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Antibiotic and Phytotoxic Activities of Ophiobolins from *Helminthosporium* Species

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Twenty isolates of *Helminthosporium* species were obtained from various grass plants and tested for controlling efficacy on the development of plant diseases. An isolate of *Helminthosporium* sp. TP-4 was chosen and six antibiotic substances were purified from cultures of the fungus by repeated silica gel column chromatography and preparative thin-layer chromatography. They were identified as ophiobolin A, 6-epiophiobolin A, 3-anhydroophiobolin A, 3-anhydro-6-epiophiobolin A, ophiobolin B, and ophiobolin I mainly by mass spectrometry and nuclear magnetic resonance spectrometry. Ophiobolins inhibited the growth of a gram-positive bacterium *Streptomyces griseus*, but were not active against gram-negative bacteria. They also showed an antifungal activity. In *in vivo* tests, ophiobolin B exhibited potent controlling activities against rice blast, tomato late blight, and wheat leaf rust with control values more than 90% and 70% at concentrations of 500 μg/ml and 100 μg/ml. Ophiobolin A and 6-epiophiobolin A controlled the development of wheat leaf rust more than 80% at concentrations of 100 /ml and 500 μg/ml, respectively. 3-Anhydro-6-epiophiobolin A was not active against any plant disease. On the other hand, the A-series ophiobolins other than 3-anhydroophiobolin A showed stronger phytotoxic activity in a leaf-wounding assay using 8 plant species than those of 3-anhydroophiobolin A, ophiobolin B, and ophiobolin I. The results indicate that there is little correlation between antifungal activity and phytotoxicity of ophiobolins.

Keywords : antibiotic activity, *Helminthosporium* sp., ophiobolins, phytotoxicity, sesterterpenes.

Helminthosporium species are distributed worldwide and common on grass plants. They cause various plant diseases such as rice leaf blight, southern corn leaf blight, Jonson grass leaf spot, and spot blotch of cereals and grasses. Graminicolous species are classified as *Drechslera* (the anamorph of *Pyrenophora*), *Bipolaris* (the anamorph of *Cochliobolus*), or *Exserohilum* (the anamorph of *Setosphaeria*) (Alcorn, 1988). Many *Helminthosporium* species produce a number of secondary metabolites including ophiobolins, HC toxins, cytochalasins, and sicanochromenes.

Ophiobolins are a group of sesterterpenes with unique chemical structures characterized by a tricyclic [5-8-5] ring system. They are produced mainly by *Helminthosporium* species and *Cephalosporium caerulens* (Itai et al., 1967) as well as *Aspergillus ustus*. Ophiobolins A (cochliobolin A) and B (cochliobolin B) were originally isolated from *H. oryzae* (syn. *B. oryzae*, the anamorph of *C. miyabeanus*), the causal agent of brown leaf spot disease of rice, as a toxic principle to rice seedlings (Nakamura and Ishibashi, 1958; Orsenigo, 1957). The ophiobolins are phytotoxic and cause inhibition of root and coleoptile elongation and leaf chlorosis on many plants including hosts and nonhosts of *H. oryzae* (Orsenigo, 1957).

In addition to phytotoxicity, ophiobolins also have nematocidal and antibiotic activities. Ophiobolins C, K, and M exhibit a potent nematocidal activity against the free-living nematode, *Caenorhabditis elegans*, but their 6-*epi* isomers are much less active (Tsipouras et al., 1996). Li et al. (1995) found that ophiobolin A had moderate to strong activity against fungi. Ophiobolins are active against gram-positive bacteria, but not active against gram-negative bacteria (Cutler et al., 1984; Li et al., 1995).

Antibiotics from microorgnisms have been extensively studied owing to the possible usage of the metabolites directly as agrochemicals or as lead molecules for the development of new fungicides. From pyrrolnitrin, a secondary metabolite of *Pseudomonas pyrrocinia*, two synthetic phenylpyrroles of fenpiclonil (Nevill et al., 1988) and fludioxonil (Gehmann et al., 1990) have been developed as seed-dressing agents against numerous fungal pathogens. Strobilurin A and oudemansin A are fungicidal natural products found in the Basidiomycete fungi *Strobilurus tenacellus* (Anke et al., 1977) and *Oudemansiella mucida* (Musilek et al., 1969), respectively. Several fungicides such as azoxystrobin (Godwin et al., 1992), kresoxim-methyl (Ammerman et al., 1992), and SSF 126 (Mizutani et al., 1995) have been synthesized by using the two fungal

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metabolites as lead molecules and commercialized.

