FK409, A NOVEL VASODILATOR ISOLATED FROM THE ACID-TREATED FERMENTATION BROTH OF *STREPTOMYCES GRISEOSPOREUS*

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

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FK409, a novel vasodilator with anti-platelet aggregation activity, has been isolated from the acid-treated fermentation broth of *Streptomyces griseosporeus* No. 16917, which was cultured on a medium containing $NaNO_3$ for 4 days. FK409 was purified from the culture-filtrate by extraction with ethyl acetate after adjusting the pH to 3.0 with HCl, followed by silica gel chromatography. The molecular formula of this compound was determined to be $C_8H_{13}N_3O_4$.

In vitro, FK409 showed a potent relaxation activity on noradrenaline induced contraction of rat aorta. In addition to the vasodilating activity, this compound also showed potent anti-aggregation activities towards rabbit platelets. *In vivo*, intravenously administered FK409 resulted in marked blood pressure lowering in rats.

In our screening program for new vasodilators, we have tested a wide range of fermentation broths for relaxation effects of the rat aorta using a superfusion technique. We have reported the discovery of the vasodilators of microbial origin, WS-1228 B[1], amauromine[2] and vinigrol[3].

In our recent screening program, we had been searching for new types of vasodilator with anti-platelet aggregation activity. As a result, FK409 was discovered from the acid-treated fermentation broth of *Streptomyces griseosporeus* No. 16917.

In this paper, we describe the taxonomic studies on the producing strain, fermentation, isolation, and physico-chemical properties of FK409. The vasodilating and anti-platelet aggregation activities of FK409 are also described.

Materials and Methods

Fermentation

A seed medium (160 ml) containing corn starch 1%, glycerol 1%, glucose 0.5%, cotton seed meal 1%, dried yeast 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% at pH 6.5 was poured into each of ten 500-ml Erlenmeyer flasks and sterilized at 121°C for 30 minutes. A loopful of slant culture of *S. griseosporeus* No. 16917 was inoculated to each of the flask contents and cultured at 30°C for 3 days. The resultant culture was inoculated to the same seed medium (80 liters) in a 200-liter jar fermenter and this was cultured at 30°C for 40 hours. Thirty two liters of the seed culture was inoculated to the production medium (1,600 liters) containing glycerol 1%, glucose 1%, cotton seed meal 0.1%, $NaNO_3$ 0.5%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.1%, $CoCl_2 \cdot 6H_2O$ 0.0004%, $CaCO_3$ 0.2% and Adekanol (Asahi Denka Co.) 0.1% at pH 7.0 in a 2,000-liter stainless steel fermenter and this was cultured at 34°C for 4 days.

Bioassay of FK409 in the Fermentation Broth

The filtrate was extracted with ethyl acetate after adjusting the pH to 3.0 with 6 N HCl and then the amount of FK409 was quantified by its relaxation activity on noradrenaline-induced contraction of rat aorta and inhibition activity on rabbit platelet aggregation induced by thrombin.

Vasodilating Activity

The vasodilating activity of FK409 was measured by the superfusion technique reported previously[1]. Briefly, male Sprague-Dawley rats of 8~10 weeks age were killed by a blow on the head. The thoracic aorta was quickly removed and spiral strips of 2 mm width and 50 mm length of the aorta were prepared. The tissues were superfused with Tyrode solution containing noradrenaline (0.03 μg/ml/minute), which increased the tension of the tissues by about 500 mg. Changes in the tension of the tissues were measured isometrically by means of force displacement transducers coupled to a Biophysiograph 180 system (SAN-EI SOKKI Co., Ltd.). For drug studies, 5~20 μl of drugs or vehicles solution were injected into the tissue. Considerable variations in individual data were observed between different preparations, but a fairly constant value was obtained for a preparation.

Anti-platelet Aggregation Activity

Platelet aggregation was measured turbidimetrically using a NKK Hema tracer (Niko Bioscience Inc.) according to the method reported previously[4]. Briefly, blood was collected through a polyethylene catheter from the carotid artery of a male Japanese white rabbit (2.5~3.0 kg body weight). After obtaining platelet rich plasma (PRP) by centrifugation, the platelet number was adjusted to 4.0×10^5 cells/mm^3 with platelet poor plasma. Platelet aggregating agents employed in this experiment were: Thrombin (Sigma) 0.3 U/ml, collagen (Tokyo Kasei Co.) 2.5 μg/ml, arachidonic acid (Sigma) 100 μM, ADP (Boehringer Mannheim) 2.5 μM and platelet activating factor (PAF) 0.1 μM, respectively. For drug studies, 0.25 ml of platelet suspension was incubated with 0.02 ml of the drug or vehicle solution for 2 minutes before the addition of 0.03 ml of the aggregating agent solution.

