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Verlamelin, an Antifungal Compound Produced by a Mycoparasite, *Acremonium strictum*

Jin-Cheol Kim*, Gyung Ja Choi, Hyun-Ju Kim, Heung Tae Kim, Jong Woong Ahn[1] and Kwang Yun Cho

Bioorganic Science Division and [1]Medicinal Science Division, Korea Research Institute of Chemical Technology, 100 Jang-Dong, Yusong-Gu, Taejon 305-600, Korea

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A strain of *Acremonium strictum*, the mycoparasite of *Botrytis cinerea*, showed strong antifungal activities both *in vitro* and *in vivo* against several phytopathogenic fungi. An antifungal substance was purified from the liquid cultures of *A. strictum* and identified as verlamelin by instrumental analyses. Verlamelin exhibited *in vitro* antifungal activity against some phytopathogenic fungi such as *Magnaporthe grisea*, *Bipolaris maydis*, and *Botrytis cinerea*, while it was not active against all the bacteria tested. *In vivo*, verlamelin exhibited strong protective and curative activities, particularly against barley powdery mildew. At 100 μg/ml, it inhibited the development of barley powdery mildew with control values of more than 90% in 7-day protective and 2-day curative applications. This is the first report on the production of verlamelin by *Acremonium* species.

Keywords : *Acremonium strictum*, antifungal substance, barley powdery mildew, mycoparasite, verlamelin.

Acremonium strictum BCP, a mycoparasite of *Botrytis cinerea* isolated in the laboratory, showed inhibition of mycelial growth of several phytopathogenic fungi including *Magnaporthe grisea, Bipolaris maydis, Curvularia inaequalis*, and *Botrytis cinerea* (Cho et al., 1999). The culture filtrate of the mycoparasite also inhibited development of several fungal diseases such as tomato gray mold, wheat leaf rust, and barley powdery mildew. In this study, isolation, structural determination, and antifungal activity of the active principle produced by the mycoparasite were investigated. This is the first report that an antifungal compound verlamelin is produced by *A. strictum*.

Materials and Methods

A. strictum BCP was cultured in potato dextrose (Difco Laboratories, Detroit, MI, USA) broth (15 liters) at 25°C and 150 rpm for 10 days. After centrifuging at 10,000×g for 10 minutes, an equal volume of acetone was added to the supernatant and then the solution was stirred. After removing the precipitate, the aqueous solution was brought to a concentration with only water remaining. The aqueous solution was extracted three times with equal volumes of ethyl acetate. On the other hand, the pellet was extracted twice with acetone (2 liters) and the acetone extract was concentrated until dried.

The residue was again dissolved in 400 ml of distilled water and then partitioned three times with equal volumes of ethyl acetate. The ethyl acetate extracts from the culture supernatant and pellet were pooled and concentrated to give 14.6 g of the residue. The concentrated extract was separated by a silica gel column [3.6 (i.d.)×60 cm, Kiesel gel 60, 260 g, 70-230 mesh; E. Merck, Darmstadt, Germany] and elution with $CHCl_3$-CH_3OH (6:1, v/v) to give an active fraction (0.4 g). The extract was further separated by a Sephadex LH-20 column [2.8 (i.d.)×45 cm, 50 g; Sigma, Mo, USA], using CH_2Cl_2-*n*-hexane-CH_3OH (10:10:1.2, v/v/v) to give an active fraction (60 mg). The fraction was finally purified by preparative silica gel TLC, using $CHCl_3$-CH_3OH (5:1, v/v) to give a pure compound (30 mg) showing antifungal activity against *B. cinerea*.

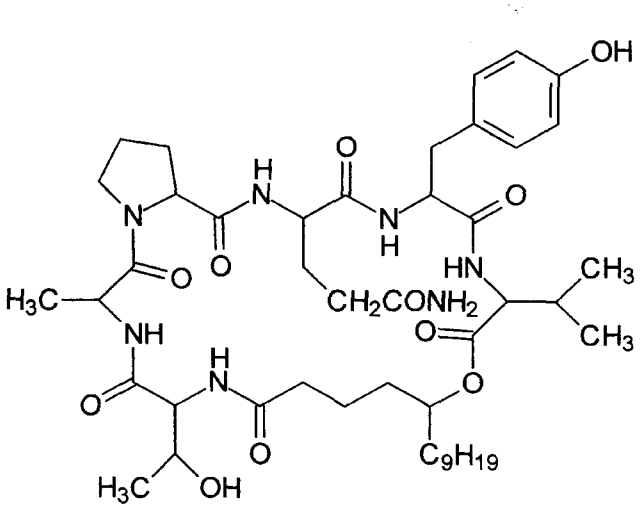

Fig. 1. Chemical structure of verlamelin isolated from *Acremonium strictum* BCP.

