A New Isopatulin Derivative Pintulin Produced by *Penicillium vulpinum* F-4148

Taxonomy, Isolation, Physico-chemical Properties, Structure and Biological Properties

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During our screening program of natural products from fungal metabolites for drugs effective against tumor cell lines, we discovered a new isopatulin derivative, pintulin, from the fermentation broth of *Penicillium vulpinum* F-4148. Pintulin shows weak activity against tumor cell lines, compared to that of adriamycin.

In the course of our screening program for low molecular weight antitumor compounds among fungal metabolites using HL-60 cells, we have discovered a new cytocidal compound pintulin from the culture broth of *Penicillium vulpinum* F-4148. Pintulin, the structure of which was determined by spectral analysis as shown in Fig. 1, is a derivative of isopatulin[1]. The present paper describes the taxonomy and fermentation of the producing organism and the isolation, physico-chemical properties, structure and biological activities of pintulin.

Results and Discussion

Taxonomy

The strain F-4148 was isolated from a soil sample collected at Towada city, Aomori prefecture in Japan. The strain F-4148 grew well on such agar media as potato-glucose agar, Czapek-Dox agar, and YpSs agar. The morphological observations of the colonies formed on potato-glucose agar for 7 days at 25°C were performed under a light microscope.

The results were summarized in Table 1. Coremia developed vertically from the surface of the colonies on all the agar media used. Many spores were formed in the apical areas of the coremia and became claviform to spathulate. Clear to yellow brownish exudate was produced on the surface of the colonies. The coremial stalks showed deep brownish color. The conidiophores were smooth and possessed septa and their diameter was 3.6~6.2 μm. Many conidiophores developed on the colony surface branched irregularly on the way and interwinded into 3~8 mm × 200~560 μm of a dense bundle of coremia. Conidiophores branched radically and irregularly to the outside on the apical areas of a bundle of coremia, where 1~5 of phialides were verticillate. The phialide was cylindrical with a size of 6.0~17.5 μm × 2.0~3.0 μm and formed phialoconidia like a chain from a slightly thin tip. Conidia was greenish gray and smooth on the surface, and the feature was globose to subglobose, slightly slender in a part and plane. The size of conidia was 3.0~5.8 μm × 3.0~4.2 μm. The strain 4148 could grow at the range of 6 to 29°C in SABROUD

Fig. 1. Structure of pintulin.

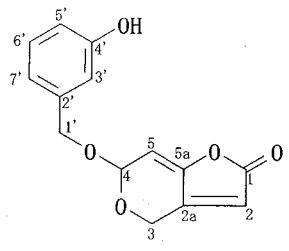


Table 1. Morphological features of *P. vulpinum* F-4148 on potato-glucose agar.

	Conidiophores (Coremia)	Phialides	Conidia
Form	(Claviform to spathulate)	Cylindric	Globose to ellipsoidal
Size	(3~8 mm × 200~560 μm)	6.0~17.5 × 2.0~3.0 μm	3.0~5.8 × 3.0~4.2 μm
Surface	Smooth	Smooth	Smooth
Color	White~reddish brown	Hyaline	Grayish green

liquid medium. The optimal temperature for the growth was 20 to 29°C in liquid medium. The strain 4148 could grow at the range of pH 3 to 10 at 26°C in YpSs liquid medium, and the optimal pH was 5 to 6.

Morphological characteristics and cultural properties of the strain 4148 indicated that it belongs to the genus *Penicillium*. Comparison of data for *Penicillium* reported by PITT[2,3] with those for the strain 4148 revealed that the strain strongly resembled *Penicillium vulpinum*. Therefore, the strain 4148 was identified as *Penicillium vulpinum* F-4148.

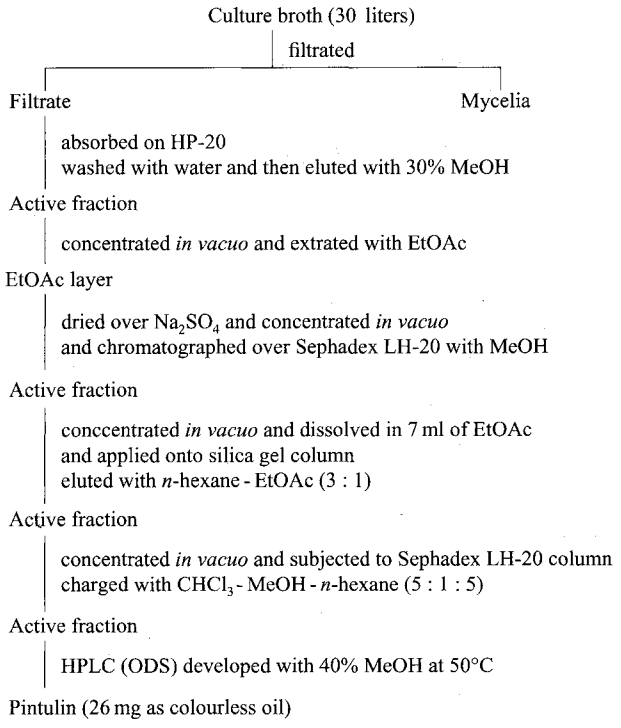
This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan with the accession No. FERM p-10944.