During our screening of new antibiotics produced by *Helminthosporium* isolates, we obtained 20 *Helminthosporium* isolates from infected tissues of various grass plants. *Helminthosporium* sp. TP-4 isolate showed the most potent antifungal activity against several plant diseases. From the cultures of the isolate, six ophiobolins with antibiotic activity were isolated and identified. This paper describes the isolation, structure determination, antibiotic activity, and phytotoxicity of the substances.

Materials and Methods

Isolation and culture of *Helminthosporium* species. Isolation of *Helminthosporium* species was attempted from 12 diseased samples of 7 grass plants, consisting of 2 samples of needlegrass (*Stipa coreana* Honda), 2 samples of corn (*Zea mays* L.), 4 samples of barnyardgrass (*Echinochloa crus-galli* (L.) Scop.), 1 sample of large crabgrass (*Digitaria sanguinalis* (L.) Scop.), 1 sample of small-flowered umbrella sedge (*Cyperus difformis* L.), 1 sample of Japanese cutgrass (*Leersia japonica* Makino), and 1 sample of zoysiagrass (*Zoysia japonica* L.). Leaves and stems showing incipient to moderately advanced lesios were cut into 1- to 2-cm sections with a sterile scalpel. The pieces were disinfested in 0.5% NaOCl for 1 min, rinsed in sterile distilled water, and placed on potato dextrose agar (PDA) amended with streptomycin (200 mg/L). Twenty isolates of *Helminthosporium* species were obtained and transferred to PDA slant tubes and distilled water tubes, which were stored at 4 °C. *Helminthosporium* species were identified to species with the criteria described by Alcorn (1988) and Ellis (1971).

Each Erlenmeyer flask (250 ml) containing 100 ml of potato dextrose broth (PDB) medium was inoculated with mycelial agar discs from a 5-day-old PDA plate of each fungus. Twenty flasks were incubated for 10 days at 25 °C with an agitation of 150 rpm, and the cultures were centrifuged at 5,000 rpm for 15 min. The supernatants were stored at 4 until use.

Evaluation of antifungal activity in greenhouse. In order to select a fungal isolate showing a potent antifungal activity, the culture supernatants of 20 *Helminthosporium* isolates were tested for the *in vivo* controlling activity against 6 plant diseases such as rice blast, rice sheath blight, tomato late blight, cucumber grey mold, barley powdery mildew, and wheat leaf rust using greenhouse tests. 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 plastic pots (4.5 cm diameter) in a greenhouse at 25±5°C for 1 to 3 weeks. The potted plant seedlings (12 pots) were sprayed with 30 ml of each supernatant containing 250 μg/ml Tween 20 and then dried overnight.

The treated rice seedlings of the 2nd leaf stage were inoculated with *Magnaporthe grisea,* a causal agent of rice blast, by spraying a spore suspension (5×10^5 spores/ml) of the fungus. Following the incubation of the seedlings in a moist chamber for 1 day at 25°C, they were transferred to an incubation room at 25°C. The disease severity on the seedlings was determined as the percentage of infected leaf area 5 days after the inoculation. For the development of rice sheath blight, the treated rice seedlings of the 3rd leaf stage were inoculated by adding 7-day-old wheat-rice bran cultures (90 g of wheat bran, 15 g of rice bran, and 100 ml of distilled water in 1L Erlenmeyer flask) of *Thanatephorus cucumeris* to soil. After incubation for 4 days in a moist chamber at 25 °C and for 4 days in an incubation room at 25°C, the disease severity were determined.

The treated tomato seedlings of the 2nd leaf stage were inoculated with *Phytophthora infestans* causing tomato late blight by spraying a spore suspension released from sporangia suspension (10^5 sporangia/ml) of the fungus. After incubation in a moist chamber for 4 days at 20 °C, the disease severity was determined. The cucumber seedlings of the 1st leaf stage were inoculated by spraying a spore suspension (10^6 spores/ml) of *Botrytis cinerea*, a causal fungus of cucumber grey mold, and incubated in a moist chamber for 3 days. Then, the disease severity was determined.

The treated wheat seedlings of the 1st leaf stage were inoculated with *Puccinia recondita,* a causal fungus of wheat leaf rust, by spraying a spore suspension (0.67 g spores/L, containing 250 μg/ml Tween 20) of the fungus. They were incubated in a moist chamber for 1 day at 20 °C and then transferred to an incubation room. The disease severity was determined by rating the infected leaf area 7 days after the inoculation. For the development of barley powdery mildew, the treated barley seedlings of the 1st leaf stage were inoculated with spores of *Blumeria graminis* f. sp. *hordei* formed on the host plants for subculture. The inoculated barley seedlings were incubated for 7 days at 20 °C in an incubation room, and then the disease severity on the seedlings was determined.

