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Funalenone, a Novel Collagenase Inhibitor Produced by *Aspergillus niger*

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Funalenone, a phenalene compound that inhibits type I collagenase (MMP-1), was isolated from mycelium of *Aspergillus niger* FO-5904 by solvent extaction, ODS column chromatography, Sephadex LH-20 column chromatography and reversed phase HPLC. Funalenone inhibited 50% of type I collagenase activity at a concentration of 170 μM, but inhibited 18.3% and 38.7% against 72 kDa and 92 kDa type IV collagenase, respectively, at a concentration of 400 μM.

Collagenase (MMP-1) is a family of matrix metalloproteinases which contain Zn^{2+} at their active center and specifically cleaves mammalian triple helical collagen at an unique cleavage site[1]. Degradation of collagen is often observed in several pathological situations such as diabetic retinopathy, psoriasis and rheumatoid arthritis, and represents a key step in the development of these pathologies[2~4]. Therefore, the discovery of potent and specific inhibitors of the enzyme will be of potential clinical value in the treatment of such diseases.

In the course of our screening program aimed at collagenase inhibitors obtained from microorganisms, a new phenalene compound, designated funalenone, was isolated from a culture broth of *Aspergillus niger* FO-5904. The present paper deals with the taxonomy of the producing organism, fermentation, isolation, physicochemical characterization, structure elucidation and biological properties of funalenone.

Materials and Methods

Instruments

^1H and ^{13}C NMR spectra were obtained with a Varian Unity 400 spectrometer using DMSO-d_6 as a solvent. The DMSO-d_6 signals (2.48 ppm of ^1H and 39.5 ppm of ^{13}C) were used as references. Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. UV and IR spectra were measured with a Beckman DU 640 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer, respectively. Melting point was measured with a Yanaco micro melting point apparatus MP-S3 and was uncorrected.

Materials

Pro-collagenase was prepared from cultured cells of human normal skin fibroblast cells, Fibrocell NHDF (Kurabou Co., Ltd.), as described by OKADA *et al.*[5] Type IV collagenase (MMP-2 and MMP-9) , FITC labeled type I collagen and type IV collagen were purchased from Cosmo Bio Co., Ltd., Japan.

Type I Collagenase Assay

The type I collagense assay was carried out as described by NAGAI *et al.*[6] with some modifications.

Pro-collagenase was converted to collagenase prior to assay by trypsin. The pro-collagenase (0.5 units) was incubated for 5 minutes at 37°C with trypsin (0.2 mg/ml of final concentration) in a total volume of 20 μl containing 50 mM Tris HCl, 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3 (pH 7.5). Soybean trypsin inhibitor (1 mg/ml of final concentration) was added and further incubated for 5 minutes at 37°C. The assay was carried out in a total volume of 150 μl containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 250 μg FITC-collagen (type I), and trypsin activated type I collagense

(0.5 unit). The mixture was incubated for 1 hour at 37°C. *O*-phenanthrolin was added in a final concentration of 2.6 mM and further incubated for 1 hour at 37°C. After cooling to room temperature, equal volume of 70% ethanol containing 15 mM Tris HCl, 45 mM NaCl, and 3 mM $CaCl_2$ (pH 7.5) was added and chilled in an ice bath for 30 minutes. Supernatant fluid (150 μl) obtained from centrifugation at 2000 rpm for 15 minutes was transferred into 96-well fluoroplate (Nunc) and the fluorescent (Ex 485 nm/Em 538 nm) was measured by Fluoroscan II (Labsystems).

Other Enzyme Assay

Type IV collagenase assay was performed by Type IV collagenase kit according to the protocol.

Antimicrobial Activity

Antimicrobial activity was measured against 14 species of microorganisms. Media for each microorganism are as follows: GAM agar (Nissui Seiyaku Co.) for *Bacteroides fragilis*; Bacto PPLO agar (Difco) supplemented with 10% horse serum and 0.1% glucose for *Acholeplasma laidlawii*; nutrient agar for the other bacteria; a medium composed of 1.0% glucose, 0.5% yeast extract and 0.8% agar for yeasts and fungi. A paper disc (i.d. 8 mm) containing 50 μg of sample was placed on an agar plate seeded with a test microorganism. Bacteria were incubated for 24 hours at 37°C except *Xanthomonas camperstris* pv. *oryzae*. Yeasts and *X. camperstris* were incubated for 24 hours at 27°C. Fungi were incubated for 48 hours at 27°C. Antimicrobial activity was expressed as diameter of inhibitory zone.