Hypotensive Activity in Experimental Animal

An 8 week-old Sprague-Dawley rat was anesthetized with urethane (700 mg/kg, ip). Mean arterial blood pressure was recorded from femoral artery using a pressure transducer coupled to a Biophysiograph 180 system. The femoral vein was cannulated for the intravenous injection of test compounds. The test compound was dissolved in saline and injected in a volume of 0.2 ml.

Results

Taxonomy of the Producing Strain

Strain No. 16917 was isolated from a soil sample obtained from Kumamoto-city, Kumamoto Prefecture. Morphological observations were made with light and electron microscopy on cultures grown at 30°C for 14 days on yeast extract - malt extract agar, oatmeal agar and inorganic salts - starch agar. The mature spores occurred in chains of more than 20 spores forming *Spira*. The spores were cylindrical or oval and 0.5~0.8 × 0.9~1.3 μm in size, spore surfaces were smooth.

Cultural characteristics were observed on ten kinds of media described by SHIRLING and GOTTLIEB[5], and WAKSMAN[6]. Colonies belonged to the gray color series when grown on oatmeal agar, yeast extract - malt extract agar and inorganic salts - starch agar. No soluble pigment was produced.

Analysis of whole cell hydrolysates of strain No. 16917 showed that it contained LL-diaminopimelic acid. Accordingly, the cell wall of this strain is believed to be of Type I.

Physiological properties of strain No. 16917 were as follows. Growth-permissible temperature was from 12 to 42°C with optimum growth temperature from 30 to 35°C. Starch hydrolysis, nitrate reduction, melanine production, H_2S production and milk peptonization were positive.

Utilization of carbon sources was examined according to the methods of PRIDHAM and GOTTLIEB[7]. The results were determined after 14 days incubation at 30°C. Almost all carbon sources were utilized except chitin, cellulose and sodium acetate.

Microscopic studies and cell wall composition analysis of strain No. 16917 indicate that this strain belongs to the genus *Streptomyces*. Accordingly, a comparison of this strain was made with the published description[8~11] of various *Streptomyces* species. As the results of comparison, strain No. 16917 is identified as a strain of *S. griseosporeus*. A culture of this strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM P-7321.

Fermentation and Isolation of FK409

FK409 production reached a maximum after 4 days of cultivation and the yield was 4 μg/ml. When $NaNO_3$ was omitted from the production medium, FK409 was not detected in the acid-treated fermentation broth of *S. griseosporeus* No. 16917.

The flow diagram of the isolation procedure described below is shown in Fig. 1. The cultured broth was filtered with the aid of diatomaceous earth (25 kg). The filtrate obtained (1,600 liters) was concentrated to a volume of 160 liters, and then the solution was adjusted to pH 3.0 with 6 N HCl. After 15 minutes, the solution was adjusted to pH 7.0 with 6 N NaOH and extracted twice with 100 liters of ethyl acetate, and the extracts were concentrated under reduced pressure. The resultant oily materials were applied to a silica gel column chromatography (3 liters) and the column was developed with a

Fig. 1. Isolation procedure of FK409.

Fermentation broth (1,600 liters)
↓
Filtrate
↓ concd to 160 liters
adjusted to pH 3.0 with 6 N HCl
stirred for 15 minutes at room temp
adjusted to pH 7.0 with 6 N NaOH
Extraction with EtOAc (100 liters × 2)
↓ concd to dryness
Silica gel column (3 liters)
↓ eluted with *n*-hexane - EtOAc (1 : 4)
concd
Silica gel column (1.5 liters)
↓ eluted with $CHCl_3$ - MeOH (25 : 1)
concd
crystallized from MeOH
FK409 (1.4 g) as colorless prisms

Fig. 2. IR spectrum of FK409 (Nujol).

