PLACETINS, PLATELET AGGREGATION INHIBITORS FROM *STREPTOMYCES* SP. Q-1043

I. FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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Placetins, platelet aggregation inhibitors were obtained from the culture broth of *Streptomyces* sp. Q-1043. These were designated placetins A, A1, B and B1, respectively. Placetins A and B showed strong cytotoxicities against P388, L1210 and HeLa cells.

In the course of a screening program to find new platelet aggregation inhibitors, we discovered from the culture broth of *Streptomyces* sp. Q-1043 inhibitors against platelet aggregation induced with platelet aggregating factor (PAF), adenosine diphosphate (ADP), or collagen. This paper describes the fermentation, isolation and biological properties of these inhibitors.

Fermentation

Microorganism

The strain Q-1043 was classified in the genus *Streptomyces* on the basis of the following characteristics: Aerial mycelia were formed on the BENNETT's agar and yeast extract - malt extract agar (the color of mature sporulated aerial mycelia was in the red-color series). Aerial mycelia used not to be formed on sucrose - nitrate agar, glucose - asparagine agar, glycerol - asparagine agar, inorganic salts - starch agar and oatmeal agar. Mature spore chains were predominantly straight to flexuous (section RF). Spore surface was smooth. Vegetative mycelial pigments ranged from brownish white to brownish black. Melanoid pigments were found. Analysis of whole cell hydrolysate of the strain Q-1043 showed that it contained diaminopimeric acid and glycine.

Fermentation

Spores of the strain of *Streptomyces* sp. Q-1043 were inoculated into 60 ml of seed medium (pH 7.0) in a 500-ml Erlenmeyer flask. This medium consisted of glucose 1.0%, potato starch 2.0%, yeast extract 0.5%, Polypepton 0.5% and $CaCO_3$ 0.4%. The seed was cultured at 27°C for 72 hours on a rotary shaker (200 rpm) and then was transferred into a production vessel containing the medium. The fermentation was carried out at 27°C for 96 hours on a rotary shaker (220 rpm).

Isolation

At harvest, the culture broth (5 liters) was adjusted to pH 7.0 with dilute HCl and centrifuged at 3,000 rpm to remove the mycelial cake. The supernatant was extracted with ethyl acetate (6 liters). The ethyl acetate extract was washed with water and concentrated under reduced pressure. The resultant mass

was diluted to 20 ml with chloroform and applied onto a 300-ml bed of silica (Silica gel 60, E. Merck) equilibrated with chloroform. After elution with 1.5 column volume of the same solvent, the column was eluted with chloroform - methanol (75 : 1). The eluate was monitored by both activity against *Bacillus subtilis* and TLC (Kieselgel 60 F_{254}, E. Merck; chloroform - methanol, 19 : 1). Fractions of each 20 g were collected. Fractions (fraction No. 27 ~ 39) were combined and evaporated to dryness under reduced pressure to give a red-brownish mass, 220 mg. This consisted mainly of placetins A and A1. The fractions (fraction No. 40 ~ 54), containing mainly placetins B and B1, were combined and evaporated to dryness under reduced pressure to give a reddish mass, 900 mg. The combined fraction containing placetins A and A1 was again chromatographed on preparative thin layer plates (Kieselgel 60 F_{254}, E. Merck) using ethyl acetate as a development solvent. Fractions containing placetins A and A1 were collected separately and extracted with ethyl acetate and each extract was concentrated to dryness under reduced pressure to give orange color crystals of pure placetin A, 13 mg and reddish color crystals of placetin A1, 23 mg. Similarly the fractions containing placetins B and B1 were chromatographed on preparative thin layer plates (Kieselgel 60 F_{254}, E. Merck) using ethyl acetate as a development solvent. Fractions containing placetins B and B1 were collected separately and each fraction was extracted with ethyl acetate and the extract was

Fig. 1. UV spectrum of placetin A.

—— MeOH, —·— 0.1 N NaOH - MeOH, --- 0.1 N HCl - MeOH.

