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# An antifungal compound from roots of Welsh onion

Nyunt Phay[a], Takako Higashiyama[a], Masahisa Tsuji[b], Hideyuki Matsuura[a], Yukiharu Fukushi[c], Atsushi Yokota[a], Fusao Tomita[a,*]

[a]*Department of Bioscience and Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo, 060-8589, Japan*
[b]*Sapporo Industrial Machinery Inc, Japan*
[c]*Department of Applied Bioscience, Faculty of Agriculture, Hokkaido University, Sapporo, 060-8589, Japan*

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**Abstract**

A novel antifungal compound, fistulosin (octadecyl 3-hydroxyindole), was isolated from roots of Welsh onion (*Allium fistulosum* L.), and its structure was elucidated by spectroscopic means. This compound showed high activity against *Fusarium oxysporum* primarily inhibiting protein synthesis. © 1999 Elsevier Science Ltd. All rights reserved.

*Keywords:* Welsh onion root; *Allium fistulosum*; Tubercullariaceas; Antifungal compound; *Fusarium oxysporum*; Protein synthesis inhibitor; Octadecyl 3-hydroxyindole; Fistulosin

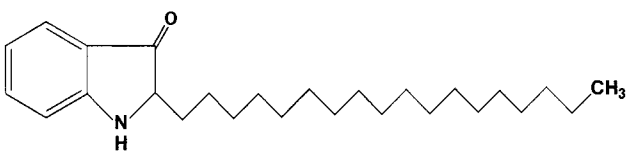
## 1. Introduction

The genus *Fusarium* is the largest in the family Tubercullariaceae. A number of Fusaria are parasitic, generally causing a wilting of the host plant. Fusaria are phytopathogenic fungi whose control is one of the most challenging problems in agriculture today. Varieties of *Fusarium oxysporum* are particularly important wilt-producing fungi and attack important crops such as tomatoes, bananas, sweet potatoes and pears (Alexopoulos & Mims, 1979). Unlike in the past, agricultural fungicides are now required not only to possess potent activity, but to be safe to animals, humans and the ecosystems in general.

Studies on utilization of by-products of agricultural crops revealed that roots of Welsh onion contained antifungal compounds against filamentous fungi, particularly various strains of *Fusarium oxysporum*.

It is a well known fact that vegetables belonging to *Allium* species are strongly resistant to diseases caused by nematodes (Tada, Hiroe, Kiyohara & Suzuki, 1988). These vegetables are characterized by a specific flavor and are used for cooking. Furthermore, it has been demonstrated that they produce protective agents, such as antimicrobial agents when traumatized. *Allium* contains pharmaceutically interesting compounds that are mainly sulfur-based compounds. To date, nematicidal and antibacterial activities of these compounds against *Bacillus* sp. and *Escherichia coli* have already been investigated (Tada et al., 1988). However, studies on their antifungal activity have not yet been undertaken. Although allelopathic substances from Welsh onion roots inhibit growth of roots of chrysanthemum cuttings (Choi, 1993), studies on their antifungal activity have yet to be done.

In Hokkaido, Japan, Welsh onion is an important vegetable crop and its annual production reaches 34,900 tons. Thus, we have had the challenge of putting to good use the huge amounts of its by-products such as roots, leaf sheaf and so on, generated annually. We therefore initiated studies on their utilization.




Fistulosin( Octadecyl 3-hydroxyindole)

* Corresponding author. Tel.: +81-11-706-2493; fax: +81-11-706-4961.

