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SF2809 Compounds, Novel Chymase Inhibitors from *Dactylosporangium* sp.

1. Taxonomy, Fermentation, Isolation and Biological Properties

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Six novel chymase inhibitors, SF2809-I, SF2809-II, SF2809-III, SF2809-IV, SF2809-V and SF2809-VI, were isolated from the fermentation broth of an actinomycete strain SF2809. The strain was identified as *Dactylosporangium* sp. by morphological, chemotaxonomical and phylogenetic studies. These six novel compounds inhibited recombinant human chymase in the range between IC_{50} of 0.014 and 7.3 μM. However, they showed little or no inhibitory activity against chymotrypsin or cathepsin G, even though these two and chymase belong to the chymotryptic serine protease family. This result indicates that these compounds work as specific chymase inhibitors.

Chymase (EC 3.4.21.39) is a chymotrypsin-like serine protease, which is synthesized as inactive precursor but stored in the secretory granules of mast cells as active enzyme. According to the previous reports, chymase mediates the processing of several neuropeptides such as angiotensin I[1], vasoactive intestinal peptide (VIP)[2], substance P[2] and endothelin[3]. Chymase has also been reported to mediate the processing of some cytokines such as interleukin-1β[4] and transforming growth factor (TGF)-β[5]. Additionally, it is pointed out that chymase promotes mast cell degranulation[6], and plays an important role in airway secretions[7] and in atopic or allergic inflammation of the skin[8,9]. Thus, it is thought that chymase inhibitors could work effectively as anti-asthma or anti-allergic drugs[10].

Recently, it was demonstrated that localized production of angiotensin II from angiotensin I by chymase in the cardiovascular system could provoke cardiomyopathy[11] and vascular proliferation[12]. Therefore, chymase inhibitors are also expected to be effective in treatment of cardiovascular diseases[13]. Furthermore, it seems that chymase induces tissue remodeling through the tissue degradation and fibrosis, which causes some chronic diseases[14,15].

From these physiological roles of chymase, the demand for development of chymase inhibitors is increasing. However, no chymase inhibitor is now available for clinical treatment. This situation prompted us to investigate for discovery of potent chymase inhibitors.

In the course of our screening program for such chymase inhibitors, we found novel compounds, SF2809-I, II, III, IV, V and VI (Fig. 1), in the fermentation broth of an actinomycete strain SF2809, which was identified as *Dactylosporangium* sp. by taxonomical and phylogenetic studies.

This paper reports on the taxonomy and fermentation of the strain SF2809, and the isolation and biological properties of SF2809-I~VI. The structural elucidation of these compounds will be described in an accompanying paper[16].

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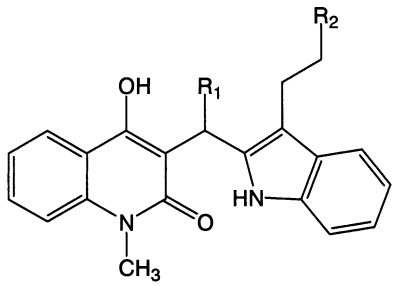
Fig. 1. Structures of SF2809 I~VI.



SF2809-I	R_1= H	R_2= $NHCOCH_3$
SF2809-II	R_1= C_6H_4-*p*-OH	R_2= $NHCOCH_3$
SF2809-III	R_1= H	R_2= OH
SF2809-IV	R_1= C_6H_4-*p*-OH	R_2= OH
SF2809-V	R_1= C_6H_5	R_2= $NHCOCH_3$
SF2809-VI	R_1= C_6H_5	R_2= OH

Materials and Methods

Taxonomy

An actinomycete strain SF2809 was isolated from a soil sample collected at Hachijo Island in Tokyo, Japan. Morphological, cultural and physiological properties of strain SF2809 were examined by the methods of SHIRLING *et al.*[17] and WAKSMAN[18]. In making the morphological observations, strain SF2809 was cultured on the following media; ISP media, yeast extract starch agar medium (yeast extract 2 g, starch 10 g, agar 18 g in 1 liter water, pH 7.0), and calcium malate agar medium[19], at 28°C for 14 days. To identify colors, Color Harmony Manual[20] was used. The amino acid composition of the cell wall and the sugar composition of the whole cell were analyzed by the method of BECKER *et al.*[21] and LECHEVALIER[22], respectively. The acyl type of peptidoglycan of the cell wall was analyzed by the method of UCHIDA[23]. Following the Identification Manual of Actinomycetes[24], phylogenetic study of the strain SF2809 was made by analysis of the 16S rRNA gene base sequence.