Results and Discussion

Taxonomy of Producing Strain FO-5904

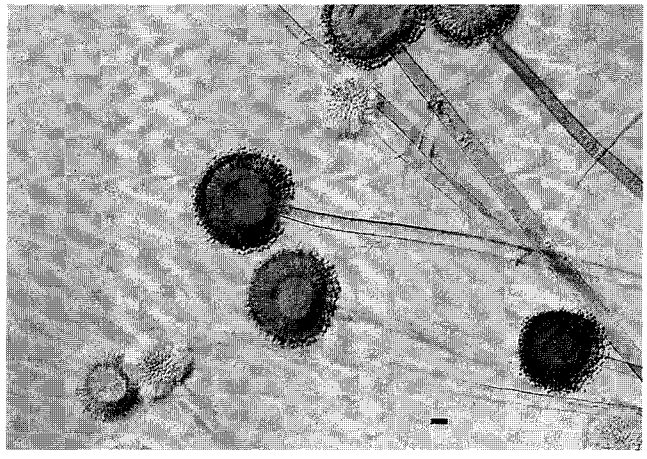
Fungal strain FO-5904 which produces funalenone was originally isolated from a soil sample collected in Funabashi City, Chiba, Japan. Czapek agar, malt extract agar, czapek yeast extract agar, Czapek yeast extract agar with 20% sucrose and oat meal agar were used for identification of the strain FO-5904.

The colonies on czapek yeast extract agar grew restrictedly, reaching a diameter of 70 mm after incubation at 25°C for 7 days. The surface of colonies was granular to velvety and dark brown in color. The reverse side of colonies was pale yellow in color. No soluble pigment was produced.

Morphological observation was carried out under a microscope (Olympus Vanox-S AH-2). When grown on malt-extract agar at 25°C for 7 days, the conidiophores were borne from substrate hyphae. Aspergilla were almost exclusively biseriate and formed metulae and phialides (Fig. 1). The metulae was 16 to 20 μm in length and 5 to 8 μm in size. The phialides were 5 to 8 μm in length and 4 to 6 mm in size. The phialidic conidia were globose or subglobose, 4 to 6 μm in diameter and brown in color.

Fig. 1. Photomicrograph of aspergilla of strain FO-5904.

Bar represents 20 μm.



From the above characteristics, strain FO-5904 was identified as *Aspergillus niger* and named *Aspergillus niger* FO-5904. This strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM P-15397.

