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Fistupyrone, a Novel Inhibitor of the Infection of Chinese Cabbage by *Alternaria brassicicola*, from *Streptomyces* sp. TP-A0569

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A new microbial metabolite, designated fistupyrone, was isolated from the culture broth of a plant-associated *Streptomyces* sp. TP-A0569. Fistupyrone inhibited the *in vivo* infection of the seedlings of Chinese cabbage by *Alternaria brassicicola* TP-F0423, the cause of Alternaria leaf spot, without any *in vitro* fungicidal activity.

Many microbes including fungi[1], bacteria[2] and actinomycetes[3] have been reported as a biocontrol agent against plant pathogens. Among them, especially, actinomycetes are known to produce a wide range of bioactive compounds. Although the role of non-pathogenic endophytes in plant protection is not well understood, it is noteworthy that some of these cases are involved with the antibiotics from the microorganisms[2]. We recently discovered endophytic *Streptomyces* from Rhododendron producing antifungal metabolites against its major fungal pathogens, *Phytophthora cinnamomi* and *Pestalotiopsis sydowiana*[4].

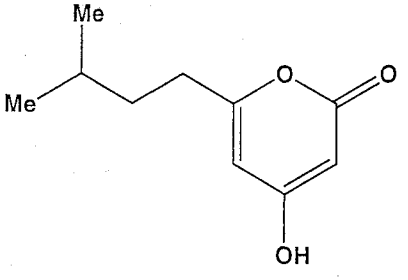
In addition to the antimicrobial metabolites, the existence of compounds which effect on the plant resistance to pathogens is speculated. As a part of our effort to find bioactive metabolites from plant-associated actinomycetes, we have screened for compounds which do not show antifungal activity but protect the plant from pathogenic fungi. In this study, the *in vivo* assay using Chinese cabbage and *Alternaria brassicicola* was employed. *A. brassicicola* is the cause of black leaf spot, major disease in cultivated *Brassica* plants worldwide. Among 375 strains tested, *Streptomyces* sp. TP-A0569 was found to produce a new compound, fistupyrone (Fig. 1), which inhibits the infection behavior of *A. brassicicola* TP-F0423 *in vivo*. We herein describe the fermentation, isolation, structure determination and biological properties of fistupyrone.

Materials and Methods

General

The melting point was determined on a Yanagimoto apparatus and is uncorrected. NMR experiments were

Fig. 1. Structure of fistupyrone.



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performed on JEOL JNM-LA400 NMR spectrometer in $CDCl_3$ with TMS as an internal standard. The FABMS spectrum was measured on a JEOL JMS-HX110A spectrometer. UV spectrum was recorded on a BECKMAN DU 640 spectrophotometer. IR spectrum was recorded on a SHIMADZU FT IR-300 spectrophotometer. Optical rotation was measured on a HORIBA SEPA-300 polarimeter.

Taxonomic Studies

Morphological, cultural and physiological characterization of strain TP-A0569 was carried out by the method of the International Streptomyces Project (ISP). The color name and color code were determined by comparing the culture with color chips according to the Manual of Color Names (Japan Color Enterprise Co., Ltd. 1987). Whole cell compositions were analyzed by the methods described by BECKER *et al.*[5], LECHEVALIER[6] and STANECK *et al.*[7].

Microorganism

The producing strain, *Streptomyces* sp. TP-A0569, was isolated from the stem of the onion *Allium fistulosum* collected in Toyama, Japan. This strain was maintained on a slant consisting of glucose 0.5%, soluble starch 0.5%, meat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.5% at 32°C.

Chemical

Daconil, 40% water dispersion of 1,3-dicyano-2,4,5,6-tetrachlorobenzene, used in this experiment, is a product of Takeda Chemical Industries, Ltd. (Japan).

Biological Assay

An isolate of *Alternaria brassicicola* (Sch.) Wiltshire TP-F0423 obtained from naturally infected Chinese cabbage (*Brassica campestris* L.) in Toyama, Japan by TAKANO was used in this experiment[8]. It was grown on a potato-dextrose agar plate at 25°C for 14 days. The plate was flooded with sterilized water and the spores were collected by pipetting. The spore concentration in the suspension was adjusted to 1×10^5 spores/ml with sterilized water.