Fermentation

A slant culture of the strain SF2809 was inoculated into a 500 ml Erlenmeyer flask containing 80 ml of a seed medium which consisted of glucose 1.0%, soluble starch 2.0%, yeast extract 0.3%, polypeptone 0.5%, wheat germ 0.6% and $CaCO_3$ 0.2% in deionized water adjusted to pH 7.0 with NaOH prior to sterilization. The flask was incubated at 28°C for 72 hours on a rotary shaker at 220 rpm. For a secondary seed culture, 8 ml of the first seed was inoculated into a 2-liter Erlenmeyer flask containing 200 ml of the seed medium, and the flask was incubated at 28°C for 48 hours on a rotary shaker at 220 rpm. Then, the second seed culture was transferred into four 50-liter jar fermentors containing 30 liters of the production medium which consisted of glucose 2.0%, soluble starch 1.0%, yeast extract 0.3%, polypeptone 0.1%, wheat germ 0.8%, Staminol 0.1%, NaCl 0.1% and $CaCO_3$ 0.2% in water (pH 8.0). The fermentation was carried out at 28°C for 5 days, stirring at 250 rpm and aerating at 17.5 liters/minute.

Isolation

The cultured broth (120 liters) was centrifuged to separate mycelial cake and supernatant. The supernatant was extracted with 100 liters of ethyl acetate. The mycelial cake was extracted with 50 liters of 66% aqueous acetone, and after removal of acetone, the resulting aqueous solution was extracted with ethyl acetate. Two ethyl acetate extracts were combined, and concentrated *in vacuo*. After washing the residue with *n*-hexane, the residue was subjected to a silica gel column (Wakogel C-300, Wako Pure Chemical Industries, Ltd.) and eluted with chloroform - methanol (100 : 1, 97 : 3 and 90 : 10). The active fractions showing inhibitory activity against chymase were collected, and applied onto a Sephadex LH-20 column (Pharmacia) after concentration. The active fractions eluted from the Sephadex LH-20 column with methanol were evaporated to dryness and loaded onto an ODS open-column (Cosmosil OPN, Nacalai Tesque, Inc.) After washing the column with 30% aqueous acetonitrile, the active components were eluted with 70% aqueous acetonitrile. The eluate was concentrated *in vacuo*, then separated by preparative HPLC (LC-6AD, Shimadzu Corporation) using a reverse phase column, Inertsil-ODS2 column (250×20 mm i.d., GL Science), and a 35-mimutes linear gradient of acetonitrile/water from 40 to 65% at a flow rate of 10 ml/minute. Eluate was monitored with a UV detector (SPD-10A, Shimadzu Corporation) at 280 nm. SF2809 I, II, III, IV, V and VI were eluted at 8, 12, 15, 16, 28 and 33 minutes, respectively.

Measurements of Protease Inhibitory Activities

Human recombinant chymase was prepared by heterologous expression in Tn5 insect cells as described by MURAKAMI *et al.*[25] The resultant chymase was purified by a Heparin-Sepharose column chromatography[25].

Bovine pancreatic α-chymotrypsin and human cathepsin G were purchased from Sigma and Athens Research & Technology, Inc., Athens, Georgia, USA, respectively.

