FR-900452[†], A SPECIFIC ANTAGONIST OF PLATELET ACTIVATING FACTOR (PAF) PRODUCED BY *STREPTOMYCES PHAEOFACIENS*

I. TAXONOMY, FERMENTATION, ISOLATION, AND PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS

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A PAF antagonist, designated as FR-900452, was isolated from fermentation products of *Streptomyces phaeofaciens* and the molecular formula was determined as $C_{22}H_{25}N_3O_3S$. The compound inhibited PAF-induced rabbit platelet aggregation with an IC_{50} of 3.7×10^{-7} M, but was much less active against collagen-, arachidonic acid- or ADP-induced aggregation (IC_{50}; 6.4×10^{-5}, $>10^{-4}$ or $>10^{-4}$ M, respectively).

PAF (1-*O*-alkyl-2-acetyl-*sn*-3-phosphorylcholine) is a putative mediator of asthma and inflammatory diseases[1]. The substance is released by a variety of immune and non-immune stimuli from macrophages[2], basophils[3], neutrophils[4] and platelets[5].

In our screening program for potential PAF inhibitors, we have tested a wide range of fermented broths for inhibitory effects on PAF-induced rabbit platelet aggregation. As a result, FR-900452 was isolated from the fermentation products of *Streptomyces phaeofaciens* No. 7739.

In this paper, we describe characterization of the producing strain and the production, isolation, and physico-chemical properties of FR-900452. The inhibitory effect against rabbit platelet aggregation *in vitro* is also described.

Taxonomy on Producing Strain

Strain No. 7739 was isolated from a soil sample obtained from Matsue-shi, Shimane Prefecture, Japan.

The aerial mycelia of the organism grown on yeast extract - malt extract agar, oatmeal agar, or inorganic salts - starch agar (incubated for 14 days at 30°C) were examined directly under the microscope, and spore surfaces were observed with electron microscope.

The mature spores developed in chains of more than 30 spores forming *Spirales* (Fig. 1). The spores were cylindrical or oval and $0.6 \sim 0.7 \times 0.7 \sim 0.9$ μm in size. Spore surfaces were smooth (Fig. 2).

For cultural and physiological characterizations, the ISP media recommended by WAKSMAN[6] and endorsed by the ISP[7] were used. Cultures were incubated for 14 days at 30°C. The color names used in this study were based on Color Standard (Nihon Shikisai Co., Ltd.). The ability of carbohy-

† FR-900452 is identical with WS7739B (NISHIKAWA, M. *et al.*: Japan Kokai 85-145,092, May 15, 1985).

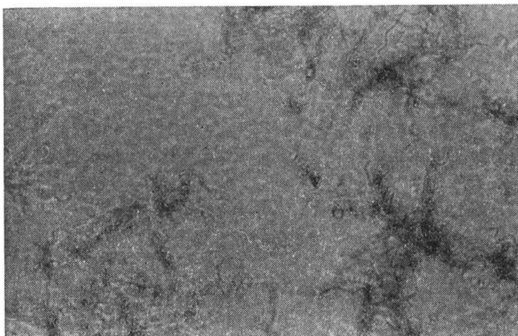

Fig. 1. Aerial mycelium of strain No. 7739 on yeast extract - malt extract agar (incubated for 14 days at 30°C).
The organism was observed with an optical microscope (×800).

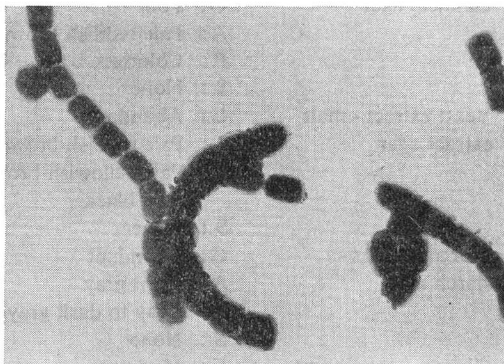

Fig. 2. Electron micrograph of spore chain of strain No. 7739 on yeast extract - malt extract agar, 10 days culture at 30°C.
Bar represents 1 μm.

drate utilization was determined by the method of PRIDHAM and GOTTLIEB[8]. Growth-permissible temperature and optimum growth temperature were determined on yeast extract - malt extract agar using a model TN-3 temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd.).

Colonies belonged to gray color series grown on oatmeal agar, yeast extract - malt extract agar or inorganic salts - starch agar. No soluble pigment was produced. Results were summarized in Table 1.

Growth-permissible temperature was from 7°C to 38°C with optimum growth temperature at around 30°C. Starch hydrolysis, melanin production, gelatin liquefaction were positive (Table 2).