*E-mail address:* ftomita@chem.agr.hokudai.ac.jp (F. Tomita)

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Table 1
$^{1}H$ and $^{13}C$ NMR spectral data (500 MHz of fistulosin in $CDCl_3$)[a]

| | $^{1}H$ | $^{13}C$ NMR |
|---|---|---|
| NH | 10.8 (1H, *brs*) | |
| C═O | | 171.5 s |
| CH | 8.1 (1H, *d*, $J$ = 8.12 Hz) | 131.9 d |
| CH | 7.1 (1H, *t*-like) | 122.7 d |
| CH | 7.6 (1H, *t*-like) | 135.8 d |
| CH | 8.8 (1H, *d*, $J$ = 8.37 Hz) | 120.7 d |
| C | | 142.5 s |
| C | | 113.7 s |
| $CH_2$ | 2.44 (2H, *t*-like) | 25.9 d |
| $CH_2$ | 1.74 (2H, *m*) | 38.9 s |
| $CH_2$ | 1.63 (2H, *m*) | 32.1 s |
| $(CH_2)_{14}$ | 1,25 (2H, *brs*) | 29.05 ~ 29.92 m |
| $CH_2$ | 1.4 (2H, *m*) | 23.4 s |
| $CH_3$ | 0.88 (3H, *t*-like) | 14.3 s |

[a] TMS (0 ppm) was used as an internal standard. $CDCl_3$ was also used as an internal standard. Assignments were based on DEPT, $^{1}H$-$^{1}H$ COSY, $^{1}H$-$^{13}C$ COSY, and selective INEPT experiments (Bax, 1984).

## 2. Results and discussion

Fistulosin was obtained as a white crystalline compound with a molecular formula of $C_{26}H_{43}NO$ at m/z 385 in the FD–MS. High resolution MS gave a molecular peak, $C_{26}H_{43}NO$ (m/z 385.6337, calcd. 385.6387), and a base peak, $C_8H_7NO$ (m/z 133).

Table 2
Antifungal effects of fistulosin

| Test organisms | MIC[a] (μg/ml) |
|---|---|
| *Fusarium oxysporum* CVF9476 | 1.62 |
| *F. oxysporum* FSK9461 | 3.25 |
| *F. oxysporum* RFT9461 | 6.5 |
| *F. oxysporum* KA37 | 3.25 |
| *F. solani* CT9472 | 3.25 |
| *F. moniliforme* | 6.5 |
| *Verticillium dahliae* | 3.25 |
| *Pencillium roqueforti* AHU8057 | 1.62 |
| *Aspergillus oryzae* AHU7135 | 1.62 |
| *Magnaporthe grisea* Ina168 | 1.62 |
| *Rhizopus oryzae* AHU6536 | 3.25 |

[a] MIC-Minimum Inhibitory Concentration; defined as lowest concentration providing complete inhibition of fungal growth.

Fistulosin has a simple $^{1}H$ NMR spectrum with four neighbouring aromatic proton signals. Fistulosin has $^{1}H$ NMR signals of $\delta$10.8 (1H, *brs*), $\delta$8.8 (1H *d* $J$ = 8.3 Hz), $\delta$8.1 (1H, *d*, $J$ = 8.12 Hz), $\delta$7.6 (1H, *t*-like), $\delta$7.1 (1H, *t*-like), $\delta$2.44 (1H, *t*-like). Unless the $^{13}C$ NMR has resonances at spectrum $\delta$171.5 *s*, $\delta$142.5 *s*, $\delta$135.8 *d*, $\delta$131.9 *d*, $\delta$122.7 *d*, $\delta$120.7 *d*, $\delta$113.7 *s*, $\delta$25.9 *d*. $^{13}C$ and DEFT experiments next revealed that fistulosin has 4-CH, 3-C, 17-$CH_2$, 1-$CH_3$ and one carbonyl carbon. The chemical shift at $\delta$171.5 ppm suggested that it possessed a carbonyl group, this