Fermentation and Isolation

A loopful of *Penicillium vulpinum* F-4148 on oat meal agar slant was inoculated in a 500-ml Erlenmeyer flasks containing 100 ml of medium consisting of glucose 2%, yeast extract 0.2%, NaCl 0.3%, polypeptone 0.5%, Mg_2SO_4 0.05% and $KHPO_4$ 0.1%. The inoculated flasks were cultured at 26°C for 96 hours on the rotary shaker. 400 ml of the cultured broth was transferred into a 50-liter jar fermenter containing 30 liters of the same medium. The fermentation was carried out at 26°C for 96 hours under aeration of 30 liter per minute and agitation speed 150 rpm.

The isolation procedure is shown in Fig. 2. The whole fractionation was guided by a bioassay for cytocidal activity against HL-60 cells. The cultured broth was separated into supernatant and mycelium by centrifugation. About 30 liters of supernatant was applied onto a HP-20 resin chromatographic column, which was washed with water and then eluted with 1 liter of 70% acetone. After removal of acetone, the aqueous layer was extracted with 1 liter of ethylacetate. The ethylacetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to give *ca.* 5.0 g of brownish syrup. The resultant material was chromatographied over a Sephadex LH 20 column prepared with MeOH. Active fractions were collected and concentrated *in vacuo* to yield *ca.* 2.0 g of brownish oil. The material was subjected to a silica gel column charged with chloroform. The column was eluted with $CHCl_3$-MeOH by a stepwise gradient of 0.05% increase in MeOH concentration from 0~2%. The active material was eluted with 0.2% of MeOH in $CHCl_3$. The active fractions were concentrated *in vacuo* and chromatographied over a Sephadex LH-20 column with $CHCl_3$-MeOH-*n*-hexane (5:1:5) to obtain about 100 mg of colorless oil. This material was further purified by HPLC (ODS) developed with 40% MeOH at 50°C. The active fractions were combined and concentrated to yield 26 mg of pintulin as colorless oil.

Fig. 2. Purification procedure of pintulin.



Physico-chemical Properties and Structural Elucidation of Pintulin

The physico-chemical properties of pintulin are described in Table 2. Pintulin is lipophilic, neutral in nature and gave positive color response to iodine, H_2SO_4 and vanillin-H_2SO_4, but negative to ninhydrin. The UV spectrum showed a maximum absorption at 270 nm, indicating the existence of an α, β, γ, δ unsaturated carbonyl in two ring system. The existence of a phenolic hydroxyl group was indicated by a bathchromic shift from 270 nm to 290 nm in the UV spectrum measured in basic solvent. The bands at 3350 cm^{-1} and 1780 cm^{-1} in the IR spectrum were ascribed to a hydroxyl group and a γ lactone, respectively. The positive FAB mass spectrum showed a pseudomolecular ion at m/z 261 $(MH)^+$. The molecular weight was determined to be 260 by the observation of its molecular ion in the EI mass spectrum. The molecular formula of pintulin was established as $C_{14}H_{12}O_5$ by its molecular ion measurement (M^+) at 260.0684 (calcd. 260.0685 for $C_{14}H_{12}O_5$)

Table 2. Physico-chemical properties of pintulin.

Appearance	Colorless oil
FAB-MS (+)	*m/z* 261 (M+H)
HR-MS	*m/z* 260.0684 (found)
	m/z 260.0685 calcd. for $C_{14}H_{11}O_5$
Molecular weight	260
Molecular formula	$C_{14}H_{11}O_5$
UV λ_{max}^{MeOH} nm (ε) +1 N NaOH	220 (9900), 270 (17500), 212 (16400), 239 (9500), 290 (3300)
IR ν^{KBr} cm^{-1}	3350, 1780
$[\alpha]_D^{20}$	0° (c=0.05 in $CHCl_3$)
Color reaction	Positive H_2SO_4, I_2
	Negative ninhydrin

Table 3. ^1H NMR of pintulin and ^{13}C NMR of pintulin, isopatulin and *m*-hydroxybenzyl alcohol.

