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SNF4435C and D, Novel Immunosuppressants Produced by a Strain of *Streptomyces spectabilis*

I. Taxonomy, Fermentation, Isolation and Biological Activities

KAZUHIKO KUROSAWA*, KOSAKU TAKAHASHI and EISUKE TSUDA

Research Institute of Life Science, Snow Brand Milk Products Co., Ltd.,
Ishibashi-machi, Shimotsuga-gun, Tochigi 329-0512, Japan

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SNF4435C and D, novel nitrophenyl pyrones, have been isolated from the culture broth of an actinomycete strain SNF4435. The strain was identified as *Streptomyces spectabilis* from its morphological and cultural characteristics. SNF4435C and D showed a potent immunosuppressive activity *in vitro* and selectively suppressed B-cell proliferation induced by LPS *versus* T-cell proliferation induced by Con A.

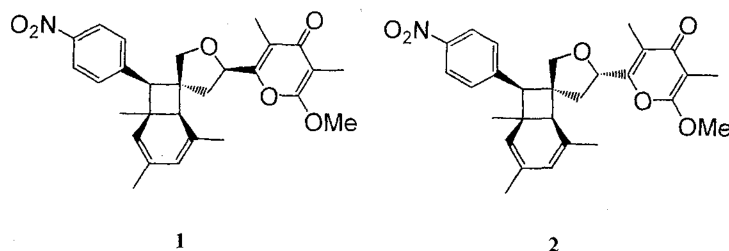
Immunosuppressants make great contributions to the prevention of the rejection against the transplantation of organs or tissues, and to the remedy for autoimmune or allergic diseases. Several immunosuppressive drugs such as cyclosporin A[1)] and tacrolimus (FK-506)[2)] have been developed and put to clinical use. Although, they are somewhat toxic and their doses are therefore limited, the treatment using them is not so satisfactory. Taking the above-mentioned situation, in the course of our screening for new immunosuppressive agents from the culture broths of microorganisms, we found that a streptomycete, strain SNF4435 produces two novel nitrophenyl pyrones designated SNF4435C and D (Fig. 1). This paper describes the taxonomy of the producing strain, fermentation, isolation and biological activities of the compounds.

Materials and Methods

Taxonomy

The producing strain SNF4435 was isolated from the soil in the main island of Okinawa, Japan by soil dilution technique. The media and procedures used for culture and physiological characterization of the SNF 4435 strain were

Fig. 1. Relative structures of SNF4435C (**1**) and D (**2**).



* Corresponding author: kurosawa@snowbrand.co.jp

those of described by International *Streptomyces* Project (ISP)[3]. Cultures were incubated at 27°C and were observed for 14~21 days. Detailed observation of mycelial and spore morphologies was performed using a scanning electron microscope (Hitachi, model S-800). Chemical composition of the cell was analyzed by the methods of HASEGAWA *et al.*[4]. Analysis of menaquinone was performed by the methods of TAMAOKA *et al.*[5].

Fermentation

A slant culture of the strain SNF4435 grown on yeast extract - malt extract agar (ISP No. 2) was inoculated into a 500-ml Erlenmeyer flask containing 70 ml of the seed medium composed of $(NH_4)_2SO_4$ 0.14%, KH_2PO_4 0.2%, $CaCl_2$ 0.03%, $MgSO_4 \cdot 7H_2O$ 0.03%, urea 0.03%, Polypepton 0.5%, yeast extract 0.1%, soybean meal 3%, glucose 1%, soluble starch 0.5%, $FeSO_4 \cdot 7H_2O$ 0.0005%, $MnSO_4 \cdot 4{\sim}6H_2O$ 0.00016%, $ZnSO_4 \cdot 7H_2O$ 0.00014% and $CoCl_2$ 0.0002%, pH 7.0 prior to sterilization. The flask was cultivated at 27°C for 4 days on a rotary shaker (230 rpm) to give a first seed culture. Approximately 2-ml portions of the culture were inoculated into each of twenty 500-ml Erlenmeyer flasks containing 70 ml of the above medium, and incubated at 27°C on a rotary shaker (230 rpm) to give a second seed culture. After 2 days, the seed culture (1.2 liters) was used to inoculate into a 200-liter fermentor containing 120 liters of a producing medium which consisted of soluble starch 1%, glucose 2%, soybean meal 2.5%, dry yeast 0.4%, meat extract 0.1%, K_2HPO_4 0.005% and NaCl 0.2%, adjusted to pH 7.2 before sterilization.

