FOLIPASTATIN, A NEW DEPSIDONE COMPOUND FROM *Aspergillus unguis* AS AN INHIBITOR OF PHOSPHOLIPASE A_2

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL PROPERTIES

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A new inhibitor of phospholipase A_2 was isolated from the fermentation broth of *Aspergillus unguis*. The structure, with a depsidone carbon skeleton, was assigned by spectroscopic experiments.

In the course of calcium blocker screening of microbial metabolites, we found that a strain of fungus, SANK 16888, produced a novel depsidone compound. It inhibited the binding of ^{3}H-nitrendipine to porcine heart microsomes potently. It also inhibited inflammatory phospholipase A_2 at both the enzyme and cellular levels. The compound, designated folipastatin (**1**), was recovered from culture broth by solvent extraction and was purified by a series of chromatographies and recrystallization. The chemical structure of folipastatin was elucidated principally by ^{1}H NMR and ^{13}C NMR spectroscopy.

This paper describes the taxonomy and fermentation of the producing organism, and the isolation, structure determination and biological properties of folipastatin.

Materials and Methods

Discovery Screen

Originally **1** was found by a radioligand binding assay of ^{3}H-nitrendipine, as described previously[1]. Contraction of taenia was also tested as described previously[1].

Assay of Phospholipase A_2

Enzyme Assay: Peritoneal exudates were collected 18 hours after intraperitoneal injection of rabbits with 2% sodium caseinate. Phospholipase A_2 was partially purified by CM-Sephadex according to the method of FRANSON *et al.*[2]. The reaction mixture (0.5 ml) contained 100 mM HEPES (pH 7.5), 5 mM $CaCl_2$, [^{14}C]-arachidonylphosphatidylcholine (52.6 mCi/nmol), and the enzyme preparations. The incubation was carried out at 37°C for 20 minutes. The reaction was stopped by adding 2 ml of DOLE's reagent[3]. One ml of *n*-heptane and 0.5 ml of H_2O were added and the radioactivity of the released [^{14}C]-arachidonic acid in the heptane layer was measured[4]. Folipastatin was dissolved in DMSO and added to the reaction mixture in such a way that the final concentration of DMSO did not exceed 1%.

Cellular Assay: Polymorphonuclear leukocytes (PMNLs) were obtained from rat peritoneal exudates after 16 hours from intraperitoneal injection with 1% sodium caseinate. The PMNLs were labeled with [^{14}C]-arachidonic acid (52.8 mCi/nmol) at 37°C in phosphate-buffered saline (PBS) containing 5.6 mM glucose, 0.1% bovine serum albumin (BSA), and 0.3 mM ethylene glycol bis(β-aminoethyl ether)-

N,N,N',N'-tetra acetic acid (EGTA). The labeled PMNLs were washed and resuspended with 450 μl of PBS containing 5.6 mM glucose, 0.1% BSA, and 0.8 mM $CaCl_2$. The reaction was started by adding 50 μl of 1 μM *N*-formyl-Met-Leu-Phe, then it was stopped after incubation for 20 minutes at 37°C by adding 500 μl of PBS containing 10 mM EDTA. The reaction mixture was centrifuged at 1,500 $\times g$ for 5 minutes and the radioactivity of the released [^{14}C]-arachidonic acid in the supernatant was measured.

Jar Fermentation and Processing

All fermentation was carried out using GPMY medium consisting of (%): Glycerol (5.0), potatoes (5.0), malt extract (0.5) and yeast extract (0.5), without adjustment of pH. 500 ml baffled flasks containing 100 ml GPMY medium were inoculated with the culture of strain SANK 16888 grown on PDA slants. The inoculated flasks were cultured at 26°C for 3 days on a rotary shaker at 200 rpm. A 300 ml seed culture was used to inoculate 15 liters of sterilized GPMY medium. The jar fermenter was stirred at 100 rpm with aeration at 1.0 v/v/minute at 26°C for 7 days when it was harvested. After filtration, **1** associated with the mycelium was recovered by successive extraction with 35 liters of acetone and 15 liters of 80% aqueous acetone, for 1 hour each with stirring at room temperature. Combined extracts were concentrated under reduced pressure. The resulting water phase was adjusted to pH 6.5 and extracted twice with equivalent volumes of ethyl acetate. Broth filtrate was adjusted to pH 7.0 and extracted with ethyl acetate. The resulting organic phases were separately concentrated to syrupy residues under reduced pressure. They were combined together to give crude extract.