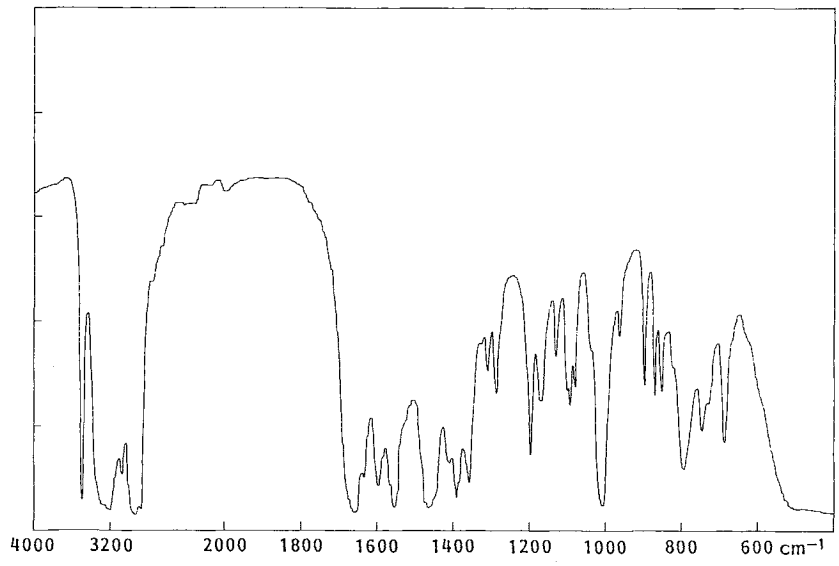


Fig. 3. 60 MHz ^1H NMR spectrum of FK409 in CD_3COCD_3.

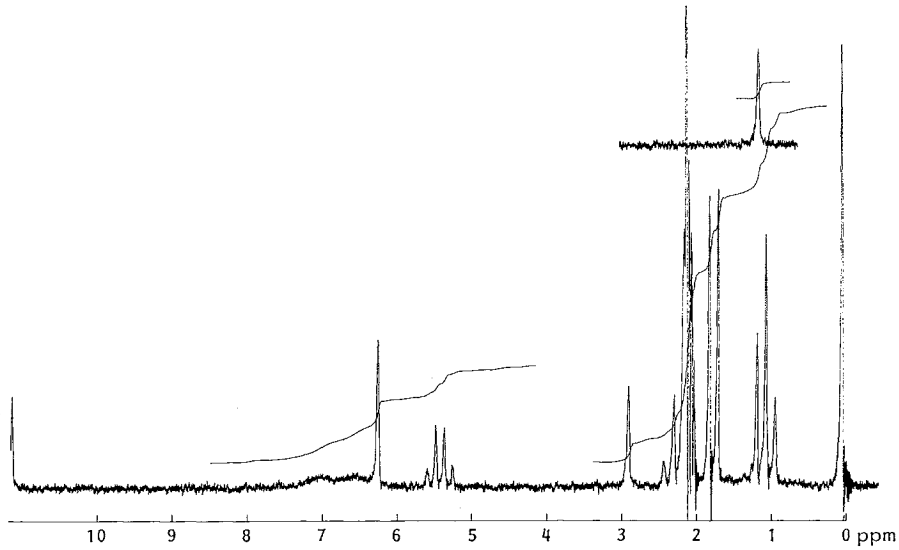
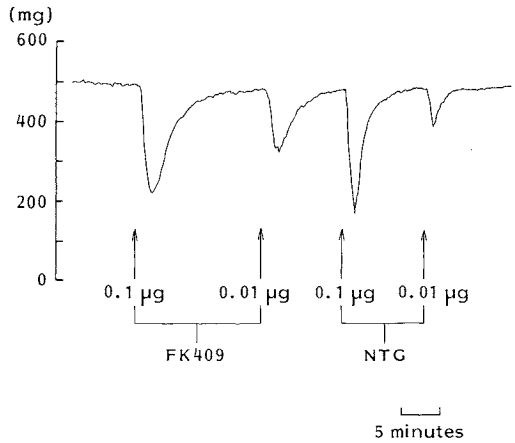


Table 1. Physico-chemical properties of FK409.

Appearance	Colorless prism
MP	140°C (dec)
Molecular formula	$C_8H_{13}N_3O_4$
FD-MS (*m/z*)	215.2
$[\alpha]_D^{25}$ (*c* 1.0, MeOH)	0°
UV λ_{max}^{MeOH} nm (ε)	240 (sh, 7,000)
$\lambda_{max}^{MeOH+NaOH}$ nm (ε)	273 (13,500)
Elemental analysis	
Calcd for $C_8H_{13}N_3O_4$:	C 44.65, H 6.09, N 19.53
Found:	C 44.64, H 5.92, N 19.60
TLC[a] (Rf)	
$CHCl_3$-MeOH (10:1)	0.30
EtOAc	0.56

[a] Stationary phase: Silica gel sheet.
FD-MS: Field desorption MS.