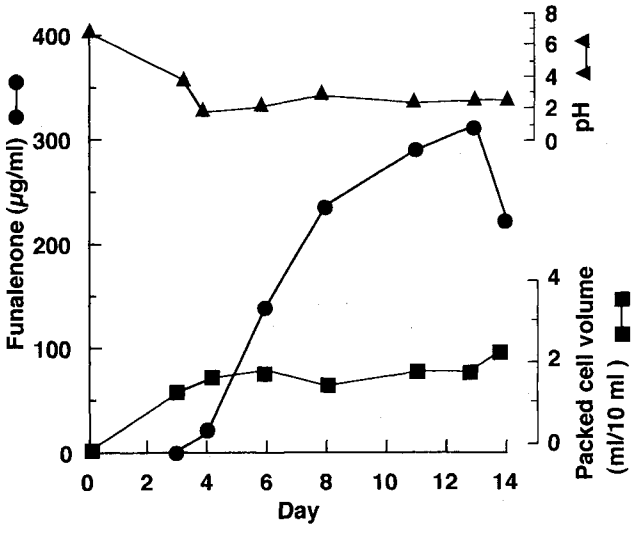
Fermentation and Isolation

A spore suspension (1×10^9 CFU/ml) of the strain FO-5904 was inoculated into each of twelve 1000-ml culture bottles containing 250 ml of the fermentation medium (sucrose 2.0%, glucose 1.0%, corn steep powder 0.5%, meat extract 0.5%, KH_2PO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.05%, $CaCO_3$ 0.3%, and agar 0.1%, pH 6.0) and the bottles were incubated at 27°C for 13 days. The production of funalenone was measured by HPLC under the following conditions: column, Senshu pak Pegasil ODS, i.d. 4.6×250 mm; mobile phase, CH_3CN - 0.5% H_3PO_4 (6 : 4); detection, flow rate, 1.0 ml/minute; UV at 210 nm. For this measurement, the culture broth was centrifuged. The precipitated mycelium was extracted with the equal volume of acetone and the extract was concentrated to one tenth

volume *in vacuo*. Ten microliters of the solution was provided for the HPLC analysis. Under these conditions, funalenone was eluted as a peak with retention time of 13.1 minutes. A typical time course of the production of funalenone is shown in Fig. 2. The production of funalenone started at day 4 and the concentrations reached maxmal level (250 μg/ml) at 11~13 days after inoculation.

Three liters of the culture broth was separated into a supernatant fluid and mycelial cake. The mycelial cake was extracted with acetone and concentrated under reduced pressure. The mycelial extract was adjusted to pH 3 with 6 N HCl and extracted three times with equal volumes of ethyl acetate. The organic layer was combined and concentrated under reduced pressure. The resulting dark brown oil was washed with *n*-hexane to remove the impurities, and the residue was dried *in vacuo* to obtain a crude material (3.03 g). The residue was dissoloved in a small volume of methanol, and applied on a ODS silica gel column (i.d. 38×400 mm, ODS R-30608, Senshu Scientific Co., Ltd.) prepared with 10% aqueous CH_3CN. The column was washed with 50% aqueous CH_3CN and funalenone was eluted with 80% and 100% CH_3CN. The active fractions were concentrated under reduced pressure to give a dark green powder (820.8 mg). The powder was dissolved in a small volume of ethanol and applied on a Sephadex LH-20 column (i.d. 45×600 mm) equilibrated with ethanol and eluted with ethanol. The active fractions were combined and concentrated under reduced pressure to afford a yellow powder (453.5 mg). It was further purified by HPLC under the following conditions: column, YMC Pack ODS (i.d. 20×250 mm, YMC Co., Ltd.); mobile phase, CH_3CN-0.1% H_3PO_4 (2 : 3); flow rate, 8.0 ml/minute; detection, UV at 220 nm. Funalenone was eluted at 17 minutes under the above conditions. The active eluate of HPLC was concentrated to remove CH_3CN, extracted with ethyl acetate at pH 3, and concentrated to dryness to give a yellow powder of funalenone (97.5 mg).

Fig. 2. A typical time course of funalenone production by *Aspergillus niger* FO-5904.



