

SPF-32629 A and B, Novel Human Chymase Inhibitors Produced by *Penicillium* sp.

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Abstract Two new human chymase inhibitors, SPF-32629A and B, were isolated from the cultured broth of *Penicillium* sp. SPF-32629. These structures were determined by spectroscopic methods and identified as new pyridone compounds. SPF-32629B was the carboxylated derivative of SPF-32629A. SPF-32629A and B specifically inhibited human chymase among four serine proteases tested with the IC₅₀ of 0.25 and 0.42 μg/ml, respectively.

Keywords chymase inhibitor, pyridone, *Penicillium*, chymostatin, SPF-32629 A and B

Introduction

Human chymase (EC 3.4.21.39) is a chymotrypsin-like serine protease stored in the secretory granules of mast cells [1]. Chymase has been implicated in the vascular remodeling by converting angiotensin I to angiotensin II in the cardiovascular tissues [2, 3]. Chymase is also shown to be involved in cholesterol efflux from macrophage foam cells by depletion of preβ1LpA1 [4] and the degranulation of skin mast cells by histamine release from mast cells [5]. It has been reported to have the abilities of processing several physiological preproteins such as big-endothelin [6], precursor interleukin-1β [7] and

interstitial procollagenase [8]. Therefore, chymase inhibitors are considered to be useful as therapeutic agents for cardiovascular diseases, arteriosclerosis and skin inflammation. Soybean trypsin inhibitor and chymostatin, which are serine protease inhibitors from natural products, are known to inhibit human chymase, with little specificity [2]. Recently SF2809 compounds have been isolated as specific human chymase inhibitors from microbial sources [9, 10].

In search for human chymase inhibitors from microorganisms, we isolated two novel compounds, SPF-32629A (**1**) and B (**2**) (Fig. 1), from the cultured broth of a fungal strain SPF-32629. In this paper, we describe the

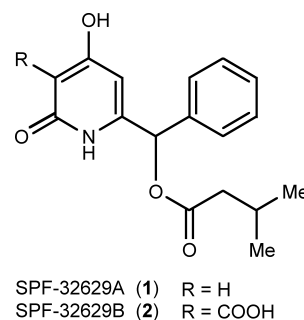


Fig. 1 Structures of SPF-32629A (**1**) and SPF-32629B (**2**).

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taxonomy and fermentation of the producing strain and the isolation, structure elucidation and biological activities of these two compounds.

Materials and Methods

General

UV spectra were recorded with a Hitachi U-2000 spectrophotometer. IR spectra were recorded with a Perkin-Elmer 1600 series FT-IR spectrometer. FAB-MS spectra were obtained with a JEOL JMS-SX102A spectrometer. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter in a 10 cm cell. NMR spectra were recorded with a JEOL JNM α -500 spectrometer and the chemical shifts are given in ppm referenced to CDCl_3 as 7.25 ppm (^1H) and 77.0 ppm (^{13}C) and to acetone- d_6 as 2.05 ppm (^1H) and 29.8 ppm (^{13}C).

Microorganism

The fungal strain SPF-32629 was isolated from a soil sample collected in Saga Prefecture, Japan. The strain has been deposited at the International Patent Organism Depository, the National Institute of Advanced Industrial Science and Technology, Japan under the accession number FERM P-18906.

Taxonomy

Taxonomic studies of the strain SPF-32629 were done according to the methods of Pitt [11]. For the evaluation of cultural characteristics, Czapek yeast extract agar (CYA, K_2HPO_4 0.1%, yeast extract 0.5%, sucrose 3%, NaNO_3 0.3%, KCl 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001%, agar 1.5%), malt extract agar (MEA, malt extract 2%, peptone 0.1%, glucose 2%, agar 1.5%), 25% glycerol nitrate agar (G25N, K_2HPO_4 0.075%, yeast extract 0.37%, glycerol 25%, NaNO_3 0.23%, KCl 0.038%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.038%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0008%, agar 1.2%), yeast extract-soluble starch agar (YSA, yeast extract 0.2%, soluble starch 1%, agar 1.5%), potato dextrose agar (PDA, Nihon Pharmaceutical, Japan) and Oatmeal agar (OA, ISP medium No. 3, Nihon Pharmaceutical, Japan) were used. Colors were determined by using the Color Tone Manual [12]. Morphology was observed under an optical microscope.