The ophiobolins isolated from *Helminthosporium* sp. TP-4 were dissolved in 10% acetone containing 200 μg/ml Tween 20 and then sprayed to run-off on the leaves of the potted plant seedlings at concentrations of 20, 100, and 500 μg/ml. The control check was 10% acetone in distilled water containing 200 μg/ml Tween 20.

Isolation of ophiobolins. A total of 4 L of 10-day-old cultures of *Helminthosporium* sp. TP-4 were filtered through four layers of cheesecloth and then the filtrates were extracted three times with equal volumes of ethyl acetate. The extracts were evaporated to dryness at 45 °C under reduced pressure. The residue (4.3 g) was subjected to silica gel (Kiesel gel 60, 70/230 mesh, 200 g; E. Merck, Darmstadt, Germany) column (3.6 cm [inner diameter] by 60 cm) chromatography, and the column was eluted with ethyl acetate-hexane (2:1, v/v). The eluate was collected in 15-ml fractions with a fraction collector. The fractions were monitored by thin-layer chromatography (TLC) and reduced to three fractions called F1, F2, and F3. Both F1 and F2 containing ophiobolins were further purified. F1 (2.5 g) was suspended in ethyl acetate-hexane (2:1, v/v) and loaded onto a silica gel column (3.2 cm [inner diameter] by 60 cm; Kiesel gel 60, 230/400 mesh, 100 g; E. Merck), which was washed with ethyl acetate-hexane (2:1, v/v) to give three fractions called F11, F12, and F13. F11 (120 mg) containing two ophiobolins was separated by preparative silica gel

TLC, using ethyl acetate-hexane (2:1, v/v) to give 3-anhydroophiobolin A (3.9 mg) and 3-anhydro-6-epiophiobolin A (27 mg). F12 (1.6 g) was finally purified by with silica gel column (2.8 cm [inner diameter] by 45 cm; Kiesel gel 60, 70 g; E. Merck) and elution of chloroform-methanol (98:2, v/v) to give ophiobolin A (1.5 g). F13 (230 mg) was separated by preparative TLC, using chloroform-methanol (98:2, v/v) to give ophiobolin I (5 mg) and 6-epiophiobolin A (55 mg). F2 (130 mg) was twice separated by preparative TLC, using chloroform-methanol (98:2, v/v) to give ophiobolin B (64 mg).

Spectral measurements. The UV spectroscopy was recorded in a methanol solution on a Shimadzu UV-2401PC spectrophotometer (Shimadzu Co., Tokyo, Japan). Mass spectra were recorded on a double-focusing high-resolution (HR) mass spectrometer (JEOL JMS-DX303; JEOL Ltd., Tokyo, Japan). ^{1}H-nuclear magnetic resonance (NMR) spedra were recorded in $CDCl_3$ on a Bruker AMX-500 (500 MHz) NMR spectrometer (Bruker Analytische Messtechnik Gmbh, Rheinstetten, Germany). Information concerning chemical structures was obtained from ^{1}H-NMR spectra by comparison with those of ophiobolins previously reported (Canonica et al., 1966a; Canonica et al., 1966b; Kim et al., 1984; Nozoe et al., 1965; Nozoe et al., 1966; Ohkawa and Tamura, 1966; Sugawara et al., 1987; Tsuda et al., 1967).

***In vitro* antibiotic activities of ophiobolins.** The antimicrobial activities of ophiobolins 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 used as test bacteria. The baterial 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 100 μl of each antibiotic solution was loaded onto a paper disc (8.0 mm diameter). After dryness, the paper discs were placed on the surfaces of NA medium seeded with bacteria. The antibacterial activity on the test microorganisms was determined according to presence or absence of an inhibition zone after incubating for 24 hr at 30°C.

The anifungal activity of ophiobolins were tested for the following fungi; *Alternaria alternnata, B. cinerea, C. heterostrophus, Fusarium oxysporium, M. grisea, Pythium graminicola,* and *T. cucumeris.* Mycelial plugs (6 mm diameter) from the margin of 5-day-old cultures of test fungi grown on PDA were seeded on the centers of PDA plates. The paper discs (8 mm diameter) impregnated with 100 μl of antibiotic solutions (1 mg/ml) were placed near the mycelial plugs of test fungi with a solvent-treated disc as control. The mycelial growth inhibition was recorded after incubation at 25°C for 5 to 7 days.