Seeds of Chinese cabbage were sworn in soil in pots and the seedlings were grown for 14 days in a temperature-controlled incubator at 25°C. *A. brassicicola* TP-F0423 was inoculated on the plants by spraying the spore suspension. After 2 hours, the plants were treated with sterilized water (control), daconil solution or fistupyrone solution. After the incubation for 22 hours at 25°C, disease symptom was evaluated based on the area of necrotic lesions and the disease severity was calculated according to the following equation.

$$\text{Disease severity} = \Sigma S_i N_i / (4 \times \text{number of plants used})$$

S_i: index of symptom which was determined on the basis of symptoms covering more than 75% of leaf (4), symptoms covering 50~75% of leaf (3), symptoms covering 25~50% of leaf (2), symptoms covering 1~25% of leaf (1) or no symptoms (0).

N_i: number of plants whose index of symptom is Si

In addition, the leaves were soaked in FAA solution (formalin/acetic acid/ethanol=1 : 1 : 1) until the chlorophyll was removed and stained with 0.1% cotton blue in lactophenol. The infection behavior of spores was observed and the number of spores which germinated or formed infection hypha was counted under a light microscope.

Results and Discussion

Taxonomy of the Producing Strain

The cultural characteristics of strain TP-A0569 are summarized in Table 1. Strain TP-A0569 grew well on both organic and synthetic media and formed open spiral spore chains at the top of aerial mycelia. The spore was globose with spiny surface (Fig. 2). The color of aerial mass was gray to brownish gray. Neither melanoid pigment nor diffusible pigment was produced. Fragmentation of vegetative mycelium, sporangium, or motile element was not observed. The whole-cell hydrolysate contained LL-diaminopimelic acid and glycine. D-Fructose, D-glucose, inositol, D-mannitol, L-rhamnose and D-xylose were utilized as a carbon source, but L-arabinose, sucrose and raffinose were not. On the basis of these characteristics, the strain was identified as *Streptomyces* sp. TP-A0569.

Fermentation

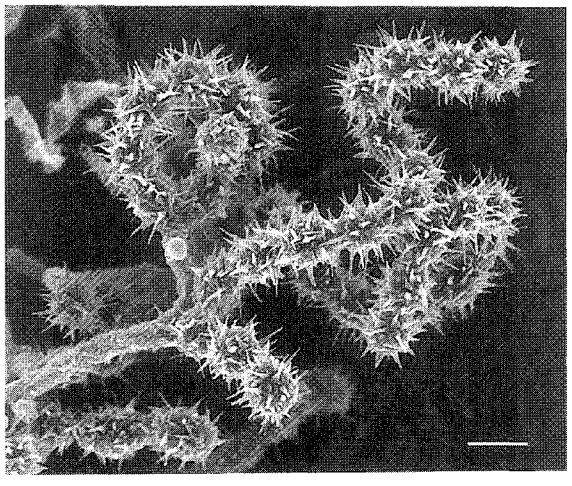
A loopful of a mature slant culture of *Streptomyces* sp. TP-A0569 was inoculated into a 500 ml K-1 flask containing 100 ml of the seed medium consisting of soluble starch 1.0%, glucose 0.5%, KH_2PO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH7.0). The flask was incubated at 30°C for 4 days on a rotary shaker (200 rpm). Three-ml aliquots of the seed culture were transferred into a hundred of 500-ml K-1 flasks each containing 100 ml of the production medium consisting of glucose 0.5%, glycerol 2.0%, soluble starch 2.0%, yeast extract (Difco Laboratories) 0.3%, Pharmamedia 1.5% and Diaion HP-20

Table 1. Cultural characteristics of strain TP-A0569.

Medium	Growth	Aerial mycelium	Reverse side	Diffusible pigment
Yeast extract-malt extract agar (ISP med. 2)	Soft yellow (147), good	Reddish gray (408), powdery	Dull yellow (150)	None
Oatmeal agar (ISP med. 3)	Grayish yellow (158), good	Brownish gray (409), powdery	Grayish yellow (156)	None
Inorganic salts-starch agar (ISP med. 4)	Soft orange (83), good	Yellowish gray (402), powdery	Grayish yellow (156)	None
Glycerol-asparagine agar (ISP med. 5)	Beige gray (401), good	Brownish gray (409), powdery	Brownish gray (409)	None
Tyrosine agar (ISP med. 7)	Beige gray (401), poor	Reddish gray (408), powdery	Brownish gray (409)	None

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. TP-A0569.

Bar represents 1 μm.



resin 1.0%. The pH of the medium was adjusted to 7.0 before sterilization. Fermentation was carried out for 8 days at 30°C on a rotary shaker (200 rpm).

