolysis-stimulating agent. IXth Int. Congress on Thrombosis and Haemostasis. Abstracts, p. 245, 1983
13) KAKINUMA, A.; G. TAMURA & K. ARIMA: Wetting of fibrin plate and apparent promotion of fibrinolysis by surfactin, a new bacterial peptidelipid surfactant. Experientia 24: 1120~1121, 1968
14) IWAKI, H.; Y. NAKAYAMA, M. TAKAHASHI, S. UETSUKI, M. KIDO & Y. FUKUYAMA: Structures of rishirilides A and B, α_2-macroglobulin inhibitors produced by *Streptomyces rishiriensis* OFR-1056. J. Antibiotics 37: 1091~1093, 1984