As shown in Table 3, almost all carbohydrates were utilized except cellulose and chitin after 14 days incubation at 30°C.

Whole-cell was analyzed according to the method of BECKER *et al.*[9]. Strain No. 7739 contained LL-diaminopimelic acid in the whole-cell hydrolysate.

From the morphological characteristics and whole-cell analysis, strain No. 7739 is classified in the genus *Streptomyces* Waksman and Henrici 1943. Therefore, the strain was compared with the published descriptions[10~13] of various *Streptomyces* species. From the results of comparison, strain No. 7739 was considered to resemble *S. phaeofaciens* Maeda *et al.* 1952[11,13] and *S. olivochromogenes* (Waksman 1923) Waksman and Henrici 1948[12,13]. Strain No. 7739 was differentiated from those two species in the morphological and physiological characteristics.

Streptomyces olivochromogenes IFO 3292

Cultural characteristics of strain No. 7739 is different from *S. olivochromogenes* on glucose - asparagine agar, glycerol - asparagine agar or potato - dextrose agar. Milk coagulation and peptonization of strain No. 7739 are negative, but those of *S. olivochromogenes* are positive. Strain No. 7739 can not grow on yeast extract - malt extract agar supplemented with 10% NaCl, but *S. olivochromogenes* can grow on it. Strain No. 7739 can utilize sucrose, D-xylose, D-fructose, rhamnose, inositol, D-mannitol, inulin, salicin, sodium citrate and sodium acetate, but *S. olivochromogenes* can not utilize those carbohydrates.

Table 1. Cultural characteristics of strain No. 7739, *Streptomyces olivochromogenes* IFO 3292 and *Streptomyces phaeofaciens* IFO 13372.

Medium		No. 7739	IFO 3292	IFO 13372
Oatmeal agar	G:	Poor	Poor	Poor
	A:	Pale reddish brown	Grayish white	Light gray
	R:	Colorless	Pale yellow	Colorless
	S:	None	None	None
Yeast extract - malt extract agar	G:	Abundant	Moderate	Moderate
	A:	Pale reddish brown	Grayish white	Pale reddish brown
	R:	Pale yellowish brown to black	Light brown	Pale yellowish brown
	S:	None	None	None
Inorganic salts - starch agar	G:	Abundant	Abundant	Abundant
	A:	Light gray	Grayish white	Pale reddish brown
	R:	Gray to dark gray	Pale yellow orange	Pale yellow
	S:	None	None	None
Glucose - asparagine agar	G:	Moderate	Abundant	Moderate
	A:	Gray	Light gray	Pale reddish brown
	R:	Pale yellow	Dark brown	Pale yellow
	S:	None	None	None
Glycerol - asparagine agar	G:	Abundant	Abundant	Abundant
	A:	Grayish yellow brown	Grayish white	Pale reddish brown
	R:	Pale yellow	Light brown	Pale yellow
	S:	None	None	None
Sucrose - nitrate agar	G:	Poor	Poor	Poor
	A:	Grayish white	None	Light gray
	R:	Colorless	Colorless	Colorless
	S:	None	None	None
Nutrient agar	G:	Moderate	Moderate	Poor
	A:	Light gray, scant	None	None
	R:	Pale yellow	Pale yellow	Colorless
	S:	None	None	None
Potato - dextrose agar	G:	Poor	Moderate	Poor
	A:	None	Grayish white	None
	R:	Colorless	Pale brown	Pale yellow orange
	S:	None	None	None
Tyrosine agar	G:	Abundant	Moderate	Abundant
	A:	Pale yellow orange to gray	Light gray	Grayish white
	R:	Dark brown	Yellowish	Brown
	S:	Dark brown	Brown	Brown
Peptone - yeast extract - iron agar	G:	Poor	Moderate	Moderate
	A:	None	None	None
	R:	Pale yellow	Pale yellow	Colorless
	S:	Brown	Brown	Dark brown

Abbreviation: G; Growth, A; aerial mass color, R; reverse side color, S; soluble pigment.

Streptomyces phaeofaciens IFO 13372

Cultural characteristics of strain No. 7739 are similar to those of *S. phaeofaciens*. Nitrate reduction and milk peptonization of strain No. 7739 are negative, but those of *S. phaeofaciens* are positive. Starch hydrolysis and gelatin liquefaction of the strain are positive, but those of *S. phaeofaciens* are negative. Strain No. 7739 can utilize sucrose, raffinose, inulin or sodium acetate, but *S. phaeofaciens* can not utilize those carbohydrates. Results were shown in Tables 1, 2 and 3.

Table 2. Physiological properties of strain No. 7739, *Streptomyces olivochromogenes* IFO 3292 and *Streptomyces phaeofaciens* IFO 13372.