Fermentation

A slant culture of the strain SPF-32629 was inoculated into a 500-ml Sakaguchi flask containing 80 ml of liquid medium composed of glucose 2%, sucrose 5%, cotton seed flour 2%, NaNO_3 0.1%, L-histidine 0.1%, K_2HPO_4 0.05%,

KCl 0.07%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0014%, pH 7.0, and cultured for 3 days at 27°C with reciprocal shaking at 130 rpm. An aliquot of 7 ml of this seed culture was transferred into 2-liter Sakaguchi flasks containing 300 ml of the same medium, and cultured for 11 days at 27°C with reciprocal shaking at 115 rpm.

HPLC Analysis

The cultured broth (10 ml) was extracted with 8 ml of *n*-BuOH. The extracts were evaporated to dryness *in vacuo*, and the residues were dissolved in 1 ml of MeOH. Samples (2 μl) were analyzed by reversed-phase HPLC using a Wakopak Wakosil-II5C18RS column (4.6 \times 150 mm, Wako Pure Chemical Industry, Japan) with aqueous 0.05% TFA - acetonitrile (60:40 to 20:80 in 20 minutes) at a flow rate of 1.0 ml/minute, and detection of UV absorption at 254 nm. Compounds **1** and **2** were eluted at 6.6 and 13.9 minutes, respectively. The optical purity of compounds **1** and **2** was analyzed by four types of chiral HPLC columns (4.6 \times 250 mm); Chiralpak AD-H, Chiralpak AS-H, Chiralcel OD-H and Chiralcel OJ-H (Daicel Chemical Industry, Japan), on four solvent systems (*n*-hexane - EtOH, *n*-hexane - *i*-PrOH, *n*-hexane - EtOH - AcOH and *n*-hexane - *i*-PrOH - AcOH) at a flow rate of 1.0 ml/minute, and detection of UV absorption at 254 nm.

Assay of Serine Protease Inhibition

Substrates and enzymes used were as follows: *N*-succinyl-Ala-Ala-Pro-Phe-MCA (Peptide Institute Inc., Japan) for human skin chymase (Elastin Product, USA), human pancreatic chymotrypsin (Athens Research Technology, USA) and human leukocyte cathepsin G (MP Biomedicals, USA) [13]; *N*-methoxysuccinyl-Ala-Ala-Pro-Val-MCA (Peptide Institute Inc., Japan) for human leukocyte elastase (Athens Research Technology, USA) [14]. Enzymatic reactions were carried out in the following buffers: 50 mM Tris-HCl buffer (pH 8.0) containing 3 M NaCl, 0.01% bovine serum albumin, 150 ng/ml enzyme and 100 μM substrate for chymase; 50 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl, 150 ng/ml enzyme and 100 μM substrate for chymotrypsin; 100 mM Tris-HCl buffer (pH 7.5) containing 1.6 M NaCl, 1 $\mu\text{g}/\text{ml}$ enzyme and 100 μM substrate for cathepsin G; 50 mM Tris-HCl buffer (pH 7.0) containing 2 mM CaCl_2 , 300 ng/ml enzyme and 100 μM substrate for elastase. Test samples (1 μl) dissolved in DMSO were added to each reaction mixture (99 μl) in 96-well microplates and incubated for 2 hours at 37°C except for chymase assay, which was carried out for 5 hours at room temperature.

Serine protease activities were measured by monitoring spectrofluorometrically the release of AMC (7-amino-4-

methyl-coumarine) at wavelengths of Ex 355 nm/Em 460 nm. Chymostatin (Peptide Institute Inc., Japan) was used as a reference compound.