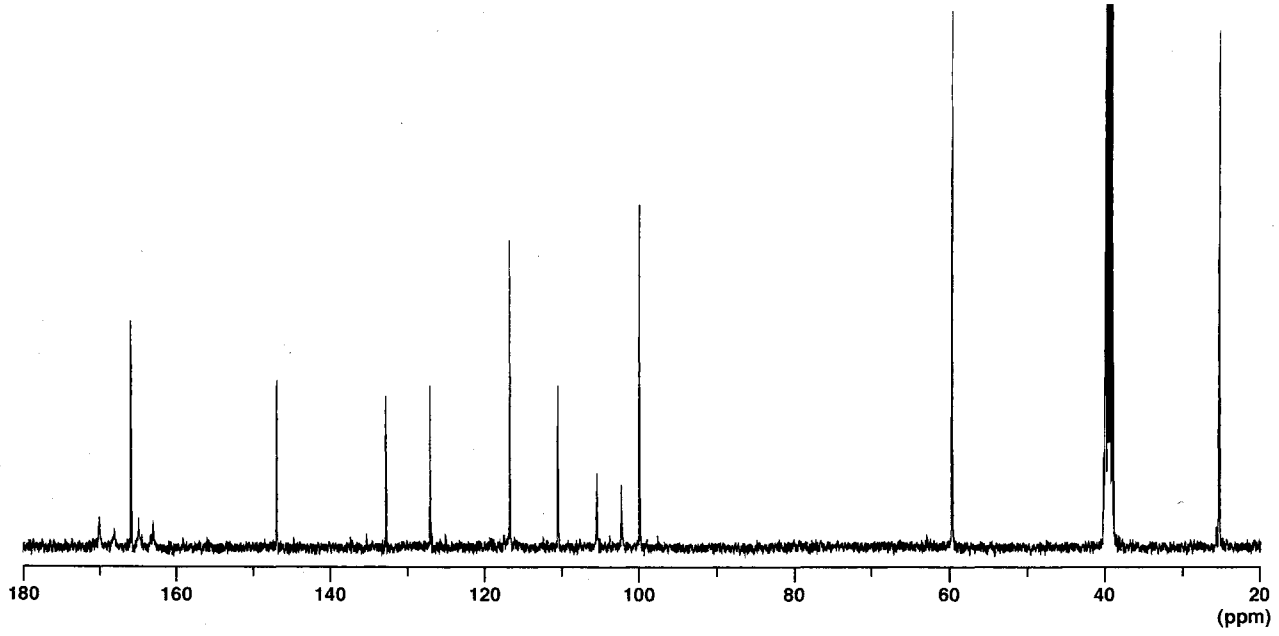
Physico-chemical Properties

The physico-chemical properties of funalenone are summarized in Table 1. The molecular formula of

Table 1. Physico-chemical properties of funalenone.

Appearance		Yellow powder
Molecular formula		$C_{15}H_{12}O_6$
Molecular weight		288
HRFAB-MS (*m/z*):	calcd	289.0712 $(M+H)^+$
	found	289.0716 $(M+H)^+$
UV λ_{max}^{MeOH} nm (ε)		254(sh, 20700), 280 (sh, 12600),
		368 (sh, 16200), 402.5 (19800)
IR ν_{max} (KBr)cm^{-1}		3160, 2990, 2360, 1616, 1500, 1460, π
		1385, 1270, 1134, 1040, 890, 830
Solubility:	soluble	MeOH, EtOH, EtOAc, DMSO
	insoluble	Hexane, $CHCl_3$, Acetone, H_2O

Fig. 3. ^{13}C NMR spectrum of funalenone (DMSO-d_6).



funalenone was deduced as $C_{15}H_{12}O_6$ by HR-FAB-MS. Funalenone was an acidic substance, soluble in dimethyl sulfoxide, ethanol and methanol, slightly soluble in ethyl acetate, and insoluble in water, hexane and chloroform. It was positive to vanilin sulfuric acid and iodine vapor color reactions but negative to ninhydrin. Chemical shifts in the ^1H and ^{13}C NMR spectra (Fig. 3) of funalenone are shown in Table 2. The HMQC experiment revealed the bonding of each proton and carbon.

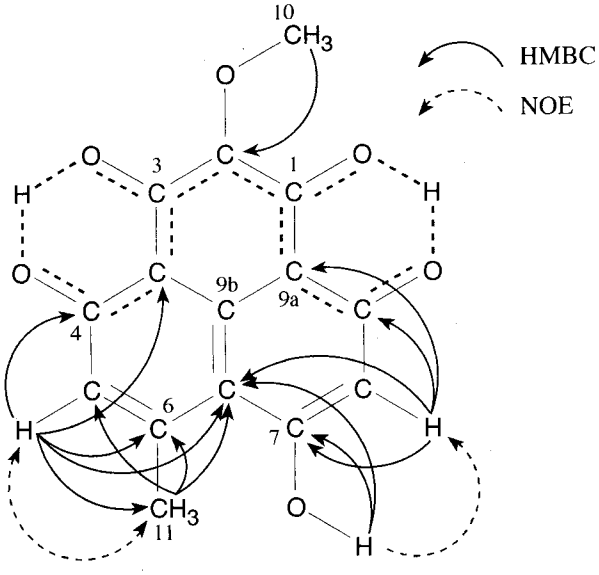
Structure Elucidation

In the DEPT spectra, funalenone showed one methyl, one methoxy, two aromatic methine, and eleven aromatic quaternary carbon signals.