No.	Pintulin		Isopatulin	*m*-Hydroxybenzyl alcohol
	^1H (δ_H)	^{13}C (δ_C)	^{13}C (δ_C)	^{13}C (δ_C)
1		168.1	165.3	
2	5.81 m	105.5	108.0	
2a		151.0	153.1	
3	4.84 dd, J=16.5, 2.2 Hz	57.3	57.0	
	4.66 dd, J=16.5, 1.3 Hz			
4	5.40 d, J=3.8	93.7	91.2	
5	5.74 dd, J=3.8, 1.8 Hz	110.2	110.5	
5a		149.5	149.3	
1′	4.54 d, J=11.5 Hz	70.3		63.5
	4.71 d, J=11.5 Hz			
2′		138.8		143.8
3′	6.78 m	114.8		114.5
4′		155.8		157.0
5′	6.72 dd, J=7.2, 1.1 Hz	115.1		114.8
6′	7.16 ddd, J=7.0, 2.5, 1.0 Hz	129.9		129.7
7′	6.84 dd, J=7.1, 0.5 Hz	120.3		117.5
4′-OH	5.03 bs			

in the high resolution EI mass spectrum in combination with the ^{13}C NMR spectrum. As the degree of unsaturation was estimated to be 9 by its molecular formula, five unsaturations were assigned to five double bonds and one to a carbonyl, leaving the final three unsaturations to accommodate three rings.

The ^{13}C NMR spectrum of pintulin showed 12 signals, consistent with its molecular formula, which were classified into the following functionalities by its DEPT spectrum; $-CH_2-\times 1$, $-OCH_2-\times 1$, $-OCH-\times 1$, $-CH=\times 6$, $-C=\times 4$ and $-CO\times 1$. It was revealed that 11 protons were bonded to 8 carbons with the existence of one hydroxyl group in the molecule. The assignment of the ^{13}C NMR spectrum is shown in Table 3. Among 12 lines given in the ^1H NMR spectrum, the line at δ_H 5.05 was exchangeable by the addition of D_2O, which was assignable to a phenolic hydroxyl group. The ^1H NMR data are summarized in Table 3.

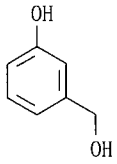
The existence of a 1,3 disubstituted benzene ring was indicated by tracing a spin network from a signal at δ_H 6.84 (H-7′) in the ^1H-^1H COSY spectrum as well as the observation of respective *meta* couplings of two lines at δ_H 6.72 (H-5′) and δ_H 6.84 (H-7′) to a resonance at δ_H 6.78 (H-3′).

The structural determination of pintulin was performed by extensive analysis of its 2D NMR spectra, especially its HMBC spectrum. The long range relationships are summarized in Table 4. Analysis of the HMBC spectrum led to the detection of a *m* substituted benzyl ring by tracing long range correlation maps from an unequiavalent methylene of H-1′ a and b to C-2′, C-3′ and C-7′, from H-3′ to C-1′, C-5′ and C-7′, from H-6′ to C-1′, C-4′ and C-2′, and from H-7′ to C-1′, C-3′ and C-5′, respectively (Table 4). The location of a hydroxyl group at C-4′ was suggested by the ^{13}C chemical shift trends of C-3′, C-4′ and C-5′ as shown in Table 3.

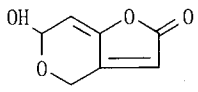
Table 4. ^{1}H-^{13}C long range coupling data of pintulin.

No.	^{13}C (δ_C)	Long range couplings (δ_H)
1	168.1	H-2 (5.81)
2	105.5	H-3a (4.84) and H-3b (4.66)
2a	151.0	H-3a (4.84) and H-3b (4.66), H-2 (5.81), H-5 (5.74)
3	57.3	H-4 (5.40)
4	93.7	H-3a (4.84) and H-3b (4.66), H-1'a (4.54) and H-1'b (4.71)
5	110.2	
5a	149.5	H-4 (5.40), H-5 (5.74)
1'	70.3	H-4a (5.40), H-3' (6.78), H-7' (6.84)
2'	138.8	H-1'a (4.54) and H-1'b (4.71), H-6' (7.16)
3'	114.8	H-1'a (4.54) and H-1'b (4.71), H-5' (6.72), H-7' (6.84)
4'	155.8	H-6' (6.72)
5'	115.1	H-3' (6.78), H-7' (6.84)
6'	129.9	
7'	120.3	H-1'a (4.54) and H-1'b (4.71), H-3' (6.78), H-5' (6.72)

Fig. 3. Structures of *m*-hydroxybenzyl alcohol and isopatulin.



m-Hydroxy benzylalcohol



Isopatulin

Table 5. *In vitro* cytocidal activity against various tumor cell lines.