Cytotoxicity Assay

HL-60 human leukemia cells (ATCC CCL-240) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum in atmosphere containing 5% CO₂ at 37°C and 95% relative humidity. HL-60 cells were plated (3×10^4 cells/well in 100 μ l) in 96-well microplates and incubated for 24 hours at 37°C with or without test compounds. After washing by centrifugation twice, fresh RPMI 1640 medium including 10% Alamar Blue (Biosource, USA) was added and incubated for 3 hours. The viability of cells was estimated by fluorescence emission at wavelengths of Ex 544 nm/Em 590 nm.

Results

Taxonomy

The cultural characteristics of the strain SPF-32629 grown at 25°C for 7 days were as follows. Colonies on MEA and PDA were white to vivid yellow and velvety with a diameter of 17~20 mm. The colony reverse was vivid yellow on MEA and bright orange on PDA. Colonies on G25N were white and thin with a diameter of 3~4 mm. The colony reverse was white. Colonies on CYA were white to deep reddish orange and velvety with a diameter of 9~10 mm. The colony reverse was deep orange. Colonies on YSA were white to vivid yellow and velvety with a diameter of 20~21 mm. The colony reverse was bright orange. Colonies on OA were white and velvety with a diameter of 26~27 mm. The colony reverse was white. Production of conidia on OA was the most abundant among these six agar media. No soluble pigment was observed at 25°C for 7 days on these agar media, but bright orange pigment and exudate were observed after 14 days of incubation on CYA.

The morphological characteristics of strain SPF-32629 on OA at 25°C for 14 days were as follows. Conidiophores were formed from substrate and aerial hyphae, and penicilli were biverticillate. Metulae were 3~5 per conidiophore. Phialides were ampulliform and 3~5 per metula. Conidia were green, globose to subglobose, smooth-walled and produced in long chains on the tip of phialide. No teleomorph was observed even after 21 days of incubation on the six agar media mentioned above. From the above characteristics, the strain SPF-32629 was identified as a strain of the genus *Penicillium* LINK and named *Penicillium* sp. SPF-32629.

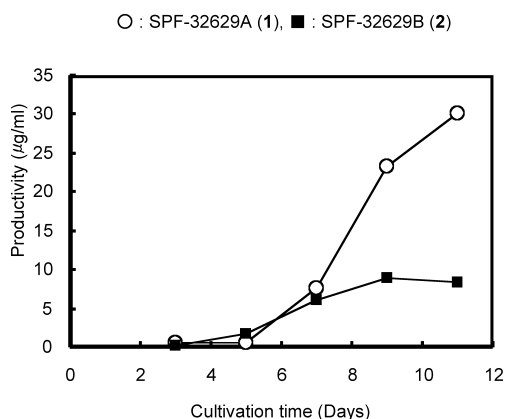


Fig. 2 Time course of production of SPF-32629A (1) and SPF-32629B (2) by *Penicillium* sp. SPF-32629 in a 2-liter Sakaguchi flask.

Fermentation

A time course of production of compounds 1 and 2 by *Penicillium* sp. SPF-32629 in a 2-liter Sakaguchi flask is shown in Fig. 2. Productivities of compounds 1 and 2 reached maximal yields of 30 μ g/ml at day 11 and 8 μ g/ml at day 9, respectively.

Isolation

After 11 days of fermentation, 5 liters of acetone was added to the cultured broth (4.8 liters). The mixture was filtered and the filtrate was evaporated *in vacuo* to remove acetone. The aqueous solution was applied to a column (500 ml) of Diaion HP-20 (Mitsubishi Chemical, Japan). The column was washed with water, 25% methanol, 50% methanol and 75% methanol, and eluted with methanol and then with acetone. The acetone eluate (1.5 liters) was evaporated *in vacuo* to give a yellow crude extract (500 mg). The crude extract was dissolved in 50 ml of chloroform, and subjected to a column (250 ml) of silica gel 60 (70~230 mesh, Merck, Germany). The column was washed with chloroform and then eluted with a mixture of chloroform-methanol (97:3~70:30, v/v). Active fractions were pooled and evaporated *in vacuo* to give a yellow crude extract (109 mg). The extract was dissolved in 3 ml of a mixture of dimethylsulfoxide-methanol (1:1, v/v) and applied to preparative HPLC equipped with Wakopak Wakosil-II5C18HG-Prep columns (30 mm i.d. \times 50 mm + 30 mm i.d. \times 250 mm). The elution was performed with aqueous 0.1% trifluoroacetic acid-acetonitrile (45:55, v/v) at a flow rate of 10 ml/minute and detection of UV absorption at 254 nm. Active fractions (Rt: 84.0 minutes) were collected and the solvent was evaporated *in vacuo* to give 21.6 mg of SPF-32629A (1) as yellow powder.