The fermentation was carried out at 27°C for 8 days under aeration of 120 liters/minute and agitation at 200 rpm.

Analytical Procedure

The content of SNF4435 compounds in the fermentation broth and purification steps was monitored by reversed phase HPLC. HPLC was performed as follows: Inertsil ODS-2 column (4.6×250 mm, GL Sciences Inc.); mobile phase, CH_3OH - H_2O (8 : 2); flow rate, 1.0 ml/minute; detection, UV (220 nm). The retention times of SNF4435C and D were 14.6 minutes and 16.6 minutes, respectively.

Antimicrobial Activity

Antimicrobial activities were determined by the conventional paper disk (ADVANTEC, 8 mm in diameter) method. Bacteria were grown on nutrient agar medium, and fungi and yeasts were grown on Sabouraud agar medium. The compounds were dissolved in MeOH and a paper disk containing each of the sample (100 μg) was placed on the agar plates. Growth inhibition was examined after 24 hours incubation at 37°C for bacteria and after 48 hours incubation at 27°C for fungi and yeasts. The antimicrobial activity was estimated by measuring the diameter of inhibitory zone.

Cytotoxicity

The cytotoxicities of SNF4435C and D against KB, K-562, HL-60 cells and rat hepatocytes were determined using the MTT colorimetric assay method[6]. Rat hepatocytes were freshly isolated from male Wistar rat (6 weeks old, Charles River Japan Inc.), according to the method of GOHDA E. *et al.*[7]. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco). To determine the cytotoxicity, cells suspended in 100 μl of the medium ($1{\sim}2\times10^4$ cells/ml) were inoculated into 96-well microtiter plates and incubated for 24 hours. The test sample, dissolved in dimethyl sulfoxide, was serially diluted with the medium and added to the culture plates. And then the mixture was incubated for 48 hours. The cells were grown in a 5% CO_2 - 95% air atmosphere at 37°C. All experiments were carried out in triplicates. The growth of cells was measured colorimetrically at 550 nm (and 660 nm as a reference) by the MTT method. Cytotoxic activities are expressed as IC_{50} values, the drug concentration required for 50% inhibition of cell growth.

Immunosuppressive Activity

The immunosuppressive activity of the compounds was assessed with suppression of blastogenesis of mouse spleen cells as described by NAKAMURA *et al.*[8]. The male BALB/c mice (6~8 weeks old, Charles River Japan Inc.) were killed and the spleens removed aseptically. Splenocytes were separated from the spleens with centrifugation on Lymphoprep (NYCOMED AS., Oslo, Norway), washed twice and resuspended in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum. Cell viability was determined by nigrosin dye exclusion[9]. Con A (Sigma) and LPS (Difco, from *E. coli*) were dissolved in RPMI-1640 medium to prepare the mitogen solutions. Each compound was dissolved in dimethyl sulfoxide and serially diluted with culture medium to prepare sample solution. The cell suspension of 10^6 cells/well (100 μl) was incubated with the sample solution (50 μl) and the mitogen solution (50 μl) in a 96-well microtiter plate at 37°C in a humidified atmosphere of 5% CO_2 - 95% air for 48 hours. At the end of the culture period, they were pulsed with [^{3}H] thymidine for 4 hours and harvested using a cell harvester (PACKARD, MICROMATE196). The amount of [^{3}H] thymidine incorporated into the cells was determined by a liquid

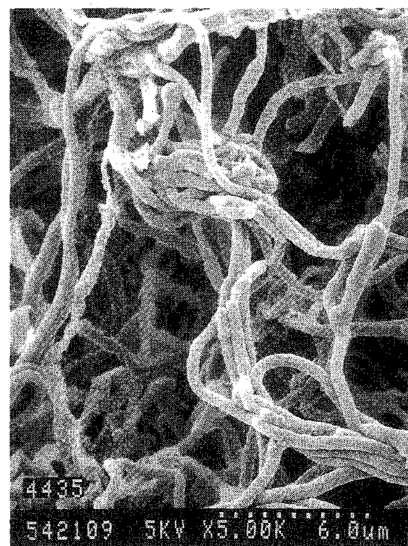
scintillation counter (PACKARD, MATRIX96). The effect of each sample on mitogen-induced and non-induced proliferations of the splenocytes was evaluated in triplicate, and expressed as a percentage of [^{3}H] thymidine incorporated into the cells with the sample to that without the sample.