Fig. 4. Vasodilating effects of FK409 and NTG.



The aorta was separated from a male SD rat weighing 270 g and superfused with Tyrode solution containing noradrenaline (0.03 μg/ml/minute) which increased the tension of the tissue by about 500 mg.

mixture of *n*-hexane and ethyl acetate (1:4). The fractions containing FK409 were collected and concentrated under reduced pressure to give an oily residue. The oily residue was rechromatographed on silica gel column and developed with a mixture of chloroform and methanol (25:1). The active fractions were concentrated under reduced pressure to give crystals. Recrystallization from methanol gave purified FK409 (1.4 g) as colorless prisms.

Physico-chemical Properties

FK409 is soluble in methanol, slightly soluble in ethyl acetate, chloroform and water. Color reactions are as follows: Positive in ceric sulfate, potassium permanganate, iodine vapor and Liebermann tests, negative in DRAGENDORFF and MOLISCH's tests. The IR (Nujol) and ^1H NMR (60 MHz, CD_3COCD_3) spectra are shown in Figs. 2 and 3, respectively. The other physico-chemical properties of FK409 are summarized

Table 2. Hypotensive activity of FK409 in a rat.

Drug	Dose (μg/kg)	Maximal decrease in mean arterial blood pressure (mmHg)	Duration (minutes)
FK409	100	40	4
	10	10	1.5
NTG	60	35	1.5

A Sprague-Dawley rat of 8 weeks age was anesthetized with urethane (700 mg/kg, ip). Drug was administered iv.

Table 3. Inhibitory effects of FK409 on rabbit platelet aggregation.

Aggregating agent	IC_{50} value (M)
Collagen	3.3×10^{-7}
Thrombin	3.3×10^{-7}
ADP	7.0×10^{-7}
PAF	3.3×10^{-7}
Arachidonic acid	3.5×10^{-6}

Each drug was added 2 minutes before the aggregation agent; collagen (2.5 μg/ml), thrombin (0.3 U/ml), ADP (2.5 μM), PAF (0.1 μM) or arachidonic acid (100 μM). Results are presented as the concentration of each drug inhibiting maximal aggregation by 50%.

in Table 1.

Biological Properties

The vasodilating activity of FK409 is shown in Fig. 4. FK409 exhibited a marked relaxation activity in doses of 0.01 and 0.1 μg on noradrenaline induced contraction of rat aorta. Nitroglycerin (NTG), a drug for angina pectoris, was also assayed in the same aorta preparation. The magnitude of the vasodilating effect of FK409 and NTG were similar. The hypotensive effect of FK409 in a rat is shown in Table 2. FK409 showed marked hypotensive effect when the compound was administered intravenously into an anesthetized rat in doses of 10 and 100 μg/kg. NTG (60 μg/kg) was also evaluated for comparison. Although, the depth of the hypotensive effect of FK409 and NTG were similar, the duration of FK409 was longer than that of NTG.

The anti-platelet activity of FK409 is shown in Table 3. FK409 showed a potent inhibitory activity on the aggregation induced by the all aggregating agents that we tested.

Discussion

It is well known that vasodilator drugs are useful therapeutic agents in patients with ischemic heart disease such as unstable angina and acute infarction. The vasodilating effect of these drugs increases peripheral blood flow to an area where perfusion is compromised by acute or chronic arterial obstruction or vasospasm[12]. On the other hand, recent pathological and clinical studies have suggested that platelets have a role in the pathogenesis of unstable angina and myocardial infarction[13]. Platelet deposition and aggregation may lead to intermittent coronary obstruction and contribute to the development of unstable angina[14,15]. These observations suggest that vasodilator drugs with an anti-platelet activity may provide an additional means of treatment for patients with ischemic heart disease. FK409 an orally active (data not shown) vasodilator with a potent anti-platelet activity seems to be promising a new drug in this area.

FK409 was isolated from the acid-treated fermentation broth of *S. griseosporeus* No. 16917, which was cultured on a medium containing $NaNO_3$ for 4 days. Without the acid treatment, this compound was not obtained, therefore we suppose that a precursor of FK409 is produced in the cultured broth, and then converted to the active compound, FK409, during the acid-treatment. The mechanism will be described in the succeeding paper[16].

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