Isolation

The fermented broth (10 liters) was centrifuged (6,000 rpm, 10 minutes) to separate the mycelia and the supernatant. The mycelia was extracted with 1.8 liters of 80% aqueous methanol and filtered. The filtrate was evaporated to remove methanol and the resultant aqueous layer was combined with the supernatant and applied on a column of Diaion HP-20 resin (310×70 mm i.d.). The column was washed with 10% aqueous methanol (2.5 liters) and eluted with 80~100% aqueous acetone (5 liters). The eluates were concentrated *in vacuo* to remove acetone and the resultant aqueous layer was extracted with ethyl acetate twice at pH 2.0. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to give a brown extract (2.0 g). One gram of the extract was fractionated by silica gel column chromatography (50 g, hexane - ethyl acetate = 10 : 1 ~ 1 : 1 and then chloroform - methanol = 10 : 1) and the fractions containing fistupyrone were pooled and concentrated *in vacuo* to give 432 mg of yellow oil. This oil was further purified by ODS column chromatography (170×55 mm i.d.) with the eluent of acetonitrile - 0.15% KH_2PO_4 buffer, pH 3.5 (30 : 70~40 : 60). Fractions containing fistupyrone were collected and evaporated to remove acetonitrile. The resultant aqueous layer was extracted with ethyl acetate twice, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to give fistupyrone (61.4 mg) as a colorless solid.

Structure Determination

The physico-chemical properties of fistupyrone (FP) are summarized in Table 2. FP was obtained from *n*-hexane-ether as colorless needles with the melting point at 73°C. It was readily soluble in $CHCl_3$ and ethyl acetate and insoluble in *n*-hexane and water. The UV-visible spectrum showed maxima at 210, 231 and 279 nm in MeOH. The IR spectrum showed the presence of hydroxyl group (2960 cm^{-1}), carbonyl group (1705 cm^{-1}) and conjugated olefin

Table 2. Physico-chemical properties of fistupyrone.

Appearance	Colorless needles
MP	73°C
HRFAB-MS	
Found:	183.1028 $(M+H)^+$
Calcd:	183.1021 (for $C_{10}H_{15}O_3$)
Molecular formula	$C_{10}H_{14}O_3$
UV λ_{max}^{MeOH} nm (log ε)	210 (4.42), 231 (3.60), 279 (3.93)
IR ν_{max} (cm^{-1})	2960, 1705, 1650
Solubility	
soluble in	$CHCl_3$, MeOH, EtOAc
insoluble in	hexane, water
TLC (Rf)[a]	0.58
HPLC (Rt)[b]	7.3 min

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=10:1)

[b] HPLC conditions: Cosmosil AR-II (250 x 4.6 mm i.d.), mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (30:70), flow rate: 0.7 ml/min, detection: UV-254 nm.

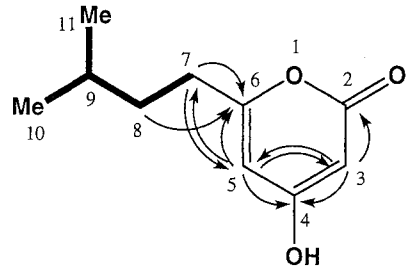
Table 3. ^1H and ^{13}C NMR data of fistupyrone.

Position	^{13}C	^1H (int, mult, *J*)
2	168.41	
3	89.73	5.58 (1H, s)
4	172.73	
5	101.23	6.01 (1H, s)
6	167.61	
7	31.63	2.49 (2H, t, 7.8 Hz)
8	35.54	1.53 (2H, m)
9	27.51	1.57 (1H, m)
10, 11	22.20	0.91 (6H, d, 6.4 Hz)

group (1650 cm^{-1}). Molecular ion $[M+H]^+$ was detected at *m/z* 183 by FAB-MS. The molecular formula was determined to be $C_{10}H_{14}O_3$ ($[M+H]^+$, *m/z*, calcd: 183.1021, found: 183.1028), based on HRFAB-MS and NMR spectra.

The assignments of ^1H and ^{13}C NMR are shown in Table 3. In combination with the HMQC and HMBC spectra, the DEPT spectra indicated the presence of two equivalent

Fig. 3. NMR analysis of fistupyrone.