Analytical HPLC

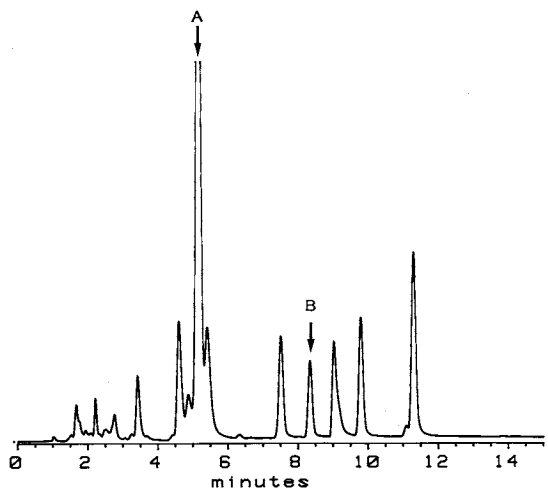
For analytical HPLC, a C_{18} reversed phase column (4 μm, 8.0 $\times$ 100 mm) was employed. A linear gradient of (A) 80% triethylammonium phosphate (0.1%, pH 3.0) - 20% acetonitrile and (B) 20% triethylammonium phosphate (0.1%, pH 3.0) - 80% acetonitrile, with 50% B at the start rising to 100% B over 10 minutes (flow rate 2 ml/minute) was used to separate all of the compounds in crude extract. The compounds were detected by UV absorption at 210 nm. Fig. 1 shows the separation of all of the components in crude extract. Two peaks indicated by arrows inhibited ^{3}H-nitrendipine binding to porcine heart membrane. For routine analysis, the column was eluted with acetonitrile - H_2O (5 : 5) at 2 ml/minute, and retention time of **1** was 8.7 minutes.

Spectroscopic Method

All NMR spectra were recorded on a Jeol JNM-GX400 spectrometer operated at 399.8 MHz for

Fig. 1. HPLC chromatogram.

A: Unguinol (**2**), B: Folipastatin (**1**).



^{1}H and 100.5 MHz for ^{13}C. Tetramethylsilane was used as an internal reference for ^{1}H NMR in the acetone-d_6 solution. For the ^{13}C chemical shift reference, the ^{13}C signal at δ 29.8 ppm of acetone-d_6 was used.

Results

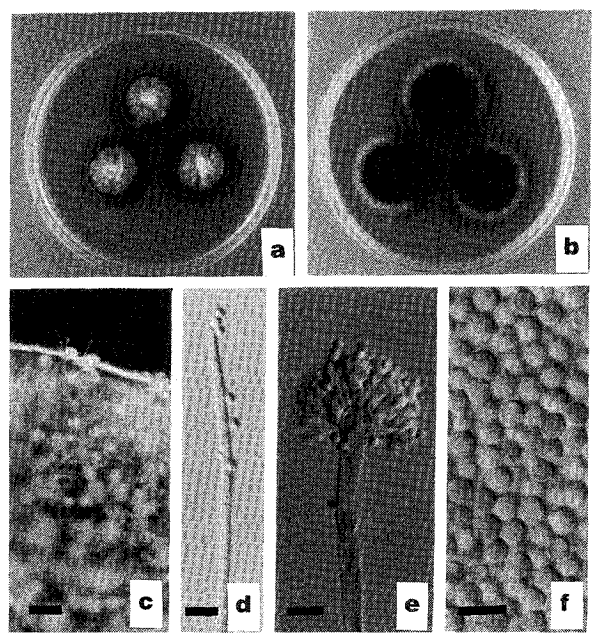
Microorganisms

The fungal strain SANK 16888 was isolated from dust collected in a building air supply duct. The strain was identified by the method described by KLICH and PITT[5]. A colony on Czapek yeast extract agar (CYA) is 2.3 cm in diameter after culturing at 25°C for 7 days as shown in Fig. 2a. The mycelium on the surface is dense and velutinous, sulcate, and slightly umbonate at the center due to floccose aerial hyphae. Production of conidia is abundant and it shows deep green color at the marginal area, light brown at the center. The color of the reverse colony is brown. A colony on malt extract agar (MEA) (Fig. 2b) is 3.2 cm in diameter (25°C, 7 days). The surface of the colony is plane, velutinous, and deep green all over, except for a white marginal area. White aerial mycelium developed at the center. The reverse colony is grayish brown. Colony on CYA with 20% sucrose is 4.0 cm in diameter (25°C, 7 days). The colony appeared to be similar to that on CYA, except for reverse color, which was pale yellow. No exudation is observed on CYA. Soluble pigment is produced on CYA at 37°C; it is pastel yellow. Colonies on CYA at 37°C are 1.5 cm in diameter.