The HMBC experiment suggested the alignment of C-5, C-6, C-6a, C-7, C-8, and C-11 as shown in Fig. 4. The long-range ^1H-^1H coupling ($J_{5,11}$=0.8 Hz) and the NOEs between 5-H and 11-CH_3 and between 7-OH and 8-H supported this alignment. The HMBC also revealed that C-10 methoxy residue (δ 59.6) were connected to C-2 (δ 132.8).

Among remaining seven quaternary carbons, four were considered as oxygenated carbons from their chemical shifts. As three hydrogens were not observed in ^1H NMR,

Fig. 4. Structure elucidation of funalenone.



they were suggested to be hydroxy residues. The remained one oxygen was considered to be a carbonyl oxygen. The index of hydrogen deficiency of funalenone was ten, and the number of double bonds was suggested to be seven.

Table 2. The ^{1}H and ^{13}C NMR data of funalenone and xanthoherquein.

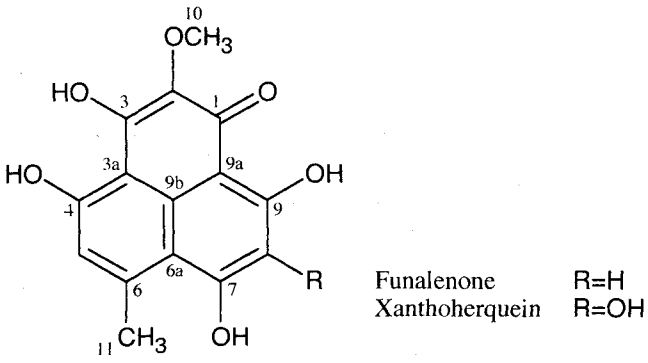
Position	Funalenone[a] ^{13}C	Funalenone[a] ^{1}H[c]	Calculated ^{13}C chemical shifts[b]	Xanthoherquein[c] ^{13}C	Xanthoherquein[c] ^{1}H
1	168.2 s		170.4	170.4	
2	132.8 s		132.7†	145.2	
3	163.0 s		160.2	160.2	
3a	105.4 s		104.3	104.3	
4	164.9 s		165.4†	158.3	
5	116.7 d	6.76 q (1H, *J*=0.8 Hz)	115.9	115.9	6.54
6	146.8 s		145.2†	132.7	
6a	110.4 s		108.9*	110.0	
7	165.9 s		165.3*	152.3	
8	99.9 d	6.41 s (1H)	100.8*	130.1	
9	170.1 s		171.3*†	165.4	
9a	102.3 s		102.1*	103.2	
9b	127.1 s		129.2*	120.4	
10	59.6 q	3.79 s (3H)	59.9	59.9	3.79
11	25.3 q	2.78 d (3H, *J*=0.8 Hz)	25.0	25.0	2.63
7-OH		11.65 br.s			

a: The spectra were obtained with Varian Unity 400 spectrometer. The DMSO-d_6 signals (2.48 ppm of ^{1}H and 39.5 ppm of ^{13}C) were used as references.
b: 8-Deoxy effects of ^{13}C chemical shifts of xanthoherquein are calculated by the substituent effects data of D. F. EWING[10]. The original xanthoherquein data are shown in right column. The data of C-2 and C-6, and C-4 and C-9 of xanthoherquein are exchanged, respectively, to show good agreement with those of funalenone. *: calculated value. †: exchanged value.
c Referred to T. SUGA *et al*[7]. Solvent, DMSO-d_6; internal reference, TMS.

Therefore funalenone was considered to have aromatic tricyclic system. Search for aromatic tricyclic systems having similar molecular formula to funalenone revealed that xanthoherquein (xanthoherqueinone[7]) had similar NMR spectra (Table 2).