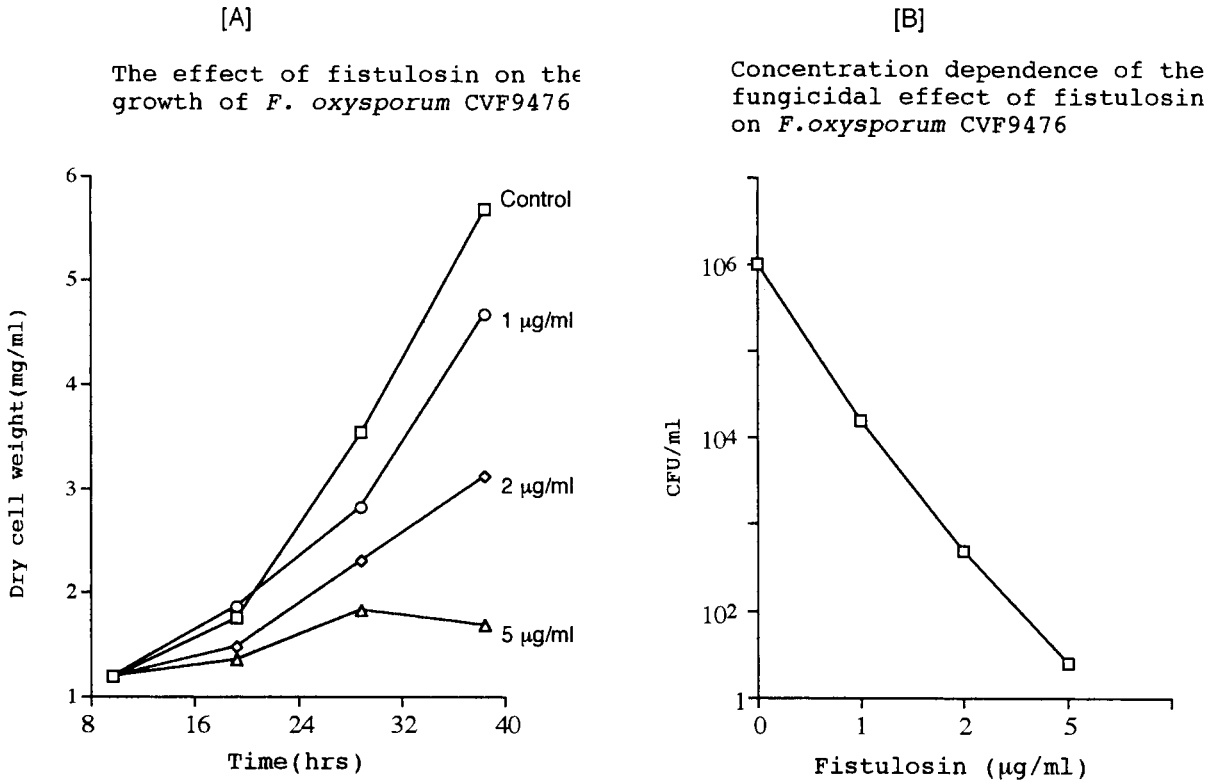


Fig. 1. The effect of fistulosin on the growth of *Fusarium oxysporum* CVF9476.