Conidial heads are columnar on MEA, radiating on CYA. On CYA, white spicular hyphae (Fig. 2d) develop. Spicular hyphae are abundant at the colony margin (Fig. 2c). The stipe wall of the conidiophore

Fig. 2. *Aspergillus unguis* SANK 16888.

a, Colony on CYA. b, Colony on MEA. c, Close up of the margin of the colony on CYA which shows extending spicular hyphae; bar = 100 μm. d, Tip of spicular hyphae. Note the sterile tip and septate hyphal structure; bar = 100 μm. e, Conidiophore with conidia, phialides, and metulae; bar = 10 μm. f, Conidia of SANK 16888; bar = 5 μm.



is smooth, thick, and brown (Fig. 2e). A vesicle is $8\sim18\,\mu m$ in diameter, ampulliform to clavate. The upper $1/3\sim2/3$ of the vesicle is covered by metulae ($7\sim10\times3\sim4.5\,\mu m$), each of which bear flask-shaped phialides ($6.5\sim7.0\times2.5\sim4\,\mu m$) (Fig. 2e). Conidia are spherical and smooth to slightly rough (Fig. 2f). No perfect state is observed in this strain.

From the characteristics stated above, the strain SANK 16888 was identified as *Aspergillus unguis* (Emile-Wiel & Gaudin) Thom & Raper.

Fermentation

Fig. 3 shows a typical time course of the fermentation of strain SANK 16888 in 30-liter jar fermenter. A maximum titer of 350 μg/ml of **1** was achieved after 168 hours.

Isolation

Crude extract (54 g from 15 liters culture) was chromatographed in approximately 20 g portions with reverse phase HPLC (Kurita Industries Co., Ltd., ODS, 100 × 500 mm) using acetonitrile-H_2O (6:4) as the eluent at 200 ml/minute. The peak with retention time 35~50 minutes was collected, evaporated and rechromatographed on Senshu ODS-H-5251 using acetonitrile-H_2O (6:4) as the eluent at 6 ml/minute. The peak with retention time 24 minutes was collected and recrystallized with a mixture of ethanol-H_2O (1:1), to yield 2.8 g of **1** as colorless crystals.

Structure and Physico-chemical Properties

EI-MS of folipastatin gave parent ion at *m/z* 380 (M^+) and HREI-MS gave M^+ at *m/z* 380.1601 ($C_{23}H_{24}O_5$ calcd 380.1579), indicating a molecular formula of $C_{23}H_{24}O_5$ for folipastatin (**1**). In the ^1H NMR spectrum of **1**, the aryl coupled methyl/olefinic proton pairs at 2.06~5.54 ppm and 1.88~5.38 ppm which are coupled with methyl protons at 1.78 and 1.68 ppm, respectively, were identified to form two sets of 1-methyl-1-propenyl groups. In addition to these substituents, were observed two singlet aromatic protons (6.62, 6.50 ppm), two phenolic protons (8.50, 9.12 ppm) and two aromatic methyl protons (2.18 ppm) with identical chemical shifts. Among 23 carbon signals identified in the ^{13}C NMR spectrum, those with directly bonded protons could be assigned straightforwardly by analysis of the ^1H-^{13}C COSY spectrum (Table

Fig. 3. Time course of folipastatin production.

● pH, □ packed cell volume, ○ folipastatin.

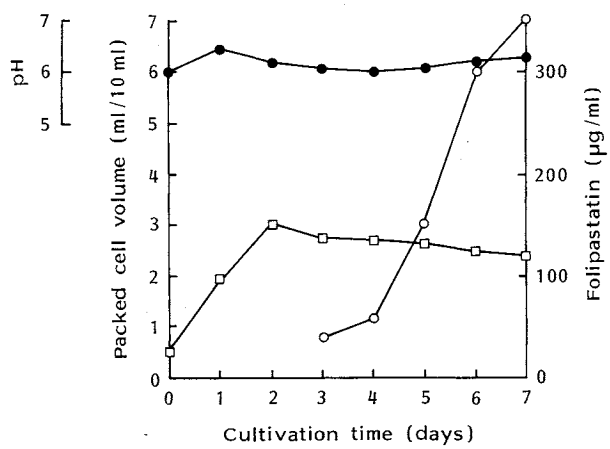


Table 1. Physico-chemical properties of folipastatin.