Results and Discussion

Taxonomy of the Producing Strain

The substrate mycelia of strain SNF4435 were well developed, branched, and did not fragment into bacillary elements or coccoid forms. Aerial mycelia were formed on yeast extract-malt extract agar (ISP No. 2), oatmeal agar (ISP No. 3), inorganic salt-starch agar (ISP No. 4) and glycerol-asparagine agar (ISP No.5). Spore-chain morphology of this strain was section *Rectiflexibiles* with straight to flexuous spore chain. Distribution of sporophores sometimes suggested verticillate morphology, but true whorls or verticils were not produced. Mature spore chains consisted of 10 to 50 or more spores. The spore was 0.4~0.5×0.8~1.0 μm in size with a smooth surface. No synnemata, sclerotia or sporangia were observed (Photo 1).

Photo. 1. Scanning electron micrograph of *Streptomyces spectabilis* SNF4435 grown on ISP-2 agar for 14 days at 27°C.



Mature aerial mycelia corresponded to the Red color series (light yellowish pink). The reverse side of the colony was reddish orange and sensitive to pH changes. Permissive temperatures for growth ranged from 12°C to 37°C, with the optimal temperature at 30°C. Soluble pigments were not produced. Melanoid pigments were produced in pepton-yeast extract iron agar (ISP 6), but not in tyrosine agar (ISP 7). Liquefaction of gelatin, peptonization of milk and hydrolysis of starch were positive, but coagulation of milk was negative. Xylose, glucose, galactose, fructose, raffinose, inositol, mannitol and mannose were utilized as a sole carbon source but not arabinose, rhamnose and sucrose. Whole-cell hydrolysates of strain SNF4435 contained LL-diaminopimelic acid, suggesting cell-wall type I. Major components of menaquinones were MK-9 (H_6) and MK-9 (H_8).

Based on the taxonomic properties described above, strain SNF4435 was considered to belong to the genus *Streptomyces*. We searched the taxonomic data of known *Streptomyces* species in the ISP descriptions by SHIRLING's reports[10~13]. The search showed that strain SNF4435 was more closely related to *Streptomyces spectabilis*[13]. Accordingly, strain SNF4435 was compared with *Streptomyces spectabilis* IFO 13424 in more detail. As shown in Table 1, the strain SNF4435 was similar to *Streptomyces spectabilis* except for small difference in the color of the aerial mycelium. Therefore, it was identified as a strain of *Streptomyces spectabilis*. The strain SNF4435 has been deposited in the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, Tsukuba City, Ibaraki Prefecture, Japan, with an accession number of FERM BP-5915 under the Budapest treaty.

Fermentation

A typical time course of fermentation by *Streptomyces spectabilis* SNF4435 in a 200-liter fermentor is shown in Fig. 2. The pH of the culture broth fell gradually from 6.8 to 5.8 for 6 days and then rose to 7.0 at day 8. The production of SNF4435C and D started between 2 and 3 days, and their concentrations reached a maximum (0.95 and 0.32 mg/liter, respectively) on day 7, then decreased slowly. Active materials were produced mainly in mycelia.

Isolation and Purification

A purification procedure for SNF4435 compounds is outlined in Fig. 3. Culture broth of SNF4435 (120 liters) was centrifuged to obtain the mycelia, and active materials were extracted from the mycelia with acetone (36 liters). The acetone was removed *in vacuo* and the aqueous residue

Table 1. Comparison of taxonomic characteristics of strain SNF4435 with *Streptomyces spectabilis*.

	SNF4435	*Streptomyces spectabilis* IFO 13424
Spore chain morphology	Rectiflexibiles	Rectiflexibiles
Spore surface	Smooth	Smooth
Spore dimensions (μm)	(0.4～0.5) x (0.8～1.0)	(0.4～0.6) x (0.7～0.9)
Aerial mass color	Light yellowish pink	Light reddish pink
Reverse color	Reddish orange	Reddish orange
Temperature for growth (℃)	12～37	12～42
Soluble pigment	Negative	Negative
Formation of melanoid pigment	Positive	Positive
Liquefaction of gelatin	Positive	Positive
Coagulation of milk	Negative	Negative
Peptonization of milk	Positive	Positive
Hydrolysis of starch	Positive	Positive
Utilization of		
L-Arabinose	−	−
D-Xylose	+	+
L-Rhamnose	−	−
D-Glucose	+	+
D-Galactose	+	+
D-Fructose	+	+
Sucrose	−	−
Raffinose	+	+
Inositol	+	+
D-Mannitol	+	+
Mannose	+	+

+: Utilization, −: no utilization.

Fig. 2. Time course of SNF4435C and D production in a 200-liter jar fermenter.