*Corresponding author.
Phone) +82-42-860-7436, FAX) +82-42-861-4913
E-mail) kjinc@krict.re.kr

The antifungal compound was isolated as a white solid soluble in chloroform, ethyl acetate, methanol, and acetone, and insoluble in *n*-hexane and water. It showed UV maxima at 225 nm, 277 nm, and 285 nm (PLS CHECK VALUES) in CH_3OH. The IR spectrum in $CHCl_3$ indicated the presence of many amide groups (3433 and 1651 cm^{-1}) in molecule. The low resolution-fast atom bombardment (FAB) mass spectrum of the antibiotic displayed strong $[M+H]^+$ and $[M+Na]^+$ ion peaks at *m/z* 887 and 909. High resolution-FAB mass spectrometry gave the molecular formula $C_{45}H_{71}N_7O_{11}$ that was supported by ^1H, ^{13}C and DEPT NMR spectra. The typical A_2M_2 two doublets (δ H 6.67 and 7.07, Δv_{AM} 200 Hz, J_{AM} 14.1 Hz) in ^1H-NMR spectrum indicated the presence of 1,4-disubstituted aromatic ring. The ^1H-NMR spectrum also showed the presence of one primary and four secondary methyls. Two ^{13}C signals (δ_C 116.4 and 131.4) from the chemically equivalent pairs of four carbons in ^{13}C-NMR spectrum supported the presence of 1,4-disubstituted aromatic ring in the antibiotic. The connectivity of the chemical shifts of all directly bonded proton and carbon pairs in the compound were determined by the ^1H-^{13}C COSY (Correlated Spectroscopy) spectrum. By compilation of all data, the antibiotic was identified as verlamelin (Fig. 1), an antifungal agent produced by *Verticillium lamellicola* (Onishi et al., 1980; Rowin et al., 1986).

The antimicrobial activities of verlamelin against five bacteria and seven fungi were estimated by the disc diffusion method. *Erwinia carotovora, Streptomyces griseus, Pseudomonas putida, Xanthomonas campestris* pv. *campestris,* and *X. c.* pv. *vesicatoria* were the bacteria tested. The cell suspension of each bacterium was seeded into molten nutrient agar (NA) medium. Each compound was dissolved in acetone at a concentration of 1 mg/ml, and 20 μl of each antibiotic solution was loaded onto a paper disc (8.0 mm diameter). After drying, the paper discs were placed on the surface of NA medium seeded with bacteria. The antibacterial activity on the test microorganisms was determined according to the sizes of inhibition zones after incubating for 24 h at 30°C.

The antifungal activity of verlamelin was tested for the following fungi *in vitro*: *M. grisea, B. maydis, B. cinerea, Fusarium oxysporum, Alternaria alternata, Thanatephorus cucumeris,* and *Pythium graminicola.* PDA plates were seeded with the test fungi by adding mycelial suspensions of the fungi to molten PDA media before pouring the media into petri dishes. The paper discs (8 mm diameter) impregnated with 20 μl of antibiotic solution (1 mg/ml) were placed at the center of each PDA plate. Control was treated with 20 μl of acetone alone. The diameters of inhibition zones were recorded after incubation at 25°C for 5 to 7 days.

Verlamelin was also tested *in vivo* for antifungal activity against the following diseases: rice blast (*M. grisea*); rice sheath blight (*T. cucumeris*); cucumber gray mold (*B. cinerea*); tomato late blight (*Phytophthora infestans*); wheat leaf rust (*Puccinia recondita*); and barley powdery mildew (*Erysiphe graminis* f.sp. *hordei*) by the methods previously described (Kim et al., 1999; Kim et al., 2001; Park et al., 2000). Rice (*Oryza sativa* L., cv. Nakdong), tomato (*Lycopersicon esculentum* Mill., cv. Seokwang), cucumber (*Cucumis sativus* L., cv. Hausbackdadagi), barley (*Hordeum sativum* Jessen, cv. Dongbori), and wheat (*Triticum aestivum* L., cv. Chokwang) plants were grown in vinyl pots (4.5 cm diameter) in a greenhouse at 25±5°C for 1-4 weeks. The plant seedlings were sprayed with verlamelin dissolved in water + methanol (95 + 5 by volume) containing Tween 20 (250 μg/ml) as wetter and allowed to stand for 24 h. The treated plant seedlings were inoculated with spores or mycelial suspensions of six plant pathogenic fungi and then disease severity was assessed 3-7 days after inoculation.

In order to investigate further *in vivo* activities against *E. graminis* f. sp. *hordei* such as the duration of protection and curative activity, foliar applications of verlamelin were made protectively (7, 4 and 1 days prior to inoculation) and curatively (2 and 1 days after inoculation).