	No. 7739	IFO 3292	IFO 13372
Temperature range for growth	7~38°C	16~36°C	11~36°C
Optimum temperature	27~32°C	27°C	25~27°C
Nitrate reduction	−	−	+
Starch hydrolysis	+	+	−
Milk coagulation	−	+	−
Milk peptonization	−	+	+
Melanin production	+	+	+
Gelatin liquefaction	+	+	−
H_2S production	−	−	−
Urease activity	+	+	+
NaCl tolerance (%)	<7%	<10%	<7%

−; Negative, +; positive.

Table 3. Carbon utilization of strain No. 7739, *Streptomyces olivochromogenes* IFO 3292 and *Streptomyces phaeofaciens* IFO 13372.

	No. 7739	IFO 3292	IFO 13372		No. 7739	IFO 3292	IFO 13372
D-Glucose	+	+	+	D-Mannose	+	+	+
Sucrose	+	−	−	D-Trehalose	+	+	+
Glycerol	+	+	+	Inositol	+	−	+
D-Xylose	+	−	±	D-Mannitol	+	−	+
D-Fructose	+	−	+	Inulin	+	−	−
Lactose	+	+	+	Cellulose	−	−	−
Maltose	+	+	+	Salicin	+	−	+
Rhamnose	+	−	+	Chitin	−	−	−
Raffinose	+	+	−	Sodium citrate	+	−	+
D-Galactose	+	+	+	Sodium succinate	+	+	+
L-Arabinose	+	±	+	Sodium acetate	+	−	−

Symbols: +; Utilization, ±; doubtful utilization, −; no utilization.

As the results of direct comparison mentioned above, cultural characteristics of the strain are in good agreement with those of *S. phaeofaciens*. However, physiological characteristics and carbohydrate utilization of strain No. 7739 are different from those of *S. phaeofaciens* in the several points. Whereas these differences do not seem to us sufficient to distinguish strain No. 7739 from *S. phaeofaciens*. Therefore, strain No. 7739 is considered a new subspecies of *S. phaeofaciens* and the strain has been designated as *Streptomyces phaeofaciens* subsp. *matsuensis* subsp. nov., referring to the soil obtained in Matsue-shi from which the organism was isolated.

Type Strain: Strain No. 7739

A culture of this strain has been deposited at the Fermentation Reseach Institute, Agency of Industrial Science and Technology, Japan, as FERM-BP-660. The descriptions of the new subspecies are the same as that given above.

Fermentation

A loopful of *S. phaeofaciens* No. 7739 on mature slant culture was inoculated to each of twenty-five 250-ml Erlenmeyer flasks containing 80 ml of sterile seed medium shown in Table 4. The flasks were shaken on a rotary shaker (220 rpm, 5.1-cm throw) for 72 hours at 30°C. The resultant seed

culture was inoculated to 160 liters of sterile fermentation medium shown in Table 4, in 200-liter stainless steel fermentor. The fermentation was carried out at 30°C for 96 hours under aeration of 160 liters/minute and agitation of 220 rpm.

Table 4. Media used for production of FR-900452.

Seed medium (%)		Production medium	
Corn starch	1.0	Glycerol	1.0%
Glycerol	1.0	Gluten meal	0.7%
Glucose	0.5	$(NH_4)_2SO_4$	0.1%
Molatein	0.5	$MgSO_4 \cdot 7H_2O$	0.05%
Corn steep liquor	0.5	$CaCO_3$	0.2%
Pharma media	1.0	$CoCl_2 \cdot 6H_2O$	4 μg/ml
		NaI	0.5 μg/ml
pH adjusted to 6.5 with 6 N NaOH then added			
$CaCO_3$	0.2		

Isolation

The cultured broth was filtered with aid of diatomaceous earth. The filtrate (160 liters) was adjusted to pH 7.0 and then extracted with 160 liters of ethyl acetate. The extract was concentrated *in vacuo*. The resultant material was applied to a silica gel column chromatography (1.5 liters) and the column was eluted with a mixture of *n*-hexane - acetone (1: 1). The fractions containing active compound were applied to pre-packed column (Li chroprep Si 60 size B, Merck) and eluted with a mixture of chloroform - methanol (100: 1). The active fractions were rechromatographed using the pre-packed column with the same condition. The active fractions were concentrated *in vacuo* to give 230 mg of pure powder.