The methanol eluate (1.5 liters) from Diaion HP-20

Table 1 Physico-chemical properties of **1** and **2**

	1	2
Appearance	Pale yellow powder	Pale yellow powder
Molecular formula	C ₁₇ H ₁₉ NO ₄	C ₁₈ H ₁₉ NO ₆
FAB-MS (<i>m/z</i>)	302 (M+H) ⁺	346 (M+H) ⁺
HRFAB-MS (<i>m/z</i>)		
Found:	302.1393 (M+H) ⁺	346.1292 (M+H) ⁺
Calcd.:	302.1392	346.1291
UV λ _{max} ^{MeOH} nm (ε)	206 (22,200), 286 (4,300)	203 (16,500), 215sh (13,900), 303 (6,300)
IR ν _{max} (KBr) cm ⁻¹	3247, 3099, 2964, 1748, 1643, 1624, 1458	3247, 3093, 2964, 1748, 1643, 1617, 1466
[α] _D ²⁵ (MeOH)	+26.4 (c 0.58)	+24.5 (c 0.40)
Solubility		
Soluble:	CHCl ₃ , Acetone	CHCl ₃ , Acetone
Insoluble:	H ₂ O, <i>n</i> -Hexane	H ₂ O, <i>n</i> -Hexane

mentioned above was evaporated *in vacuo* to give a yellow crude extract (4.0 g). The crude extract was dissolved in 50 ml of methanol and applied to a column (800 ml) of Sephadex LH-20 (Amersham Biosciences, USA), and the column was eluted with methanol. Active fractions (1.5 g) were evaporated *in vacuo*, dissolved in 15 ml of chloroform, subjected to a column (200 ml) of silica gel 60 (70~230 mesh) and washed with chloroform and then eluted with a mixture of chloroform-methanol (98:2~80:20, v/v). Fractions obtained with chloroform-methanol (95:5, v/v) were evaporated *in vacuo* to give a yellow solid (150 mg). The solid was dissolved in 2 ml of methanol and applied to preparative HPLC mentioned above. The elution was performed with aqueous 0.1% trifluoroacetic acid-acetonitrile (63:37, v/v) at a flow rate of 10 ml/minute and detection of UV absorption at 254 nm. Active fractions (Rt: 69.5 minutes) were evaporated *in vacuo* to give 29.8 mg of SPF-32629B (**2**) as yellow powder.

Structure Elucidation

The physico-chemical properties of **1** and **2** are summarized in Table 1. These data suggest that they are structurally related. The molecular formula of **1** was established as C₁₇H₁₉NO₄ by HRFAB-MS and ¹H and ¹³C NMR. The IR spectrum of **1** showed the presence of hydroxyl (3247 cm⁻¹) and ester (1748 cm⁻¹) groups. A 4-hydroxy-2-pyridone chromophore was suggested in the IR (1643 and 1624 cm⁻¹) and UV (λ_{max} 286 nm) spectra. The ¹H and ¹³C NMR spectral data of **1** are summarized in Table 2.