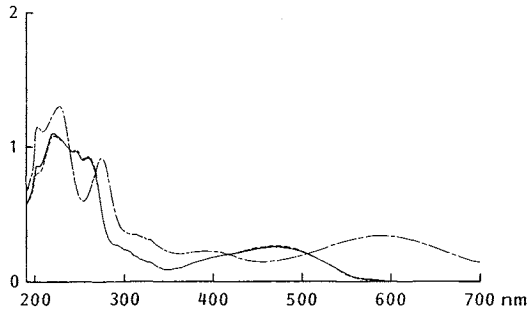

Fig. 2. UV spectrum of placetin A1.

—— MeOH, —·— 0.1 N NaOH - MeOH, --- 0.1 N HCl - MeOH.

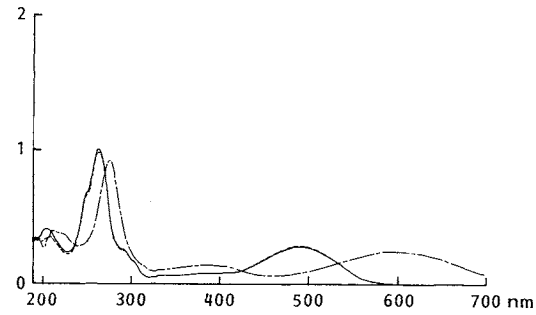

Fig. 3. IR spectrum of placetin A.

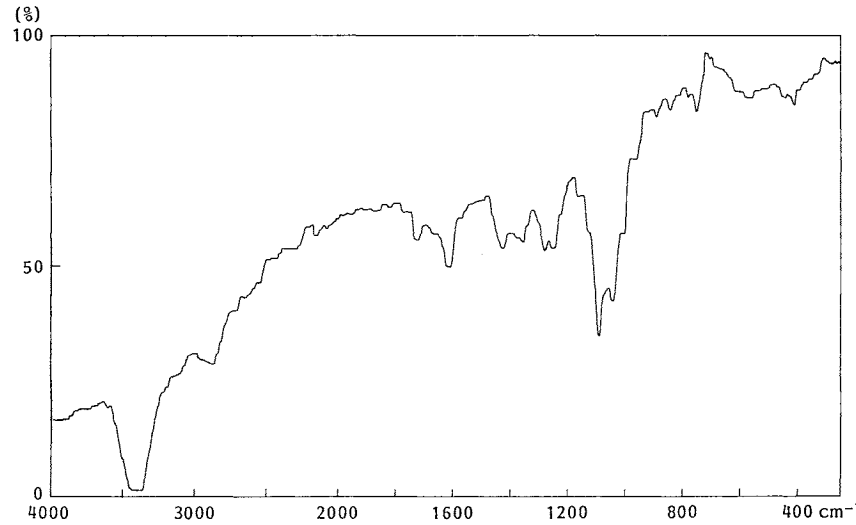

Fig. 4. IR spectrum of placetin A1.

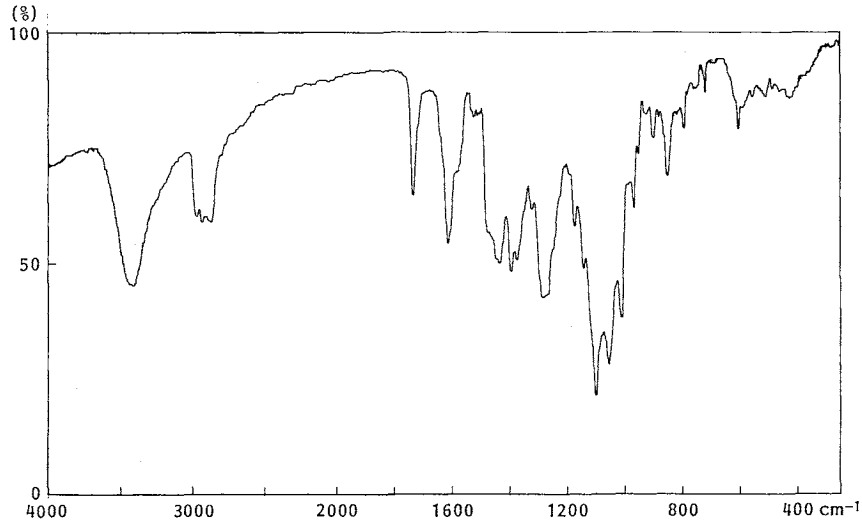

Table 1. Physico-chemical properties of placetins.