Xanthoherquein (Fig. 5) was an acid hydrolysis product of herqueinone, a metabolite of *Penicillium herquei*[8,9]. The molecular formula of xanthoherquein ($C_{15}H_{12}O_7$) showed that it had one more oxygen than that of funalenone. Comparing ^{13}C NMR data of xanthoherquein with those of funalenone, funalenone was suggested as 8-deoxyxanthoherquein. Since the reported ^{13}C chemical shifts of xanthoherquein were simply assigned by comparison with the data of herqueinone[7], the chemical shifts of C-2 and C-6, and C-4 and C-9 of xanthoherquein were exchanged, respectively, to show good agreement with those of funalenone. Then 8-deoxy effects of ^{13}C chemical shifts of xanthoherquein were calculated by the substituent effects data of D. F. EWING[10] and shown in Table 2. The calculated value was quite similar to the funalenone data. The structure of 8-deoxyxanthoherquein was consistent with the partial structure elucidated by the HMBC experiment. The broad signals of C-1, C-3, C-3a, C-4, C-9, and C-9a (Fig. 3) suggested that they were in an equilibrium of tautomers as *nor*xanthoherquein (10-demethylxanthoherquein)[9].

Fig. 5. Structure of funalenone and xanthoherquein.

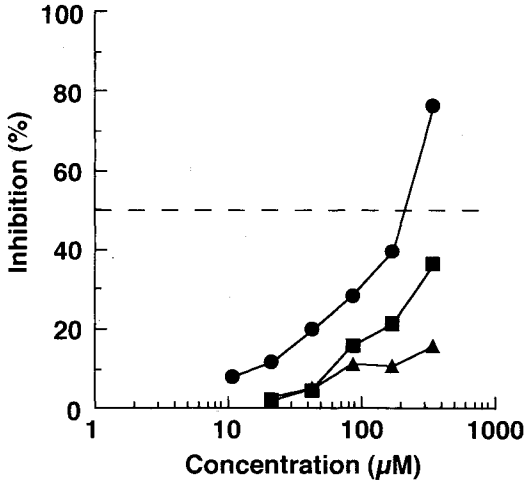


The structure is the one shown above or equivalent tautomer.

Consequently, the structure of funalenone was elucidated as 3,4,7,9-tetrahydroxy-2-methoxy-6-methyl-1*H*-phenalen-1-one or its equivalent tautomer (Fig. 5).

Fig. 6. Inhibition of metalloproteinases by funalenone.

●, Type I collagenase (MMP-1)
■, Type IV collagense (MMP-9)
▲, Type IV collagenase (MMP-2).



Biological Properties

Effect of Funalenone on Matrix Metalloproteinases

The activity of funalenone toward type I collagenase, and type IV collagenases (72 kDa and 92 kDa) is shown in Fig. 6. Funalenone inhibited type I collagenase activity dose-dependently at an IC_{50} value of 170 μM, but the inhibitory activity of funalenone for type IV collagenases (72 kDa and 92 kDa) are 18.3% and 38.2%, respectively, at a concentration of 400 μM. Furthermore, 7-methoxy and 7-(*p*-bromobenzoyloxy) derivatives showed no effect against type I collagenase at 400 μM, indicating that hydrophilic properties of funalenone is neccesary for the activity.

Other Biological Activity

Funalenone showed no antimicrobial activity at the concentration of 50 μg/disc (paper disc method) against the following microorganisms: *Staphylococcus aureus* KB210 (ATCC 6538 P), *Micrococcus luteus* KB40 (ATCC 9341), *Bacillus subtilis* KB27 (PCI 219), *Bacteroides fragilis* KB169 (ATCC 23745), *Pyricularia oryzae* KF 180, *Mycobacterium smegmatis* KB42 (ATCC 607), *Pseudomonas aeruginosa* KB115 (IFO 3080), *Escherichia coli* KB8 (NIHJ), *Xanthomonas camperstris* vp. *oryzae* KB 88, *Acholeplasma laidlawii* KB174 (PG 8), *Candida albicans* KF 1, *Saccharomyces cerevisiae* KF26, *Mucor racemosus* KF223 (IFO 4581), and *Aspergillus niger* KF103 (ATCC 6275).

Acknowledgment

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