Analysis of the COSY and HMBC spectra of **1** revealed

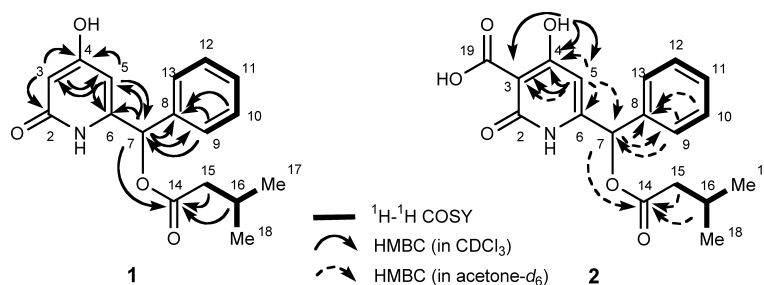
the presence of phenyl and isovaleryl ester groups (Fig. 3). The 4-hydroxy-2-pyridone moiety was confirmed on the basis of ¹H and ¹³C chemical shifts and the HMBC correlations observed from the two aromatic protons at H-3 and H-5 in **1**. The methine group (C-7: δ_H 6.63, δ_C 72.8) was connected to the C-6 of the pyridone ring on the basis of the HMBC correlations of H-7 with C-5 and C-6 and of H-5 with C-7. The HMBC correlations of H-7 with the phenyl carbon C-8 and C-9 and of the phenyl proton H-9 with C-7 revealed the connection of the phenyl group and the 4-hydroxy-2-pyridone *via* C-7 methine group. The remaining isovaleryl ester group was located at C-7 on the basis of the chemical shifts for C-7 (δ_H 6.63, δ_C 72.8) and the HMBC correlations of H-7 with isovaleryl carbonyl carbon C-14. Using the combined data, the structure of **1** was determined to be (1,6-dihydro-4-hydroxy-6-oxo-2-pyridinyl)(phenyl)methyl isovalerate as shown in Fig. 3.

The molecular formula of **2** was established as C₁₈H₁₉NO₆ by HRFAB-MS and ¹H and ¹³C NMR, differing from that of **1** by the addition of CO₂ unit. The ¹H and ¹³C NMR spectra of **2** were very similar to those of **1** except that **2** showed signals for an additional carbonyl group (δ_C 171.8) and a quaternary carbon (δ_C 96.9) in place of the C-3 methine of the pyridone ring observed in **1**. These ¹H and ¹³C NMR data, together with the molecular formula, suggested that **2** was a carboxylated derivative of **1**. The 4-OH and 19-OH resonances at δ_H 13.46 and δ_H 14.19 strongly suggested a peri-location hydrogen-bonding and this corroborates the bathochromic shift data (λ_{max} **2**: 303 nm, **1**: 286 nm) that suggested the location of the carboxylic moiety at C-3 of the 4-hydroxy-2-pyridone moiety. This was confirmed by the HMBC correlations of

Table 2 ^1H and ^{13}C NMR chemical shifts of **1** and **2**

No.	1 (in CDCl_3)		2 (in CDCl_3)		2 (in $\text{acetone-}d_6$)	
	^{13}C	^1H ($J=\text{Hz}$)	^{13}C	^1H ($J=\text{Hz}$)	^{13}C	^1H ($J=\text{Hz}$)
2	164.4		166.0		166.4	
3	97.7	6.09 (1H, s)	96.9		96.7	
4	171.4		174.9		175.1	
5	104.1	6.23 (1H, s)	101.4	6.26 (1H, s)	100.2	6.38 (1H, s)
6	148.4		151.1		153.2	
7	72.8	6.63 (1H, s)	72.7	6.63 (1H, s)	73.4	6.76 (1H, s)
8	135.3		134.7		136.8	
9, 13	127.2	7.34 (2H, m)	127.3	7.38 ^a (2H, br s)	128.1	7.57 (2H, d, 6.3)
10, 12	129.1	7.33 (2H, m)	129.3	7.38 ^a (2H, br s)	129.5	7.43 ^h (2H, d, 6.3)
11	129.5	7.32 (1H, m)	129.9	7.38 ^a (1H, br s)	129.8	7.43 ^h (1H, d, 6.3)
14	172.1		171.3		171.4	
15	43.0	2.32 (2H, d, 7.0)	42.9	2.32 (2H, br s)	43.3	2.41 (2H, d, 6.8)
16	25.6	2.08 (1H, m)	25.7	2.10 (1H, br s)	26.3	2.15 (1H, m)
17	22.3 ^a	0.89 ^b (3H, d, 6.7)	22.3 ^d	0.91 ^f (3H, br s)	22.5 ^g	0.92 ⁱ (3H, d, 6.4)
18	22.2 ^a	0.88 ^b (3H, d, 6.7)	22.3 ^d	0.91 ^f (3H, br s)	22.5 ^g	0.92 ⁱ (3H, d, 6.4)
19			171.8		172.8	
1-NH		ND ^c		11.10 (1H, br s)		ND
4-OH		ND		13.46 (1H, br s)		13.51 (1H, br s)
19-OH				14.19 (1H, br s)		ND