Phytotoxic activities of ophiobolins. The phytotoxic activities of ophiobolins to various plants were tested by a leaf-wounding assay. Rice, barley, corn, soybean (*Glycine max* L.), grain sorghum (*Sorghum bicolar* Moench), goose grass (*Eleusine indica* (L.) Gaertn.), cocklebur (*Xanthium strumarium* L.), and velvetleaf (*Abutilon theophrasti* Medik) were used. The plants (2-weeks old) were grown in a greenhouse and centers of detached plant leaves were nicked with a pin. The damaged leaf surfaces immediately covered with 5 μl of each toxin solution. Ophiobolins were dissolved in methanol and diluted with distilled water to give final toxin concentration of 1 mM. The control used was 2% methanol in distilled water. The leaf blades treated were subsequently incubated in a sealed Petri dish containing moistened filter paper. After 72 hr incubation at 24°C, phytotoxicity was assessed.

Results and Discussion

Fungal isolation and *in vivo* antifungal activity. Twenty isolates of *Helminthosporium* sp. were recovered from 12 samples of 7 grass plant species. Representatives of five species were identified: *C. sativus* (the teleomorph of *B. sorokiniana*; 2 isolates from zoysiagrass), *C. miyabeanus* (the teleomorph of *B. oryzae*; 2 isolates from rice), *C. heterostrophus* (the teleomorph of *B. maydis*; 1 isolate from needlegrass and 2 isolates from corn), *Pyrenophora avenae* (the teleomorph of *Drechslera avenae*; 1 isolate from needlegrass), and *Setosphaeria monoceras* (the teleomorph of *Exserohilum monoceras*; 6 isolates from barnyardgrass); 6 isolates could not be identified.

Sixteen (80%) of the 20 *Helminthosporium* isolates showed disease-controlling activities more than 70% against rice blast (Table 1). The other plant diseases were controlled more than 70% by 1 isolate (5%) for rice sheath blight, 2 isolates (10%) for cucumber grey mold, 1 isolate (5%) for tomato late blight, 4 isolates (20%) for barley powdery mildew, and 7 isolates (35%) for wheat leaf rust. Especially, *Helminthosporium* sp. TP-4 controlled 5 plant diseases more than 70%. Since the isolate exhibited the most potent antifungal activity, it was chosen for the further study on the isolation of antibiotic substances.

Isolation and identification of ophiobolins. A series of antibiotic sesterterpenoids was isolated from 10-day-old cultures of *Helminthosporium* sp. TP-4. Six active compounds were identified as ophiobolins by spectroscopic procedures of ^{1}H-NMR spectroscopy and mass spectrometry (Fig. 1). The major metabolite was ophiobolin A, and ophiobolin B, 6-epiophiobolin A, and 3-anhydro-6-epiophiobolin A were isolated in minor amounts. In addition, ophiobolin I and 3-anhydroophiobolin A were purified in trace amounts.

Ophiobolins are a family of naturally occurring sesterterpenes characterized by a tricyclic ring system and known as phytotoxins, antibiotics, or nematocidal agents. *C. miyabeanus, C. heterostrophus, B. sorghicola, B. zizaniae, B. leersiae, S. turcica,* and *H. panici-miliacei* have been reported to be producers of ophiobolins (Ishibashi, 1961; Ishibashi, 1962; Nozoe et al., 1966; Scheffer, 1983). *Cephalosporium caerulens* (Itai et al., 1967) and *A. ustus* (Cutler et al., 1984; Singh et al., 1991) were also reported to produce the secondary metabolites; the former produces ophiobolin D and the latter ophiobolins G, H, and K, and 6-

Table 1. Disease controlling activity of 20 *Helminthosporium* isolates from infected tissues of various grass plants against six plant diseases[a]

Organism	Source	Control value (%)[b]					
		RCB[c]	RSB	CGM	TLB	BPM	WLR
Cochliobolus heterostrophus TP-K	Needlegrass	96	30	60	60	50	96
C. heterostrophus B-23	Corn	76	0	70	26	58	66
C. heterostrophus B-24	Corn	71	0	20	33	80	86
C. miyabeanus B-31	Rice	83	5	70	40	0*	88
C. miyabeanus B-32	Rice	95	0	20	66	16	83
C. sativus L-08	Zoysiagrass	63	0	20	0	0	0
C. sativus L-09	Zoysiagrass	66	5	20	0	0	0
Pyrenophora avenae 12042	Needlegrass	86	0	0	33	0	33
Setosphaeria monoceras H-06	Barnyardgrass	83	0	20	0	0	0
S. monoceras H-07	Barnyardgrass	71	10	40	0	0	0
S. monoceras H-09	Barnyardgrass	86	0	0	0	0	0
S. monoceras H-12	Barnyardgrass	86	0	20	0	0	0
S. monoceras H-13	Barnyardgrass	58	0	0	0	0	0
S. monoceras H-882	Barnyardgrass	80	0	0	0	0	0
Helminthosporium sp. TP-4	Needlegrass	90	70	40	76	80*	93
Helminthosporium sp. L-09	Corn	58	0	20	40	80	94
Helminthosporium sp. L-10	Barnyardgrass	86	0	20	6	0	0
Helminthosporium sp. L-11	Large crabgrass	86	0	20	66	43	86
Helminthosporium sp. L-13	Small-flowered umbrella sedge	90	0	0	0	0*	0
Helminthosporium sp. L-14	Japanese cutgrass	91	0	40	6	71	66