: DQF-COSY and proton homodecoupling

: ^1H-^{13}C long range couplings

methyl groups, two methylenes, one sp^3 methine, two sp^2 methines and three oxygenated sp^2 carbons. Only 13 protons were accounted for from the ^1H and ^{13}C NMR data suggesting the presence of one exchangeable proton in the molecule. Decoupling experiments and the DQF-COSY spectrum revealed a spin system: H-7/H-8/H-9/H-10/H-11 due to an isoamyl substituent. HMBC correlations from H-5 to C-3, C-4, C-6 and C-7 and from H-3 to C-2, C-4 and C-5 established the presence of 4-hydroxy-2-pyrone moiety. Substitution of the isoamyl group at C-6 was confirmed by the long-range couplings from H-7 and H-8 to C-6. Thus the gross structure of FP was determined to be 4-hydroxy-6-(3′-methyl)-butyl-2*H*-pyran-2-one (Fig. 3). ^1H and ^{13}C chemical shifts of the α-pyrone moiety showed the good accordance with those of 4-hydroxy-6-methyl-α-pyrone[9].

Biological Properties

Effects of fistupyrone (FP) on the infection of Chinese cabbage by *A. brassicicola* TP-F0423 were evaluated macroscopically and microscopically (Table 4). Disease severity of the plants treated with 100 ppm FP was reduced to 12% of the untreated (control). This effect on the disease suppression was at the same level with 400 ppm daconil, an antifungal agrochemical. At the lower concentrations (4~20 ppm), the effect of FP was slightly weaker with the disease severity of 33.3~41.7.

The infection behavior of the spores observed under a light microscope is shown in Fig. 4. Once settled on the leaf, the spore germinates, forms the infection hypha and penetrates through the cuticle into the host plant. Penetration of the infection hypha into the leaf was seen with the spores which had infected (Fig. 4a) whereas the

Table 4. Effect of fistupyrone on the infection of Chinese cabbage by *A. brassicicola*.

Test sample	Disease severity*	Infection behavior**		
		Spore germination (%)	Infection hypha formation (%)	Infection (%)
Control	66.7	89.8±4.5	60.0±4.7	53.8±2.5
Daconil (400 ppm)	8.3	14.3±8.7	7.0±6.1	1.1±0.2
Fistupyrone (100 ppm)	8.3	24.3±7.5	19.5±2.7	4.7±1.1

* Each disease severity was determined using three seedlings.

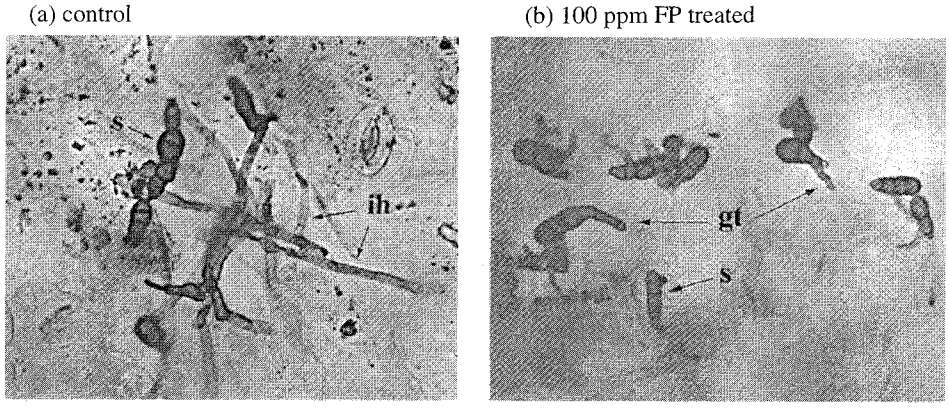
** Each value is the average of four replications and the standard deviation.

Spore germination (%) = germinated spores / total spores

Infection hypha formation (%) = infected spores / germinated spores

Infection (%) = infected spores / total spores

Fig. 4. Infection behavior of *A. brassicicola* on the leaf of Chinese cabbage.



s: spore, ih: infection hypha, gt: germination tube

spores which had failed in infection formed germination tube but not the infection hypha (Fig. 4b). In control, 90% of the inoculated spores had germinated and 60% of the germinated spores succeeded in infection. The rate of infection was thus 54%. On the other hand, when treated with 100 ppm FP, both of the rate of spore germination and infection hypha formation was suppressed and the infection rate was 9% of the control. Similar result was obtained by the treatment with 400 ppm daconil. On the agar plate, FP showed no growth inhibition of *A. brassicicola* TP-F0423 at 1000 μg/ml.

The molecular mechanism of the infection by *A. brassicicola* is not elucidated yet. Recent study suggests that surface-bound lipases of spores interact with the plant surface molecules for adhesion and penetration during the early stages of infection[10]. It is also suggested that host-specific toxins, produced during the spore germination, cause the necrosis of the host plant to facilitate the infection[11]. Our observations on the effect of FP on *A. brassicicola* TP-F0423 indicates the interaction of FP with the molecules responsible for the infection. Study on the mode of action of FP is in progress.

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