●: SNF4435C, ○: SNF4435D, □: pH.

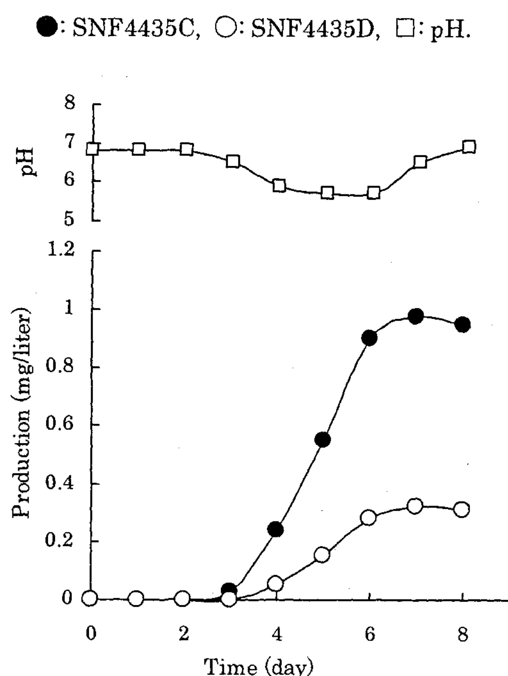


Fig. 3. Isolation of SNF4435C and D.

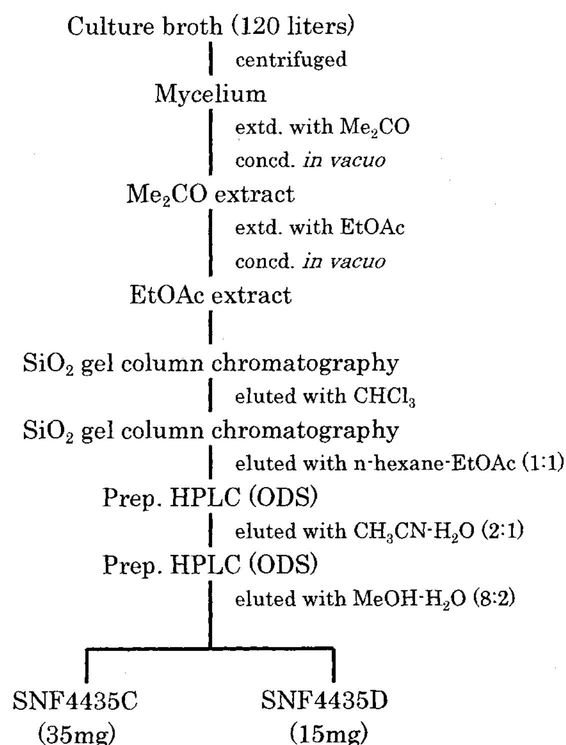


Table 2. Antimicrobial activities of SNF4435C and D.

Organisms	Diameter of inhibition zone (mm)	
	SNF4435C	SNF4435D
Escherichia coli BE 1186	0	0
Salmonella typhimurium TV 119	0	0
Pseudomonas aeruginosa IFO 13130	0	0
Xanthomonas oryzae IFO 3312	0	0
Xanthomonas citri IFO 3781	0	0
Erwinia carotovora IFO 12380	0	0
Staphylococcus aureus 209P	0	9
Bacillus subtilis H17 Rec^+	0	10
Micrococcus luteus IFO 12708	0	0
Mycobacterium phlei IFO 3158	14	18
Alternaria mali IFO 8984	0	0
Botryotinia fuckeliana IFO 5365	0	0
Glomerella lagenaria IFO 7513	0	0
Pyricularia oryzae IFO 5994	18	16
Fusarium oxysporum IFO 9761	0	0
Trichophyton rubrum IFO 6203	0	27[a]
Aspergillus fumigatus IFO 9733	0	0
Candida albicans IFO 1594	9	9
Schizosaccharomyces pombe IFO 0638	0	0

[a] Hazy zone.