	A	A1	B	B1
Nature	Orange	Reddish	Orange	Reddish
MP (°C)	245~250	206~208	261~263	252~256
$[\alpha]_D^{21}$ (c 0.5, $CHCl_3$)	−22°	+6°	−12°	+42°
Anal				
Calcd for	$C_{49}H_{60}O_{19}\cdot H_2O$:	$C_{49}H_{58}O_{18}\cdot 1\frac{1}{2}H_2O$:	$C_{55}H_{70}O_{21}\cdot H_2O$:	$C_{55}H_{68}O_{20}\cdot 4H_2O$:
	C 60.61	C 61.17	C 60.87	C 58.91
	H 6.44	H 6.39	H 6.09	H 6.83
Found:	C 60.33	C 60.98	C 60.58	C 58.88
	H 6.09	H 6.11	H 6.16	H 6.52
MS (M^-) m/z	952	934	1,066	1,048
Rf value[a]				
Solvent 1	0.34	0.48	0.24	0.34
Solvent 2	0.38	0.51	0.30	0.41

[a] Kieselgel 60 F_{254}, (E. Merck). Solvent 1: benzene-EtOAc-MeOH (1:1:0.1), solvent 2: EtOAc.

concentrated to dryness under reduced pressure to give orange color crystals of pure placetin B, 120 mg and reddish color crystals of placetin B1, 86 mg.

Physico-chemical Properties

Placetins A and B are easily soluble in chloroform, methanol, *N,N*-dimethylformamide and dimethyl sulfoxide, but practically insoluble in water and hexane. Placetins A1 and B1 are soluble in *N,N*-dimethylformamide and dimethyl sulfoxide, slightly soluble in methanol and chloroform, but almost insoluble in water and hexane. UV spectrum of placetin A in methanol is identical to that of placetin B. Similarly the UV spectra of placetins A1 and B1 are the same. Placetin A exhibits UV absorptions at 220, 260, 310, 420, 445 and 470 nm as shown in Fig. 1. While placetin A1 exhibits UV absorption at 250, 262, 290, 310 and 490 nm in methanol as shown in Fig. 2. The IR spectra of placetins A and A1 are shown in Figs. 3 and 4, respectively. A definite difference between placetins A and A1 is an absorption at 1675 cm^{-1} present in IR spectrum of the former. The physico-chemical properties of placetins A, A1, B and B1 are summarized in Table 1.

Biological Properties

Antimicrobial Activity

The MICs of placetins A and B were determined against various bacteria by the 2-fold dilution method. As shown in Table 2, placetins A and B exhibited weak inhibitory activities against Gram-positive bacteria, but placetins A1 and B1 did not exhibit antimicrobial activity against *B. subtilis* ATCC 6633 at a concentration of 1,000 μg/ml.

Cytotoxicities

1) Placetins were tested for *in vitro* cytotoxicity against HeLa cells. HeLa cells (1×10^5 cells/ml) in the EAGLE's minimum essential medium (MEM) containing 10% calf serum and the test sample solutions were planted into the wells of 96-well microtiter plates and incubated at 37°C for 72 hours with 5% CO_2 under high humidity. The cytotoxicity of the test samples was determined by counting the viable cells. The cytotoxity is shown in Table 3.

2) Placetins A and B were furthermore tested for *in vitro* cytotoxicities against L1210 and P388. The cytotoxicities of placetins A and B compared to that of mitomycin C in Table 4.