Inhibitory activity against chymase was measured as

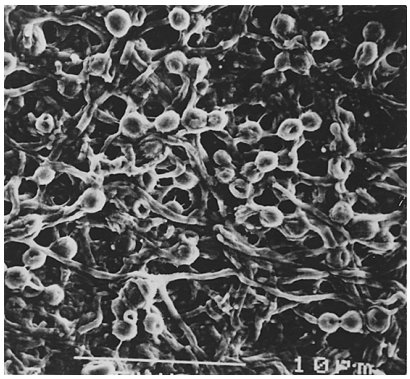
described by EDA *et al.*[26] A sample for determination of inhibitory activities against proteases was dissolved in dimethylsulfoxide, and 2 μl of the solution was added to 50 μl of buffer A (50 mM Tris-hydrochloric acid buffer, pH 8.0, containing 3 M sodium chloride and 0.001% PEG6000) which contained recombinant human chymase. Then, 50 μl of 0.5 mM N-succinyl-Ala-His-Pro-Phe-*p*-nitroanilide (Bachem AG, Bubendorf, Switzerland) in buffer A was added to the solution as a substrate. The resultant mixture was incubated for 5 minutes at room temperature, and the release of *p*-nitroaniline was monitored spectrophotometrically at 405 nm. Inhibitory activities against chymotrypsin and cathepsin G were measured according to the method of NAKAJIMA *et al.*[27] using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and N-succinyl-Phe-Pro-Phe-*p*-nitroanilide (Bachem AG, Bubendorf, Switzerland), respectively.

Results

Taxonomic Studies

Strain SF2809 developed vegetative mycelia with a diameter of 0.4~0.5 μm. The mycelia branched irregularly without fragmentation. Aerial mycelium was not observed. Many spore-like structures with a diameter of 1.0~1.6 μm were formed in the vegetative mycelia (Fig. 2). They were independent and spherical, and their surface was smooth or slightly rough. Whereas, finger-shaped or rod-shaped sporangia extending from the vegetative mycelia were not observed. Table 1 shows the cultural characteristics of strain SF2809 on various media. The growth of this strain was abundant in some media, but poor in many media. Spore-like formations were observed in all the media. The color of vegetative mycelia was orange or

Fig. 2. Scannning electron micrograph of *Dactylosporangium* sp. SF2809.



(Bar: 10 μm).

Table 1. Cultural characteristics of strain SF2809.

Medium	Growth	Reverse color	Aerial mycelium	Spherical structure	Soluble pigment
Yeast extract-malt extract agar (ISP medium 2)	Moderate	Apricot (4ga)	None	None	None
Oatmeal agar (ISP medium 3)	Abundant	Russet orange (4nc)	None	Abundant	None
Inorganic salts-starch agar (ISP medium 4)	Abundant	Dk Luggage Tan (4pg)	None	Abundant	None
Glycerol-asparagine agar (ISP medium 5)	Poor	Lt Melon Yellow (3ea)	None	Abundant	None
Peptone-yeast extraction iron agar (ISP medium 6)	Poor	Bright Melon Yellow (3ia)	None	Poor	None
Tyrosine agar (ISP medium 7)	Poor	Lt Melon Yellow (3ea)	None	Abundant	None
Calcium malate agar	Poor	Melon Yellow (3ga)	None	Abundant	None
Sodium malate	Poor	Lt Melon Yellow (3ea)	None	Abundant	None
Sodium succinate agar	Poor	Lt Melon Yellow (3ea)	None	Abundant	None
Yeast extract-starch agar	Abundant	Orange Rust (4pe)	None	Abundant	None
Bennett agar	Abundant	Cinnamon Yellow Maple (3le)	None	None	None

( ): Color number designations taken from Color Harmony Manual[18]

Table 2. Physiological properties of strain SF2809.

Condition	Properties
Temperature range for growth	15-37°C
(Optimum)	(28°C)
Liquegition of gelatin	±
Coagulation of milk	±
Peptonization of milk	±
Hydrolysis of starch	+
Reduction of nitrate	+
Production of melanoid pigment	−
Sodium chloride tolerance	≦1%

+: Positive, ±: Slightly positive, −: Negative

Table 3. Carbohydrate utilization of strain SF2809.