[a] The plant seedlings were inoculated with spores or mycelial suspensions of the test organisms 1 day after the culture supernatants of 20 *Helminthosporium* isolates were 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
[c] RCB, rice blast; RSB, rice sheath blight; CGM, cucumber grey mold; TLB, tomato late blight; BPM, barley powdery mildew; WLR, wheat leaf rust.
*Phytotoxicity

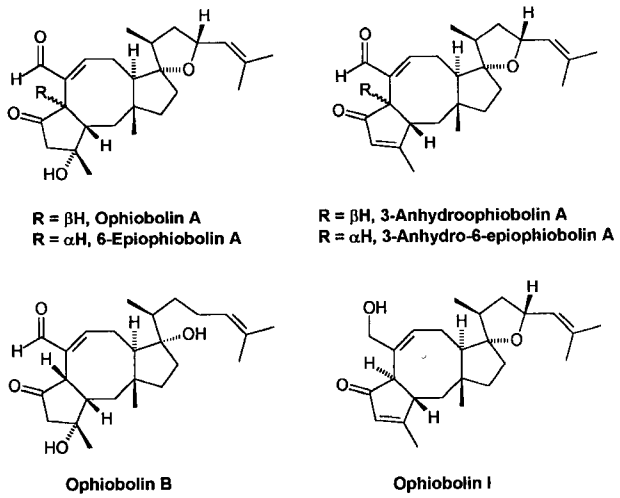

Fig. 1. Chemical structures of six ophiobolins isolated from *Helminthosporium* sp. TP-4.

piophiobolin K. In the present study, six antibiotic substances belonging to ophiobolin series were detected as metabolites of *Helminthosporium* sp. TP-4. *C. miyabeanus* was also reported to produce the six substances (Xiao et al., 1991). Sugawara et al. (1987) reported that *C. miyabeanus* produced ophiobolin A, 6-epiophiobolin A, 3-anhydro-6-epiophiobolin A, ophiobolin C, ophiobolin I, while *B. sorghicola* did ophiobolin A, 6-epiophiobolin A, ophiobolin I, and 25-hydroxyophiobolin I. Ophiobolin A and its analogs including 6-epiophiobolin A, 3-anhydroophiobolin A, and 3-anhydro-6-epiophiobolin A were produced by an unidentified isolate of *Helminthosporium* (Kim et al., 1984).

Antibiotic activities of ophiobolins. Ophiobolins inhibited the growth of a gram-positive bacterium *Streptomyces griseus*, while they were not active against all of the gram-negative bacteria tested at a rate of 100 μg/paper disc (Table 2). Such a characteristic was also previously reported (Cutler et al., 1984; Li et al., 1995); ophiobolins A and B inhibited the growth of a gram-positive bacterium *Staphylococcus aureus*, but they did not inhibit that of a gram-negative bacterium *Escherichia coli* (Li et al., 1995). Ophiobolins G and H showed the antibiotic activity against *Bacillus substilis*, while neither inhibit the growth of *E. coli* (Cutler et al., 1984).

Of the 4 ophiobolins tested, ophiobolin B showed the strongest *in vitro* antifungal activity followed by ophiobolin A and 3-anhydro-6-epiophiobolin A. Ophiobolin B inhibited mycelial growth of all of the fungi tested, while 6-epiohiobolin A did not show any antifungal activity at the treated amount. Li et al. (1995) also reported that ophiobolin A had moderate to strong activity against fungi with

Table 2. Antibiotic activity of ophiobolins from *Helminthosporium* sp. TP-4 against various bacteria and fungi by the disc diffusion method[a]