Cell line	IC_{50} (μg/ml)	
	Pintulin	Adriamycin
P388	0.8	0.02
L1210	0.8	0.06
HL-60	0.8	0.02
KB	0.25	0.02

The partial structure of –O–CH–CH= was deduced by tracing a network from a signal at δ_H 4.42 (H-4) to a line at δ_H 4.74 (H-5) in the ^{1}H-^{1}H COSY spectrum. Similarly unequivalent methylene protons at δ_H 4.66 and 4.75 was assigned, which coupled to a line at δ_C 67.5 in the ^{1}H-^{13}C COSY spectrum. A pyran ring was assigned by the observation of long range couplings from H-3a and b to C-2a and C-2, from H-3b to C-4 and C-5a and from H-4 to C-3 and C-5a (Table 4). Two cross peaks from H-4 to C-1' and from H-1'a and b to C-4 in the HMBC spectrum allowed a *m*-hydroxyl benzyl group to be substituted at C-4 as shown in Fig. 1. The HMBC spectrum showed long rang correlations from H-4 to C-5a, from H-3a and b to C-2a and C-2, from H-3b to C-5a, from H-5 to C-5a and C-2a and from H-2 to C2a, C-5a and C-1 as shown in Table 4. Taking into consideration the absorption at 1780 cm^{-1} due to a γ lactone system in the IR spectrum and the unsaturated degrees, the structure of pintulin was established as shown in Fig. 1.

As the structure of pintulin was reminiscent of isopatulin and *m*-hydroxybenzyl alcohol, the structure was also confirmed by comparison of the ^{13}C NMR spectrum of pintulin with those of isopatulin and *m*-hydroxybenzyl alcohol as shown in Table 3. They showed compatibility of their chemical shift values and functionalities except for downfield shift of a oxymethylene of C-1' by *ca.* 6.8 ppm. SEKIGUCHI *et al.*[1] reported that isopatulin was optically inactive. The $[\alpha]_D$ of pintulin was 0°, indicating that pintulin was in a racemic form as well as isopatulin.

It is reported that isopatulin is a intermediate of patulin[4,5] and biosynthesized *via* oxidative cleavage of a polyketide-derived aromatic intermediate 6-methylsalicylic acid[6], which is derived from *m*-hydroxybenzyl alcohol[7], in *Penicillium urticae*[7] and *Penicillium patulum*[8]. Although we isolated isopatulin and *m*-hydroxybenzyl alcohol but not patulin from the fermentation broth of *Penicillium vulpinum* F-4148, our producing organism might have the same biosynthetic pathway as *Penicillium urticae* in the production of isopatulin. The structure of pintulin led to the speculation that pintulin is produced by coupling of isopatulin with *m*-hydroxybenzyl alcohol through some transferase.

Biological Activities of Pintulin

As described in Table 5, pintulin exerted ten to forty times weaker cytocidal activity against various cultured cell lines than adriamycin.

Administrations of 1.25 mg/kg of pintulin at days 1 and 3 showed a little effect against mice transplanted with p388 leukemia cells (i.p.-i.p.) (data not shown). And pintulin did not prolong the survival time of mice and showed toxicity in the experiment of continuous administrations for 5 days at the concentration of 3.13 mg/kg (data not shown). Pintulin showed little inhibitory activity against all microbe tested.

Experimental

General

Melting point was determined with a Yanagimoto micro-melting apparatus and uncorrected. Optical rotation was measured on a Jasco DIP-360 polarimeter in 10 cm tube. IR spectrum was recorded on a Perkin-Elmer 1760 FT-IR spectrophotometer. UV spectrum was measured on a Hitachi 220 spectrophotometer. EI-MS, HR-EI and FAB-MS spectra were determined with a Jeol JMX-SX 102 mass spectrometer. NMR spectra were obtained in $CDCl_3$ with a Jeol JMN-GX400 at ambient temperature with ^1H NMR at 400 MHz and ^{13}C at 100 MHz using solvent peaks as internal references downfield of TMS at 0 ppm.

Taxonomy

The media used for the identification of the fungus were; CZAPEK yeast extract agar, Czapek-Dox agar, YpSs agar, oat meal agar, potato-glucose agar, MIURA agar, SABROUD agar and malt extract agar. Morphological observations were made on potato-glucose agar after 7 days incubation at 26°C unless stated otherwise.

Color names and hue numbers indicated in Table 1 are adopted from the Book of JIS (Japanise Industrial Standard) Color Standards (JISZ 8721).

Cytocidal Activity

HL-60, P388 and L1210 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. KB cells were grown in DULBECO's modified EAGL's medium supplemented with 10% calf serum. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded into 96-well microtiter plates (2×10^4 cells/well) and incubated for 24 hours. The test sample, dissolved in MeOH, was added in serial dilution. After addition, the plates were incubated for 72 hours. For the evaluation of *in vitro* cytocidal activity, a miroculture tetrazolium assay (MTT assay) method was used. The IC_{50} value was calculated with PROBIT's method.

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