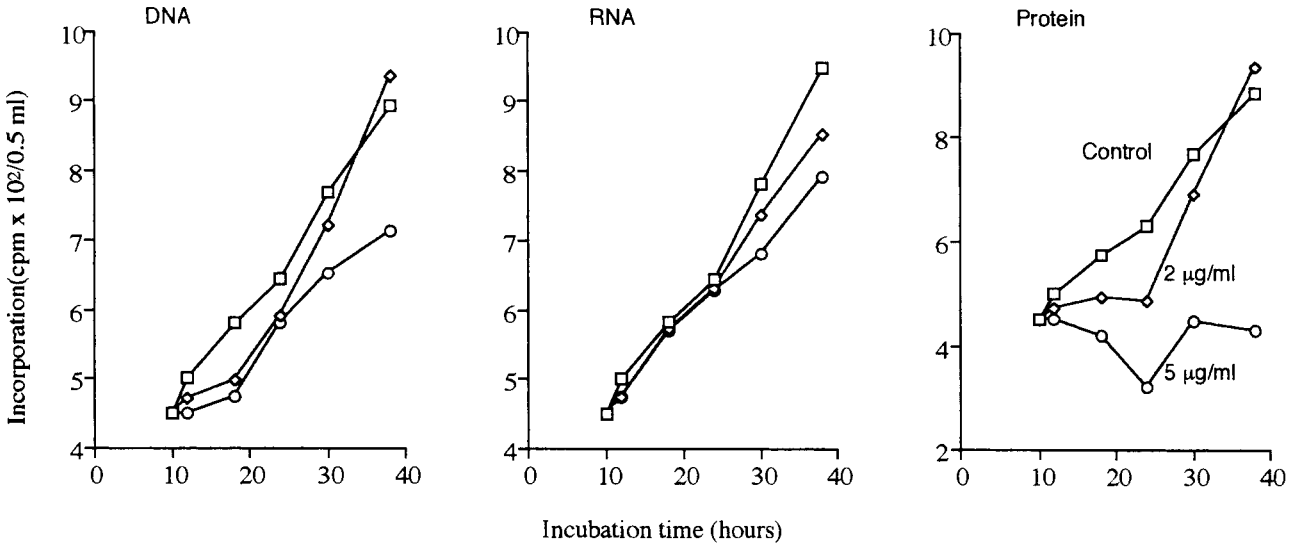


Fig. 2. Effects of fistulosin on macromolecular synthesis of *Fusarium oxysporum* CVF9476.

being supported by an absorption at 1684 $cm^{-1}$ on the IR spectrum. These data thus suggested that the compound had partial structures of 3-hydroxyindole. Based on these NMR results (Table 1), it was concluded that fistulosin is octadecyl 3-hydroxyindole.

Fistulosin exhibited antifungal activities against different phytopathogenic filamentous fungi, especially varieties of *Fusarium oxysporum* at 1.62–6.5 μg/ml of MICs and other filamentous fungi at 1.62–3.25 μg/ml of MICs (Table 2). In contrast, no activity was observed against *Saccharomyces cerevisiae* and bacteria such as *Salmonella typhimurium* SL3770, *E. coli* K-12 and *Bacillus subtilis*.

Fig. 1A shows the effect of fistulosin on the growth of *Fusarium oxysporum* CVF9476 which was inhibited at a concentration of 2 μg/ml; any further increase in its concentration above 2 μg/ml resulted in a more suppressive effect on growth. At a concentration of 5 μg/ml, cell lysis was observed indicating that fistulosin acts as a fungicidal compound (Fig. 1A). The effect of fistulosin on the growth of *F. oxysporum* cells was also examined by cell viability (Fig. 1B).

The mode of action of fistulosin is shown in Fig. 2. Fistulosin inhibited protein synthesis and had a slight inhibitory effect on DNA synthesis, but no inhibitory effect on RNA synthesis (Fig. 2).

## 3. Experimental

### 3.1. General

UV spectra were recorded using a Hitachi U-3210 recording spectrometer and IR spectra were obtained on a Perkin Elmer system 2000 FT-IR. FD–MS was measured on a JOEL JMS-SX102A mass spectrometer. EIMS and HR-EIMS were measured on a JOEL JMS-AX500 mass spectrometer. NMR data were taken on a Bruker AM-500 FT–NMR spectrometer. Silica gel column chromatography was carried out on Wakogel C300 at amounts equivalent to 100× the sample amount. Merck Kieselgel $60F_{254}$ pre-coated plates were utilized for PTLC.

### 3.2. Plant materials

Roots of Welsh onion (*Allium fistulosum* L.) were collected from the agricultural farm of Ono-town, Hokkaido, Japan.