Carbon source	Utilization
D-Glucose	+
L-Arabinose	±
D-Xylose	+
myo-Inositol	−
D-Fluctose	+
L-Rhamnose	+
Sucrose	+
Raffinose	+

+: Utilizable, ±: Slightly utilizable, −: Not utilizable, Basal medium: ISP medium No.9

orange-brown. Blackening and wetting of colonies, which are characteristic of typical strains of the genus *Micromonospora*, were not observed for this strain. Tables 2 and 3 show summaries of the physiological properties and the utilization of carbon sources of this strain, respectively. The cell wall hydrolysate contained *meso*-diaminopimelic acid and glycine. The whole cell hydrolysate contained arabinose and xylose. These data indicate that strain SF2809 has a cell wall chemotype IIID in the classification of LECHEVALIER *et al.* The acyl type of peptidoglycan of the cell wall was determined to be a glycolate type, and mycolic acid was not detected. MK-9 (H_6) and MK-9 (H_8) were the major components of menaquinone. Cellular fatty acids consisted of branched fatty acids, *i*-16:0, *i*-15:0, *antei*-17:0, *antei*-15:0 and *i*-17:0 as major components and mono-unsaturated branched fatty acids and linear saturated fatty acids as minor components. Hydroxyfatty acid and 10-methylfatty acid were not detected.

On the basis of morphology and chemotaxonomic characteristics described above, strain SF2809 was considered to belong to the family *Micromonosporaceae*.

Phylogenetic Study

Almost complete 16S rRNA gene base sequence of strain SF2809 was analyzed (accession number; AB017374) and compared with the NCBI data base. From these comparisons, strain SF2809 was classified into the cluster of the genus *Dactylosporangium*. The strain has been deposited to the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Japan, as *Dactylosporangium* sp. SF2809 with an accession number FERM P-16975.

Isolation and Purification

Inhibitory activity against chymase was detected both in mycelial cake and supernatant of the cultured broth of the strain SF2809. The active components were extracted with ethyl acetate from the mycelial cake and the supernatant of 120 liters of the broth. The ethyl acetate extracts were combined, then active compounds were purified by silica gel, Sephadex LH-20, ODS and preparative HPLC. Consequently, six compounds having chymase inhibitory activity were isolated, *i.e.*, SF2809-I (2.3 mg), II (1.3 mg), III (2.3 mg), IV (2.7 mg), V (1.1 mg) and VI (1.0 mg).

Biological Activities

Biological activities of SF2809-I~VI are shown in Table 4. In addition to their inhibitory activities against human chymase, their effects on human cathepsin G and bovine pancreatic chymotrypsin, which belong to the chymotryptic serine protease family as chymase, were measured for the evaluation of their specificity. As a result, SF2809-II, IV, V and VI inhibited chymase at the IC_{50} of 0.014~0.081 μM, whereas they inhibited cathepsin G at the IC_{50} values of 1.0~6.3 μM, and they did not inhibit chymotrypsin at the concentration of 20 μM. SF2809-I and III showed relatively weak activities against chymase with the IC_{50} values of 7.3 and 2.1 μM, respectively, and these two compounds

Table 4. Enzyme inhibitory activities of SF2809 compounds.

	IC_{50}, μM		
	Chymase	Cathepsin G	Chymotrypsin
SF2809-I	7.3	>20	>20
SF2809-II	0.041	3.0	>20
SF2809-III	2.1	>20	>20
SF2809-IV	0.081	6.3	>20
SF2809-V	0.043	1.0	>20
SF2809-VI	0.014	1.3	>20

did not inhibit either cathepsin G or chymotrypsin at the concentration of 20 μM. Furthermore, all SF2809 compounds did not show cytotoxicity against mammalian cells, bacteria and fungi at the concentration of 100 μM (data not shown). Consequently, it was shown that SF2809 compounds were potent and specific chymase inhibitors.

Discussion

We have isolated novel compounds, SF2809-I~VI, and found that these compounds are potent chymase inhibitors showing specific activities and no cytotoxicities.

SF2809 compounds are produced by an actinomycete strain SF2809. The strain SF2809 was identified as a member of the genus *Dactylosporangium*, mainly based on the chemotaxonomic characteristics and 16S rRNA gene base sequence. However, the formation of finger-like sporangia, which is characteristic of the genus *Dactylosporangium*, was not observed. Instead of sporangia, strain SF2809 formed a unique spherical structure, which was closely resemble to the globose body. It has been reported that globose body could be found in some strains of *Dactylosporangium*, and according to ENSIGN[28], globose body could germinate like a spore. Therefore, it could be considered that strain SF2809 is a unique strain of *Dactylosporangium* which has lost the capability of producing sporangia and has obtained a spherical structure for germination. Subsequent taxonomical study of strain SF2809 will be the subject for a future report.

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