Physico-chemical Properties

FR-900452 was obtained as a pale yellow powder which decomposes at 112～120°C. It is soluble in methanol, acetone and ethyl acetate and insoluble in water. The specific rotation is $[\alpha]_D^{23}$ +97.0° (*c* 0.5, $CHCl_3$). Color reactions are as follows: Positive to Dragendorff, Ehrlich and ceric sulfate, negative to ninhydrin. Thin-layer chromatography was carried out on a silica gel sheet (Merck chromatogram sheet 60 F254) using the solvent systems of chloroform - methanol (50: 1) and *n*-hexane - acetone (1: 1). The Rf values of FR-900452 were 0.4 and 0.3, respectively. The molecular formula was determined as $C_{22}H_{25}N_3O_3S$ by HR-MS (obsd 411.1567; calcd for $C_{22}H_{25}N_3O_3S$ 411.1618). The UV and IR absorption spectral data of FR-900452 were summarized in Table 5. The structure of FR-900452 is currently under study, and the results will be published in due course.

Table 5. UV and IR absorption spectra of FR-900452.

UV (MeOH) nm (ε):	246 (13,600), 347 (14,500)
IR ($CHCl_3$) cm^{-1}:	3350, 3000, 2900, 1670, 1610, 1595, 1490, 1470, 1445, 1380, 1350, 1340, 1310, 1297, 1220

Biological Properties

The active compound present in the fermentation broth or in preparations obtained during the purification process was detected by its inhibitory activity against rabbit platelet aggregation induced by PAF. The methods were reported previously[14]. Briefly, the platelet count in the incubation mixture used for aggregation studies was 4.0×10^5 platelets/mm^3. Aggregometry was performed using NKK Hema tracer (Niko Bioscience Inc.) at 37°C, with the following platelet aggregating agents: PAF, collagen (Tokyo Kasei), arachidonic acid (Sigma) and ADP (Boehringer Mannheim). For drug studies, 0.3 ml of platelet suspension was incubated with drugs or vehicles for 2 minutes before the addition of aggregating agent at a concentration which gave maximum aggregation (PAF, 0.1 μM; collagen, 2.5 μg/ml; arachidonic acid, 100 μM; ADP, 2.5 μM). FR-900452 was prepared in ethanol. The small amount of ethanol (final concentration of 0.05～0.1%) employed as a vehicle had no effect

Table 6. Inhibition of rabbit platelet aggregation by FR-900452 and tiaramide.

Inducer	IC_{50} value (M)	
	FR-900452	Tiaramide
PAF	3.7×10^{-7}	7.9×10^{-5}
Collagen	6.4×10^{-5}	3.3×10^{-6}
Arachidonic acid	$>10^{-4}$	3.7×10^{-5}
ADP	$>10^{-4}$	3.3×10^{-5}

Each drug was added 2 minutes before an aggregating agent, PAF (0.1 μM), collagen (2.5 μg/ml), arachidonic acid (100 μM) or ADP (2.5 μM). Results (n=4) are presented as the concentration of each drug inhibiting maximal aggregation by 50%.

on platelet aggregation. Tiaramide (Fujisawa) was dissolved in saline. PAF was synthesized by chemists of Research Laboratories of Fujisawa Pharmaceutical Co., Ltd.[15].

FR-900452 inhibited PAF-induced platelet aggregation with an IC_{50} of 3.7×10^{-7} M, and slightly (6.4×10^{-5} M) inhibited collagen-induced aggregation, but had no effect (10^{-4} M) on the aggregation induced by arachidonic acid and ADP (Table 6). Tiaramide was also examined for comparison. This drug inhibited not only PAF-induced aggregation (IC_{50}; 7.9×10^{-5} M) but also collagen-, arachidonic acid- and ADP-induced aggregation (IC_{50}; 3.3×10^{-6}, 3.7×10^{-5} and 3.3×10^{-5} M, respectively).

Discussion

Table 6 shows, FR-900452 in IC_{50} of 3.7×10^{-7} M inhibited PAF-induced platelet aggregation and it also inhibited collagen-induced aggregation, but the IC_{50} is 6.4×10^{-5} M, which is much higher than that of PAF-induced aggregation. Whereas, it showed no effect against arachidonic acid- and ADP-induced aggregation up to 10^{-4} M. The experimental results presented here that FR-900452 is a specific inhibitor of PAF. We presume that PAF might play some role in collagen-induced aggregation. Tiaramide also inhibited PAF-induced aggregation but the effect was not PAF specific. Tiaramide is a non-steroidal and basic anti-inflammatory drug. Its anti-anaphylactic action has also been reported[16]. However, whether the anti-anaphylactic effect of this drug depends on its PAF inhibition is still unclear.

PAF is produced by a range of inflammatory cells. This material has been considered as a potential mediator of both allergic[17,18] and non-allergic[20] inflammation. The availability of FR-900452, selective PAF antagonist will allow a critical testing to this association, and it seems to be promising as a new drug in these area, though further examination of anti-PAF activity in animal model is needed.

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