Appearance	Colorless needles
MP (°C)	246~248
Molecular formula	$C_{23}H_{24}O_5$
Elemental analysis	
Calcd:	C 72.61, H 6.36
Found:	C 72.55, H 6.40
HREI-MS (*m/z*) Calcd:	380.1579
Found:	380.1601 (M^+)
UV λ_{max} nm (ε)	
in EtOH	210 (43,900), 278 (10,074)
in 0.1 N NaOH+EtOH (1:100)	207, 320
IR (KBr) cm^{-1}	1670, 1423, 1276, 1266, 1131

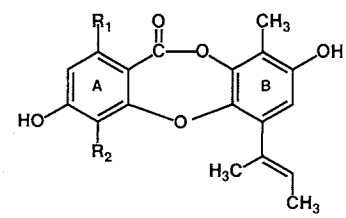
Table 2. Proton chemical shifts and coupling constants of folipastatin.

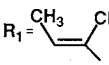
Assignment	δ (ppm)	Multiplicity (J=Hz)	No. of proton
2	6.62	s	1
4-Me	2.18	s	3
7	6.50	s	1
9-Me	2.18	s	3
2′	5.54	q, q (6.9, 1.4)	1
3′	1.78	d, q (6.9, 1.1)	3
4′	2.06	quintet (1.2)	3
2″	5.38	q, q (6.9, 1.4)	1
3″	1.68	d, q (6.9, 1.1)	3
4″	1.88	quintet (1.2)	3
OH	8.50, 9.12	br	1, 1

Table 3. ^{13}C NMR spectral data of folipastatin.

Assignments	δ (ppm)	Multiplicity
1	149.3	s
2	112.9	d
3	159.9	s
4	114.8	s
4a	163.0	s
5a	143.6	s
6	137.2	s
7	112.1	d
8	153.2	s
9	116.0	s
9a	144.8	s
11	164.3	s
11a	113.4	s
1′	135.1	s
2′	126.1	d
3′	14.1	q
4′	18.1	q
1″	136.9	s
2″	124.7	d
3″	14.2	q
4″	17.8	q
4-Me, 9-Me	8.9, 9.3	q, q

Fig. 4. Structure of folipastatin (**1**) and unguinol (**2**).



Folipastatin (**1**) R_1 =  $R_2 = CH_3$

Unguinol (**2**) $R_1 = CH_3$ $R_2 = H$

3). Based on these NMR data and the other physico-chemical properties summarized in Table 1, **1** was suggested to be a novel depsidone that has an additional 1-methyl-1-propenyl group as compared to unguinol[3], **2**.

The location of substituents on each aromatic ring could be determined by the analysis of ^1H-^{13}C long range couplings obtained from the COLOC spectrum (Fig. 5), with the consideration of the effect of phenolic and/or ether substituents on the ^{13}C chemical shifts of the benzene ring. As the observable ^1H-^{13}C long range correlations are practically restricted to the proton-carbon pairs that are separated by two or three bonds, the presence of long range coupling between C-1″ (136.9 ppm) and 2-H (6.62 ppm) indicated the location of one 1-methyl-1-propenyl group at the ortho position to 2-H. The carbon signals at 159.9 and 114.8 ppm, which were long range coupled with 2-H, could be assigned to C-3 and C-4, respectively, to account for the upfield shifts of C-2 (112.9 ppm) and C-4 (114.8 ppm) caused by the phenolic or ether substituent on C-3 (159.9 ppm). Thus the remaining carbon signal at 113.4 ppm, which was also long range coupled with 2-H was assigned to C-11a. One of the methyl groups resonating at 2.18 ppm could be located at C-4 due to the ^1H-^{13}C long range correlations with C-3 and C-4. The assignment of the C-4a carbon

Fig. 5. Summary of ^{1}H-^{13}C long range correlations from a COLOC spectrum of folipastatin.

The arrow (←) points from ^{13}C to ^{1}H.

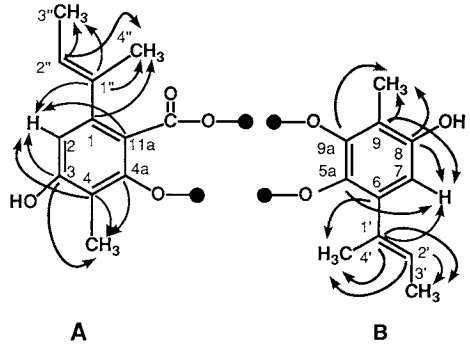
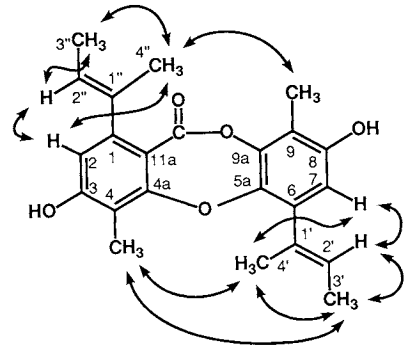


Fig. 6. The key NOE from a NOESY spectrum of folipastatin.