Test organism	O–A[b]	EO–A	AEO–A	O–B
Bacteria				
Erwinia carotovora	–[c]	–	–	–
Streptomyces griseus	+	+	+	+
Pseudomonas putida	–	–	–	–
Xanthomonas campestris pv. *campestris*	–	–	–	–
X. c. pv. *vesicatoria*	–	–	–	–
Fungi				
Alternaria alternata	–	–	–	+
Botrytis cinerea	+	–	–	+
Cochliobolus heterostrophus	+	–	+	+
Fusarium oxysporium	–	–	–	+
Magnaporthe grisea	–	–	–	+
Pythium graminicola	+	–	+	+
Thanatephorus cucumeris	+	–	+	+

[a] Each compound was dissolved in acetone at a concentration of 1 mg/ml and treated 100 μl of toxin solutions per paper disc.
[b] O–A, ophiobolin A; EO–A, 6-epiophiobolin A; AEO–A, 3-anhydro-6-epiophiobolin A; O-B, ophiobolin B.
[c] Evaluated by inhibitory activity against microbial growth: –, inactive; +, active.

minimum inhibitory concentrations of 25 μg/ml for *A. flavus*, 12.5 μg/ml for *Candida albicans* and *Trichophyton mentagrophytes*, 1.56 μg/ml for *Torulopsis petrophilum*, and 0.20 /ml for *Torulopsis cremoris*.

Ophiobolin B exhibited a potent controlling activity against rice blast, tomato late blight, and wheat leaf rust with control values more than 90% and 70% at concentrations of 500 μg/ml and 100 μg/ml, respectively (Table 3). However, it was not active against the other plant diseases such as rice sheath blight, cucumber grey mold, and barley powdery mildew. Ophiobolin A and 6-epiophiobolin A controlled the development of wheat leaf rust alone more than 80% at concentrations of 100 μg/ml and 500 μg/ml, respectively. 3-Anhydro-6-epiophiobolin A was hardly active against the 6 plant diseases. Three A-series substances showed phytotoxicity on barley at a concentration of 500 μg/ml, but ophiobolin B did not exhibit evident phytotoxicity on any plants.

Phytotoxic activities of ophiobolins. Ophiobolins showed varying degrees of phytotoxicity in the leaf-wounding assay (Table 4) and they caused reddish brown lesions, sunken lesions, necrotic lesions, and dark necrotic lesions on the leaves. Of the 6 ophiobolins tested, 3-anhydroophiobolin A and ophiobolin A appeared to be most phytotoxic and both substances caused necrosis on the leaves of all plants. 6-Epiophiobolin A was moderately active, and the other three ophiobolins of 3-anhydro-6-epiophiobolin A, ophiobolin B and ophiobolin I exhibited relatively low phytotoxicity.

Ophiobolins have a broad spectrum of biological activity and are active to plants, fungi, bacteria (Cutler et al., 1984; Li et al., 1995), and nematodes (Singh et al., 1995; Tsipouras et al., 1996). Ophiobolin A interacts with the maize calmodulin (Ca^{2+}-binding protein), resulting in the loss of

Table 3. Disease controlling activity of ophiobolins from *Helminthosporium* sp. TP-4 against six plant diseases[a]

Compounds	Conc. (μg/ml)	Control value (%)[b]					
		RCB[c]	RSB	CGM	TLB	BPM	WLR
Ophiobolin A	500	0	15	13	50	0*	93
	100	0	20	25	0	0	87
	20	0	25	0	0	0	0
6-Epiophiobolin A	500	0	0	25	50	0*	80
	100	0	0	0	50	0	0
	20	0	0	0	0	0	0
3-Anhydro-6-epiophiobolin A	500	0	10	0	20	0*	0
	100	0	10	0	0	0	0
	20	0	5	0	0	0	0
Ophiobolin B	500	93	0	19	95	0	97
	100	80	0	13	80	0	73
	20	25	0	0	50	0	67

[a] The plant seedlings were inoculated with spores or mycelial suspensions of the test organisms 1 day after the toxin solutions of ophiobolins were 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
[c] RCB, rice blast; RSB, rice sheath blight; CGM, cucumber grey mold; TLB, tomato late blight; BPM, barley powdery mildew; WLR, wheat leaf rust.
*Phytotoxicity

Table 4. Phytotoxicity of ophiobolins from *Helminthosporium* sp. TP-4 by the leaf-wounding assay[a]

Plant	O-A[b]	EO-A	AO-A	AEO-A	O-B	O-I
Rice	++(a)[c]	++	+++	+	+	+
Barley	++++(b)	+++	+++	-	+	+
Corn	+++(b)	++	+++	+	+	+
Soybean	++(b)	++	++	-	++	+
Grain sorghum	+++(a)	++	+++	++	+++	+
Goose grass	++(b)	+++	++	++	++	++
Cocklebur	+(d)	++	++	–	+	+
Velvet-leaf	+(d)	–	++	–	–	+