^{a,b} Assignments may be interchanged. ^c Not determined. ^{d-i} Overlapped.

**Fig. 3** ^1H - ^1H COSY and HMBC correlations observed in **1** and **2**.

H-5 with C-3 and C-4 and of C-4 hydroxyl proton with C-3, C-4 and C-5 in CDCl_3 and of H-5 with C-3, C-4, C-6 and C-7 in $\text{acetone-}d_6$. Thus, **2** was determined to be the 3-carboxylated derivative of **1**.

Compounds **1** and **2** showed optical rotations of $+26.4^\circ$ and $+24.5^\circ$, respectively. Both compounds possess an asymmetric carbon at C-7. To analyze the optical purity, we used four types of chiral HPLC columns; Chiralpak AD-H, Chiralpak AS-H, Chiralcel OD-H and Chiralcel OJ-H, on several solvent systems. Both **1** and **2** exhibited a single peak under all conditions tested, suggesting compounds **1** and **2** are optically pure. The absolute configurations of **1** and **2** remain to be determined.

Biological Activities

Compounds **1** and **2** inhibited human chymase with the IC_{50} values of 0.25 and 0.42 $\mu\text{g/ml}$, respectively (Table 3). Compound **1** inhibited both human cathepsin G and human elastase with the IC_{50} value of 4.9 $\mu\text{g/ml}$ and showed 30% inhibition against human chymotrypsin at the concentration of 10 $\mu\text{g/ml}$. Compound **2** inhibited human elastase with the IC_{50} value of 4.5 $\mu\text{g/ml}$ but did not inhibit human chymotrypsin or human cathepsin G at the concentration of 10 $\mu\text{g/ml}$. Therefore, **1** and **2** were specific chymase inhibitors. Moreover, **1** and **2** did not show cytotoxicity against HL60 human leukemia cells at the concentration of 10 $\mu\text{g/ml}$. Chymostatin, a known inhibitor of serine

Table 3 Inhibitory activities of **1** and **2** against four human serine proteases

	IC ₅₀ (μg/ml)			
	Chymase	Chymotrypsin	Cathepsin G	Elastase
1	0.25	>10	4.9	4.9
2	0.42	>10	>10	4.5
Chymostatin	0.005	0.003	0.057	>10

proteases, was more potent than **1** and **2**, but less specific.

Discussion

In the course of our screening for human chymase inhibitors, we discovered two novel compounds, SPF-32629A (**1**) and B (**2**), from the cultured broth of *Penicillium* sp. SPF-32629. Although chymostatin showed very potent inhibition against human chymase with the IC₅₀ value of 0.005 μg/ml, it also inhibited human chymotrypsin to the same degree. Compounds **1** and **2** showed at least 20 times higher selectivity against human chymase than human chymotrypsin. Compound **1** showed relatively higher potency to inhibit human chymase than **2**, which was the carboxylated derivative of **1**.

Recently, SF2809 compounds have been isolated as specific human chymase inhibitors from *Dactylosporangium* sp. [9, 10]. Compounds **1** and **2** structurally resemble SF2809-V and VI in that they possess a phenyl group and a hydroxypyridone moiety and an asymmetric methine. SF2809-V has been reported to be racemic. Our compounds **1** and **2** seem to be optically pure as analyzed by chiral HPLC though their absolute configurations remain to be established.

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