Inhibitory Activities on Platelet Aggregation

Nine volumes of blood were drawn from the central ear artery of male rabbit (Japan white, 3 kg) directly into plastic syringe containing 1 volume of 3.8% sodium citrate. The blood was centrifuged at $270 \times g$ for 10 minutes at room temperature and the platelet rich plasma (PRP) was removed. The pellet was further centrifuged at $1{,}100 \times g$ for 15 minutes. The supernatant was used as platelet poor plasma (PPP). The platelet concentration was adjusted to 5×10^5 cells/μl with PPP. Platelet aggregation was measured by the method[1] of BORN and CROSS using a HEMA TRACER (Nikon Bio Science, Japan). The extent of platelet aggregation was determined by the maximum change of light transmission, assigning the transmission of unstimulated PRP to be 0% and that of PPP to be as 100%. The inhibitory activity of placetins on a rabbit platelet aggregation induced by various aggregating agents (ADP, platelet

Table 2. Antimicrobial activities of placetins A and B.

Organism	MIC (μg/ml)		Organism	MIC (μg/ml)	
	A	B		A	B
Staphylococcus aureus FDA 209P JC-1	25	12.5	*Corynebacterium xerosis* CAY 76-1	12.5	12.5
S. aureus Smith	100	25	*Micrococcus luteus* ATCC 9341	12.5	12.5
S. aureus Terashima	>100	>100	*Bacillus subtilis* ATCC 6633	25	12.5
S. epidermidis IID 866	100	100	*Escherichia coli* NIHJ	>100	>100
Streptococcus pyogenes Cook	25	12.5	*Klebsiella pneumoniae* ATCC 10031	>100	>100
S. faecalis IID 682	50	25	*Pseudomonas aeruginosa* NCTC 10490	>100	>100

Table 3. Cytotoxicity of placetins against HeLa cells.

	IC_{50} (μg/ml)
A	0.05
A1	10
B	0.05
B1	5

Table 4. Cytotoxicities of placetins A and B.

	IC_{50} (μg/ml)	
	L1210	P388
A	0.064	0.031
B	0.066	0.021
Mitomycin C	0.066	0.011

aggregating factor (PAF) or collagen) is shown in Table 5.

Placetins A1 and B1 did not show an inhibitory activity on aggregation induced by the above agents at a concentration of 30 μg/ml.

Table 5. Effects of placetins on platelet aggregation.

P.A.A.	IC_{50} (μg/ml)	
	A	B
PAF (10 nM)	6.4	6.7
ADP (3 μM)	>10	>10
Collagen (10 μM)	9.7	9.4

P.A.A.: Platelet aggregating agents.

Discussion

Streptomyces sp. Q-1043 produced new biological active antibiotics, placetins A, A1, B and B1. Placetins A and B are active against Gram-positive bacteria and show an inhibitory activity toward platelet aggregation induced by various platelet aggregating agents.

Furthermore placetins A and B exhibit strong cytotoxicities against P388, L1210 and HeLa. The physico-chemical properties of placetins show some similarities to those of kerriamycin[2], urdamycin[3], aquayamycin[4] and OM-4842[5]. However their structures are different from these known antibiotics.

Acknowledgments

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References

1) Born, G. V. R. & M. J. Cross: The aggregation of blood platelets. J. Physiol. 168: 178~195, 1963
2) Hayakawa, Y.; K. Adachi, T. Iwakiri, K. Imamura, K. Furihata, H. Seto & N. Ōtake: Kerriamycins A, B and C, new isotetracenone antibiotics. Agric. Biol. Chem. 51: 1397~1405, 1987
3) Drautz, H.; H. Zähner, J. Rohr & A. Zeeck: Metabolic products of microorganisms. 234. Urdamycins, new angucycline antibiotics from *Streptomyces fradiae*. I. Isolation, characterization and biological properties. J. Antibiotics 39: 1657~1669, 1986
4) Sezaki, M.; S. Kondo, K. Maeda, H. Umezawa & M. Ohno: The structure of aquayamycin. Tetrahedron 26: 5171~5190, 1970
5) Ōmura, S.; A. Nakagawa, N. Fukamachi, S. Miura, Y. Takahashi, K. Komiyama & B. Kobayashi: OM-4842, a new platelet aggregation inhibitor from *Streptomyces*. J. Antibiotics 41: 812~813, 1988