[a] The leaves on filter papers moistened in petri dishes were incubated at 24 for 72 hr.
[b] Each compound was dissolved in 2% methanol in distilled water and 5 μl of each toxin solution was applied onto each pin hole. O-A, ophiobolin A; EO-A, 6-epiophiobolin A; AO-A, 3-anhydroophiobolin A; AEO-A, 3-anhydro-6-epiophiobolin A; O-B, ophiobolin B; O-I, ophiobolin I.
[d] Lesion size: ++++, >4.5 mm; +++, 3.0 mm to 4.5 mm; ++, 1.5 mm to 3.0 mm; +, 0.5 mm to 1.5 mm; –, <0.5 mm.
(a) Reddish brown lesion; (b) Sunken lesion; (c) Necrotic brown lesion; (d) Dark necrotic lesion.

ability of the calmodulin to activate the phosphodieasterase (Leung et al., 1985). The calmodulin interaction compound ophiobolin A inhibits the sporangium production in *Phytophthora palmivora* which requires calcium (Elliot, 1986). Ophiobolins C, K, and M have much stronger nematocidal activity than their 6-*epi* isomers. The nematocidal activity of ophiobolins is known to correlate with their affinity for the receptor of ivermectin, which is a widely used anthelmintic agent known to modulate an invertebrate-specific glutamate-gated chloride channel (Tsipouras et al., 1996).

In this study, ophiobolin B showed stronger antifungal activity than A-series ophiobolins, while the former was less active to plants than the latters exception that 3-anhydro-6-epiophiobolin A in the leaf-wounding assay. There was little correlation between antifungal activity and phytotoxicity of ophiobolins. On the other hand, ophiobolin B showed clear disease controlling activity without any phytotoxicity on whole plants. The compound was particularly active against rice blast, tomato late blight, and wheat leaf rust. It may play a significant role as a lead compound in the development of new fungicides.

References

Alcorn, J. L. 1988. The taxonomy of "Helminthosporium" species. *Annu. Rev. Phytopathol.* 26:37-56.

Ammerman, E., Lorenz, G., Schelberger, K., Wenderoth, B., Sauter, H. and Rentzea, C. 1992. BAS 490F-a broad-spectrum fungicide with a new mode of action. *Brighton Crop Prot. Conf. Pests Dis.* 1:403-410.

Anke, T., Oberwinkler, F., Steglich, W. and Schramm, G. 1977. The strobilurins-new antifungal antibiotics from the Basidiomycete *Strobilurus tenacellus*. *J. Antibiot.* 30:806-810.

Canonica, L., Fiecchi, A., Galli Kienle, M. and Scala, A. 1966a. The constitution of cochliobolin. *Tetrahedron Lett.* 11:1211-1218.

Canonica, L., Fiecchi, A., Galli Kienle, M. and Scala, A. 1966b. Isolation and constitution of cochliobolin B. *Tetrahedron Lett.* 13:1329-1333.

Cutler, H. G., Crumley, F. G., Cox, R. H., Springer, J. P., Arrendale, R. F., Cole, R. J. and Cole, P. D. 1984. Ophiobolins G and H: new metabolites from a novel source, *Aspergillus ustus*. *J. Agric. Food Chem.* 32:778-782.

Ellis, M. B. 1971. Dermatiaceous Hypomycetes. Commonwealth Mycological Institute, Kew, England. 608pp.

Elliot, C. G. 1986. Inhibition of reproduction of *Phytophthora* by the calmodulin-interacting compounds trifluperazine and ophiobolin A. *J. Gen. Microbiol.* 132:2781-2785.

Gehmann, K., Nyfeler, R., Leadbeadter, A. J., Nevill, D. and Sozzi, D. 1990. CGA 173506: a new phenylpyrrole fungicide for broad-spectrum disease control. *Proc. Brighton Crop Conf. Pests Dis.* 2:399-406.

Godwin, J. R., Anthony, V. M., Clough, J. M. and Godfrey, C.R.A. 1992. ICIA 5504: a novel broad spectrum, systemic-methoxyacrylate fungicide. *Proc. Brighton Crop Conf. Pests Dis.* 1:435-442.

Ishibashi, K. 1961. Studies on antibiotics from *Helminthosporium* sp. fungi. Part III. Ophiobolin production by *Helminthosporium turcicum*. *Nippon Nogeigaku Kaishi* 35:323-326.

Ishibashi, K. 1962. Studies on antibiotics from *Helminthosporium* sp. fungi. Part IV. Ophiobolin production by *Ophiobolus heterostrophus, Helminthosporium leersi, H. panici-miliacei and H. zizaniae*. *Nippon Nogeigaku Kaishi* 36:226-228.