was reextracted twice with ethyl acetate. The ethyl acetate soluble portion was concentrated *in vacuo* to give a brown oil. The resulting oily material was fractionated with a silica gel column (Merck 60; 10×50 cm) using $CHCl_3$ as mobile phase and the active fraction was collected. After concentration, the active fraction was further applied onto a silica gel column (Merck 60; 10×50 cm) and developed with *n*-hexane - ethyl acetate (1 : 1). The fractions containing active substrate were combined and concentrated *in vacuo*. The obtained orange material was dissolved in CH_3CN, and subjected to reverse-phase HPLC (CAPCELL PAK C_{18} SG120, Shiseido Co. Ltd., 10×50 cm) using CH_3CN - H_2O (2 : 1) as isocratic mobile phase at a flow rate of 200 ml/minute. The fraction containing SNF4435C and D was concentrated *in vacuo* to give a yellowish powder. The powder was dissolved in a small amount of MeOH and repeatedly purified by ODS-HPLC (CAPCELL PAK C_{18} SG120, Shiseido Co. Ltd., 20×250 mm, flow rate; 10 ml/minute) with MeOH - H_2O (8 : 2). SNF4435 compounds were eluted at 27.1 (C) and 29.4 (D) minutes. The fractions containing SNF4435C or D, respectively, were collected separately and concentrated *in vacuo*. As a result, SNF4435C (35 mg) and D (15 mg) were obtained as a pale-yellow powder.

The structures of SNF4435C and D are shown in Fig. 1. The studies on the structure determination of these compounds will be reported in an accompanying paper.

Biological Activities

Antimicrobial Activity

The antimicrobial activities of SNF4435C and D are summarized in Table 2. These compounds showed no antimicrobial activity against Gram-negative bacteria tested but had activity against *Mycobacterium phlei*, *Pyricularia oryzae* and *Candida albicans*. SNF4435D also exhibited an activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Trichophyton rubrum*, although SNF4435C was inactive. The spectrum of SNF4435C was somewhat less than that of SNF4435D.

Cytotoxic Activity

The cytotoxic activities of SNF4435C and D are shown in Table 3. They did not show remarkable cytotoxicities against KB, K-562, HL-60 and rat hepatocytes. IC_{50} values of the compounds against KB, K-562 and HL-60 cells ranged from 40 μM to 50 μM and those against rat hepatocytes were 10 μM.

Suppression of Lymphocyte Proliferation

The immunosuppressive activity of SNF4435C and D was evaluated by mitogen induced lymphocyte blastogenesis. As shown in Fig. 4, the LPS-induced response of splenocytes was more significantly inhibited than the Con A-induced responses in a dose-dependent manner. The IC_{50} values of SNF4435C and D were calculated to be 0.8 μM and 0.2 μM against Con A-induced proliferation, and 0.08 μM and 0.02 μM against LPS-induced proliferation of mouse spleen lymphocytes, respectively. These facts suggest that the compounds selectively suppress B-cell proliferation *versus* T-cell proliferation. Showing no suppressive activity against rat hepatocytes at a concentration of 1 μM, it appeared that these compounds have interest specificity of suppressive activity against lymphocytes.

In conclusion therefore, SNF4435C and D are expected to show different pharmaco-kinetic behaviors from cyclosporin A and FK-506. We consider that SNF4435 compounds are worthy of further evaluation as immmunotherapeutic agents. Detailed studies on biological activities *in vitro* and *in vivo* will be published elsewhere.

Table 3. Cytotoxicities of SNF4435C and D.

Cells	IC_{50}(μM)	
	SNF4435C	SNF4435D
KB	40	45
K-562	45	50
HL-60	40	40
Hepatocyte (rat)	10	10

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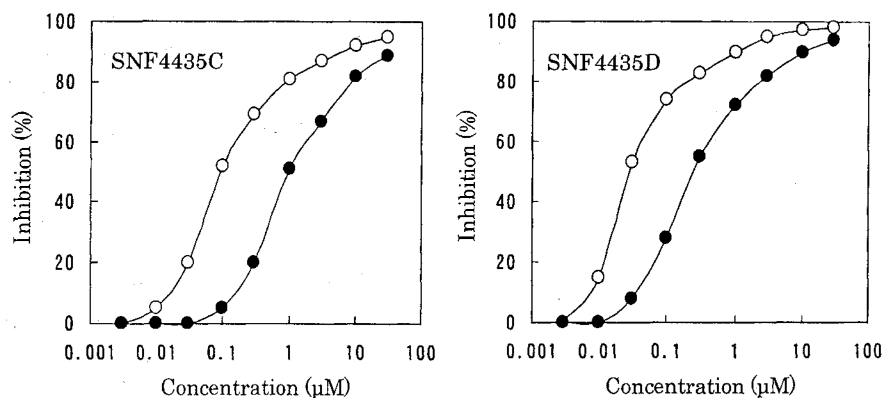
Fig. 4. Effect of SNF4435C and D on mitogen-induced stimulation of murine splenocyte.



The mouse spleen cells were cultured with Con A (●) or LPS (○) in the presence of indicated amount of SNF4435C and D.

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