The double-headed arrow indicate NOE correlation.



signal based on the long range correlation with methyl protons at 2.18 ppm alone was ambiguous due to the coexistence of the 9-Me signal at the same position. However it seemed relevant to assign a carbon signal at 163.0 ppm to C-4a, considering the *meta*-disubstitution of ether and phenolic groups on ring A. The introduction of the ether substituent on C-4a would explain the upfield shifts of C-4 and C-11a. Thus, the ring A of **1** could be established as shown in Fig. 5. The ring B of **1** could be derived from analysis of the ^{1}H-^{13}C long range correlation essentially in the same fashion as described above. The *ortho*-disubstitution of –OR and –O(C=O)R groups at C-5a and C-9a was suggested by their chemical shifts.

Table 4. Biological activities of folipastatin *in vitro*.

	IC_{50}: μM (μg/ml)	
	Folipastatin	Unguinol
^{3}H-Nitrendipine binding	0.39 (0.15)	1.69 (0.55)
Contraction of taenia	>100	>100
Phospholipase A_2	39 (15)	104 (34)
Arachidonate release	24 (9)	98 (32)

As shown in Fig. 6, the A and B rings of **1** could be connected by the NOE cross peaks between 4-Me and 4′,3′-Me and between 9-Me and 4″-Me observed on the NOESY spectrum. The *trans* configurations of 1-methyl-1-propenyl groups were also derived from the NOEs observed between 3′-Me and 4′-Me, and between 3″-Me and 4″-Me.

Biological Properties

Folipastatin inhibited the binding of ^{3}H-nitrendipine to porcine heart membrane potently, with an IC_{50} value of 0.39 μM (0.15 μg/ml). Unguinol inhibited the binding with an IC_{50} value of 1.69 μM (0.55 μg/ml). The contraction of taenia induced by 1 mM Ca^{2+}, however, was only slightly inhibited by folipastatin or unguinol, and the potencies were less than 1% of dilitiazem. Blood pressure of spontaneous hypertensive rats was also unaffected by iv injection of 300 μg/kg folipastatin.

On the other hand, folipastatin inhibited the phospholipase A_2 purified from rabbit peritoneal exudate with an IC_{50} value of 39 μM (15 μg/ml), and it suppressed the release of arachidonic acid from rat polymorphonuclear leukocytes (IC_{50} = 24 μM, 9 μg/ml). These data are summarized in Table 4. Folipastatin exhibited only a weak antibacterial activity against *Bacillus subtilis*. No acute toxicity was observed up to 100 mg/kg ip in mice.

Discussion

Folipastatin was found by ^{3}H-nitrendipine binding assay. Using the same method, we previously

isolated leualacin, a new cyclic peptide[1]. Leualacin inhibited the contraction of taenia with similar potency to diltiazem. Folipastatin inhibited ^{3}H-nitrendipine binding more potently than leualacin, as indicated by the IC_{50} value of 0.39 μM (0.15 μg/ml) *versus* 1.75 μM (1.0 μg/ml) for leualacin. The contraction of taenia, on the other hand, was negligibly affected by folipastatin, suggesting that the binding affinity to the L-type Ca channel does not correlate with the pharmacological potency of the compounds. On the other hand, the compound was shown to inhibit phospholipase A_2.

Among microbial metabolites, duramycins and cinnamycins are reported to be inhibitors of phospholipase A_2[7]. Folipastatin is a depsidone, and it has nothing in common with these peptide antibiotics. The inhibition of secreted phospholipase A_2 by folipastatin was reversible and noncompetitive, with a *Ki* value of 35 μM. When administered topically, folipastatin showed anti-inflammatory activity to the 12-*O*-tetradecanoylphorbol 13-acetate-induced ear edema at 0.5 mg per rat or mouse (HISAMOTO *et al.*, manuscript in preparation). Folipastatin is more potent than unguinol in inhibiting phospholipase A_2 as well as ^{3}H-nitrendipine binding (Table 4). This potency is attributed to the additional 1-methyl-1-propenyl group in its structure, and the introduction of this group contributes to increased hydrophobicity. There are several depsidones besides unguinol, such as 2-chlorounguinol[8], nidulin[9] and emeguisins[10]. Therefore, more detailed study on the structure-activity relationship will be informative in evaluating the anti-inflammatory effects of depsidones.

Phospholipase A_2 used in the present study is 14 kdaltons enzyme. The specificity of folipastatin to this enzyme and the 100 kdaltons enzyme recently described[11] is a subject of current interest.

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