Itai, A., Nozoe, S., Tsuda, K. and Okuda, S. 1967. The structure of cephalonic acid, a pentaprenyl terpenoid. *Tetrahedron Lett.* 42:4111-4112.

Kim J. M., Hyeon, S. B., Isogai, A. and Suzuki, A. 1984. Isolation of ophiobolin A and its analogs as inhibitors to photosynthesis. *Agric. Biol. Chem.* 48:803-805.

Leung P. C., Taylor, W. A., Wang, J. H. and Tipton, C. L. 1985. Role of calmodulin inhibition in the mode of action of ophiobolin A. *Plant Physiol.* 77:303-308.

Li, E., Clark, A. M., Rotella, D. P. and Hufford, C. D. 1995. Microbial metabolites of ophiobolin A and antimicrobial evaluation of ophiobolins. *J. Nat. Prod.* 58:74-81.

Mizutani, A., Miki, N., Yukioka, H. and Masuko, M. 1995. Mechanism of action of a novel alkoxyaminoacetamide fungicide SSF-126. *Proc. Brighton Crop Conf.-Weeds, 1995.* 1: 89-96.

Musilek, V., Cerna, J., Sasek, V., Semerdzieva, M. and Vondracek, M. 1969. Antifungal antibiotic of the Basidiomycete *Oudemansiella mucida*. *Folia Microbiol.* (Prague) 14:377-387.

Nakamura, M. and Ishibashi, K. 1958. New antibiotics "ophiobolins", produced by *Ophiobolus miyabeanus*. *Nippon Nogeikagaku Kaishi* 32:739-744.

Nevill, D., Nyfeler, R. and Sozzi, D. 1988. CGA 142705: a novel fungicide for seed treatment. *Proc. Brighton Crop Conf. Pests Dis.* 1:65-72.

Nozoe, S., Hirai, K. and Tsuda, 1966. The structure of zizanin-A and -B, C_{25} terpenoids isolated from *Helminthosporium zizaniae*. *Tetrahedron Lett.* 20:2211-2226.

Nozoe, S., Morisaki, M., Tsuda, K., Takahashi, N., Tamura, S., Ishibashi, K. and Shirasaka, M. 1965. The structure of ophiobolin, a C_{25}-terpenoid having a novel skeleton. *J. Am. Chem. Soc.* 87:4968-4970.

Ohkawa, H. and Tamura, T. 1966. Studies on the metabolites of *Cochliobolus miyabeanus*. Part I. Ophiobolin A and ophiobolin B. *Agric. Biol. Chem.* 30:285-291.

Orsenigo, M. 1957. Estrazine e purificazione della Cochliobolina, una tossina prodotta da *Helminthosporium oryzae*. *Phytopathol. Z.* 29:189-196.

Scheffer, R. P. 1983. Toxins as chamical determinants of plant disease. In: *Toxins and Plant Pathogenesis*, ed by J. M. Daly and B. J. Deverall, pp.1-40. Academic Press, Australia.

Singh, S. B., Smith, G. S., Dombrowski, A. W., Schaeffer, J. M., Goetz, M. A. and Bills, G. F. 1991. Structure and conformation of ophiobolin K and 6-epiophiobolin K from *Aspergillus ustus* as a namatocidal agent. *Tetrahedron* 47:6931-6938.

Sugawara, F., Strobel, G., Strange, R. N., Siedow, J. N., Van Duyne, G. D. and Clardy, J. 1987. Phytotoxins from the patho-

genic fungi *Drechslera maydis* and *Drechslera sorghicola*. *Proc. Natl. Acad. Sci., USA*. 84:3081-3085.

Tsipouras, A., Adefarati, A. A., Tkacz, J. S., Frazier, E. G., Rohrer, S. P., Birzin, E., Rosegay, A., Zink, D. L., Goetz, M. A., Singh, S. B. and Schaeffer, J. M. 1996. Ophiobolin M and analogues, noncompetitive inhibitors of ivermectin binding with nematocidal activity. *Bioorganic Med. Chem.* 4:531-536.

Tsuda, K., Nozoe, S., Morisaki, M., Hirai, K., Itai, A., Okuda, S., Canonica, L., Fiecchi, A., Galli Kienle, M. and Scala, A. 1967. Nomenclature of ophiobolins. *Tetrahedron Lett.* 35:3369-3370.

Xiao, J.Z., Tsuda, M., Doke, N. and Nishimura, S. 1991. Phytotoxins produced by germinating spores of *Bipolaris oryzae*. *Phytopathology* 81:58-64.