Results and Discussion

The *in vitro* antimicrobial activities of verlamelin are presented in Table 1. It was not active against all the bacteria tested at a rate of 20 μg/paper disc (Table 1). In contrast, it showed a strong activity against several fungi including *M. grisea, B. maydis,* and *B. cinerea. F. oxysporum* and *A. alternata* were much less sensitive to the antibiotic. However, it was virtually inactive against *T. cucumeris* and *P. graminicola.*

In *in vivo* tests, verlamelin exhibited potent control activities against wheat leaf rust and barley powdery mildew with control values of more than 90% at a concentration of 100 μg/ml (Table 2). It almost inhibited the development of barley powdery mildew even at a concentration of 10 μg/ml. It showed weak *in vivo* antifungal activities against cucumber gray mold and tomato late blight. However, it hardly controlled the development of rice blast and rice sheath blight even at a concentration of 100 μg/ml. Foliar infections of *E. gaminis* f. sp. *hordei* were almost completely controlled with 100 μg/ml of verlamelin applied up to 7 days before inoculation; it showed a strong control activity in 7, 4, and 1-day protective applications (Table 3). Verlamelin also showed a strong curative activity against barley powdery mildew, almost inhibiting the development of barley powdery mildew in 2 and 1-day curative appli-

Table 1. Antimicrobial activity of verlamelin from *Acremonium strictum* BCP against various bacteria and fungi by the disc diffusion method[a]

Test organism	Diameter of inhibition zone (mm)[b]
Bacteria	
Pseudomonas putida	–[c]
Xanthomonas campestris pv. *vesicatoria*	–
X. campestris pv. *campestris*	–
Erwinia carotovora	–
Bacillus subtilis	–
Streptomyces griseus	–
Fungi	
Magnaporthe grisea	35
Bipolaris maydis	34
Botrytis cinerea	30
Fusarium oxysporum	20
Alternaria alternata	15
Thanatephorus cucumeris	–
Pythium graminicola	–

[a] The antibiotic was dissolved in acetone at a concentration of 1 mg/ml and treated with 20 μl of the antibiotic solution per paper disc.
[b] Includes the diameter of paper disc (8 mm) used.
[c] – : no inhibition.

Table 2. *In vivo* antifungal activity of verlamelin isolated from *Acremonium strictum* BCP against six phytopathogenic fungi[a]

Disease	Pathogen	Dose (μg/ml)	
		20	100
Rice blast	*Magnaporthe grisea*	0[b]	0
Rice sheath blight	*Thanatephorus cucumeris*	5	10
Cucumber gray mold	*Botrytis cinerea*	0	28
Tomato late blight	*Phytophthora infestans*	15	36
Wheat leaf rust	*Puccinia recondita*	0	95
Barley powdery mildew	*Erysiphe graminis* f. sp. *hordei*	80	98

[a] The plant seedlings were inoculated with spores or mycelial suspensions of the test organisms one day after the antibiotic solution was sprayed to run-off on the leaves.
[b] Control value (%) = 100 × (disease severity of untreated plants – disease severity of treated plants) ÷ disease severity of untreated plants.

cations.

The antibiotic was originally reported from the liquid cultures of *Verticillium lamellicola* as an antifungal agent. Even though *in vitro* antibiotic activities of verlamelin as reported by Rowin et al. (1986) were similar to the results of this study, *in vivo* activities were much different. Rowin et al. (1986) reported that verlamelin was particularly active against rice blast with control values of more than 60% even at a concentration of 0.01 μg/ml using their standard greenhouse tests. Such difference in *in vivo* activity appears to be caused by different test methods including inoculum concentration, plant stage, and rating method.

On the other hand, verlamelin showed strong protective and curative activities against barley powdery mildew. At present, two major chemical classes inhibiting sterol biosynthesis are generally being used for the control of barley powdery mildew. One chemical class including cyproconazole, epoxiconazole, and tebuconazole (azoles) inhibits sterol biosynthesis through C14 demethylation inhibition (DMIs) (Koller, 1992), while the other class including fenpropimorph, tridemorph, and fenpropidin (morpholines and piperidines) works through the inhibition of $\Delta^{87}\rightarrow\Delta^{7}$-isomerase (Baloch and Mercer, 1987). Recently, other broad-spectrum compounds, such as the synthetic strobilurins affecting mitochondrial transport, and anilinopyrimidines inhibiting amino acid biosynthesis, have been developed and commercialized (Godwin et al., 1992; Heye et al., 1994). However, resistance of *E. graminis* f. sp. *hordei* to DMIs, morpholines, and synthetic strobilurins has reduced the usefulness of the fungicides (Gisi et al., 1986; Gilmour, 1994; Hardwick, 1994). The problem prompted the need for new fungicides with different chemical skeletons. In this respect, verlamelin with its strong protective and curative activities against barley powdery mildew is very important for the development of such fungicides, even though chemical synthesis of the lipopeptolide seems to be sophisticated. This is the first report on the production of verlamelin by *Acremonium* species.

Table 3. *In vivo* control of *Erysiphe graminis* f. sp. *hordei* with protective and curative spray applications of verlamelin

Chemical	Concentration (μg/ml)	Control of affected leaf area (%) (±SD)[a]				
		Days prior to inoculation			Days after inoculation	
		7	4	1	1	2
Verlamelin	100	92±7.6	92±2.7	95±3.9	93±6.7	92±4.7
Fenarimol	30	100±0	100±0	100±0	100±0	100±0

[a] Disease rating was carried out 7 days after inoculation.

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