### 3.3. Extraction and isolation

The roots of Welsh onion (*Allium fistulosum* L.) (50 kg) were extracted with MeOH (100 l). MeOH extracted residue (2610 g) was dissolved in water (1:20) at pH 2.0, extracted ×3 with an equal volume of EtOAc. The concentrated EtOAc extract (522 g) was chromatographed on silica gel column eluted with $CHCl_3$ and $CHCl_3$–MeOH (9:1). The concentrated active $CHCl_3$ residue (141 g) was then rechromatographed on the same column eluting with *n*-hexane and *n*-hexane–$Me_2CO$ (9:1). The concentrated active *n*-hexane–$Me_2CO$ fractions (28 g) were separated on PTLC with $C_6H_6$–EtOAc (8:2). The active portion (374 mg) was subjected to silica gel column chromatography and eluted with *n*-hexane–EtOAc–$Me_2CO$ (10:10:1), followed by *n*-hexane–EtOAc–$Me_2CO$–$CHCl_3$ (5:5:0.5:4.5). This eluate was crystallized from $CHCl_3$ at 4° for about 36 h in order to obtain fistulosin (51 mg).

### 3.4. Fistulosin (octadecyl 3-hydroxyindole)

White crystal, mp 80–83°; $UV_{max}$ (EtOH)nm: 220, 237, 252, 277, 302; $IR_{vmax}$ (film)$cm^{-1}$: 3333, 2919, 2850, 1684, 1415, 1264, 755; FD–MS m/z 385 $[M]^+$; EIMS 385 $[M]^+$, 133. NMR spectral data are listed in Table 1.

### 3.5. Determinations of the antifungal spectrum

Minimum inhibitory concentrations (MICs) of fistulosin were determined by serial dilution assay in MPG liquid medium (malt extract 1%, Polypepton 1%, glucose 1%, pH 5.5) inoculated with approximately $10^6$ CFU/ml of the respective test organisms. After incubation at 27° for 24 h, the MICs were determined by selecting the lowest concentration of antibiotic which caused complete inhibition of fungal growth. Experiments were done in triplicate.

### 3.6. Fungicidal effect

The test organism (*F. oxysporum* CV9476) was inoculated in 50-ml Erlenmeyer flasks containing 15 ml of PG liquid medium (Polypepton 1%, glucose 1%, pH 5.5) and incubated at 27° on a reciprocal shaker (100 strokes per min). After incubation for 10 h, 1, 2 and 5 μg of fistulosin were added into each flask inoculated with test organisms. Following the addition of fistulosin, 0.5 ml of the samples were taken at a set interval and poured into 2.5 ml of ice-cold 5% TCA and placed for 1 h in the ice bath. The mixture was filtered through a millipore filter paper (0.45 μ) and washed with 15 ml of cold 5% TCA. The filters were dried and the dry cell weight was measured.

After incubation, as described above, quantitative measurement of fungicidal activity was done by the determination of viable fungal colony counts. Experiments were done in triplicate.

### 3.7. Macromolecular syntheses assay

The procedure for macromolecular syntheses assay is almost as described under 'Fungicidal effect'. The test organism (*F. oxysporum* CVF9476) was inoculated in 50-ml Erlenmeyer flasks containing 15 ml of PG liquid medium (Polypepton 1%, glucose 1%, pH 5.5) and incubated at 27° on a reciprocal shaker (100 strokes per min). After incubation for 10 h, 2 and 5 μgs of fistulosin were added and the effects of fistulosin on the synthesis of DNA, RNA and protein were determined by measuring the incorporation of labelled [methyl-$^3$H]thymidine, [5,6-$^3$H]uracil and [4,5-$^3$H]L-leucine into acid-insoluble precipitates. Following the addition of radioactive precursors, 0.5 ml of the samples were removed at a set interval and poured into 2.5 ml of ice-cold 5% TCA and placed for 1 h in the ice bath. They were filtered through millipore filter paper (0.45 μ) and washed with 15 ml of cold 5% TCA. The filters were dried, and the dry cell weight was measured and counted in vials containing toluene scintillation fluid consisting of 4 g 2,5-diphenyloxazole and 0.1 g 2,2-*p*-phenylene-bis-(5-phenyl-oxazole) per liter of toluene. Experiments were done in triplicate.

## Acknowledgements

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