Journal of Bioscience and Bioengineering
VOL. 117 No. 5, 557–562, 2014

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# Production of antioomycete compounds active against the phytopathogens *Phytophthora sojae* and *Aphanomyces cochlioides* by clavicipitoid entomopathogenic fungi

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Received 19 April 2013; accepted 18 October 2013
Available online 21 November 2013

**A total of 412 strains belonging to 14 genera of clavicipitoid entomopathogenic fungi (EPF) were screened for activities against two economically important plant pathogenic oomycetes, *Phytophthora sojae* and *Aphanomyces cochlioides*. To identify the antioomycete compounds produced by EPF, the extracts of 13 highly active EPF strains were characterized in detail by high performance liquid chromatography with diode array detection and high-resolution mass spectrometric detection and antioomycete assay. The antioomycete activity of several *Metarhizium* extracts was associated with previously isolated aurovertins, fungerin, *N*-(methyl-3-oxodec-6-enoyl)-2-pyrroline, and *N*-(methyl-3-oxodecanoyl)-2-pyrroline. The depsipeptide beauvericin was confirmed to be one of the active principles of three strains of *Isaria tenuipes*, which strongly inhibited mycelial growth of both *P. sojae* and *A. cochlioides*. Two known bioactive metabolites, paecilosetin and aranorosinol A, together with a novel and potent antioomycete compound, farinomalein, were isolated from the extracts of *Isaria farinosa* and all compounds were confirmed to have antioomycete activity. Identification of 8 antioomycete compounds from 13 clavicipitioid EPF demonstrated a new potential use of EPF as a source of compounds for the control of soil-borne plant pathogenic oomycetes.**



[**Key words:** Antioomycete compounds; *Phytophthora sojae*; *Aphanomyces cochlioides*; Clavicipitoid entomopathogenic fungi; Bioactive compound]

*Aphanomyces cochlioides* is the pathogen that causes the damping-off disease in spinach, sugar beet and some other members of Chenopodiaceae and Amaranthaceae, whereas *Phytophthora sojae* is responsible for root- and stem-rot disease in soybean (1,2). Both pathogens belong to Oomycetes, a class that includes some of the world's most destructive plant pathogens, which are collectively responsible for multibillion dollar losses in agriculture (3,4). Therefore, considerable attention has been paid over the last two decades to the biological control of these plant pathogens (3,5,6). Although synthetic chemicals have been effective to prevent the outbreak of such plant diseases for many years, their use has been significantly reduced either because of potential risks to human health, environmental pollution, negative effects on non-target organisms or the development of pest resistance (7–9). Thus, there is an urgent need to develop alternative control systems to replace or complement conventional pesticide usage, such as by finding new and safer natural control agents (7,10–12).

In this study aimed at identifying natural agents as alternatives to conventional synthetic agrochemicals, we focused on clavicipitoid entomopathogenic fungi (EPF). EPF are well-known for their ability to produce various bioactive compounds during infection and proliferation in insects (13–15). In addition to their agricultural use as biological control agents against insect pests, EPF have been utilized as the bioresource of antibacterial, antiinsectan, antimalarial, antituberculous, anticancer, and neuritogenic compounds (9,16–18). However, there had been no studies on the antioomycete activity or the production of antioomycete metabolites of EPF until a recent report by our group (19). We herein describe the first systematic study of the antioomycete activity of a wide range of clavicipitoid EPF, with particular emphasis on the identification of compounds active against *P. sojae* and *A. cochlioides*. Four hundred and twelve strains of EPF were screened for their biological activity against two economically important plant pathogenic oomycetes. Mycelial growth inhibition of *A. cochlioides* and *P. sojae* was determined using a dual culture assay in the preliminary screening and led to the selection of 13 isolates of EPF for further study.

## MATERIALS AND METHODS

**Fungal strains and culture conditions** All chemicals, media and reagents were purchased from Wako, Japan, unless stated otherwise. Strains of

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1389-1723/$ – see front matter 
http://dx.doi.org/10.1016/j.jbiosc.2013.10.014

entomopathogenic fungi were isolated from larval cadavers collected from various sites in Japan. Fungal conidia that developed on the surface of the dead larva were transferred to SMY medium and incubated at 25°C for several days. After the conidia had developed on SMY slants, the strain was kept at −30°C as a stock culture (18). The isolated strains were identified according to its morphology by one of the authors (F.I.). The fungi are deposited at the culture collection of National Institute of Fruit Tree Science, Japan. A total of 412 strains of clavicipitoid EPF (38 species under 14 genera; Table S1) were used for initial screening. For the first screening, the fungi were cultivated on potato dextrose agar (PDA) plates supplemented with 2% powdered silkworm pupa Sanagiko (Maruq Co., Japan) at 25°C for 5–21 days. Forty-seven strains that exhibited strong antioomycete activity in the first screening were grown in liquid SMY+HP-20 [maltose 4%, yeast extract 1%, peptone 1%, adsorption resin Diaion HP-20 (Mitsubishi Chemical Co.) 1%] at 25°C for 7–21 days without shaking for the second screening. The indicator strain, *A. cochlioides* AC-5 was grown at 25°C on PDA while *P. sojae* P6497 was grown at 25°C on V8-juice agar [vegetable juice without NaCl (COOP, Japan) 20% v/v, $CaCO_3$ 0.2%, agar 1.8%].

**Antioomycete assay** The first screening was done by a dual culture agar assay and the second screening was performed by a cup plate diffusion test. The antioomycete activity of the extracts and purified compounds was measured by a disk diffusion susceptibility test.

For dual culture agar assay, entomopathogenic fungi were cultivated on PDA supplemented with 2% Sanagiko (Maruq Co., Japan) at 25°C for 5–21 days until they formed spores. The dual culture agar assay was performed by the previously described method (1) with several modifications. The lawn of the indicator strain was punched out as an agar plug (8.0-mm diameter) from the leading edge of the actively growing culture. The agar plug was placed on a new plate and was incubated at 25°C for 3–5 days until the diameter of the lawn reached 2 cm. Similar agar plugs of entomopathogenic fungi (7-, 14-, or 21-day-old) were then placed 3 cm away from the center of the indicator strain. The diameter of the inhibition zone was measured after the indicator strain in the control plate (without EPF agar plugs) had fully grown to the edge of the plate.

Antioomycete activity in the culture supernatant was measured by the cup plate diffusion method in duplicate. A culture grown in liquid SMY+HP-20 medium was centrifuged at 14,000 rpm for 5 min and the supernatant was filtrated with a 0.2-μm cellulose acetate filter (Advantec, Japan) to remove spores and mycelia. Two hundred microliters of each culture filtrate was dispensed into a stainless steel cup ($\varphi$ = 8.0 mm; TOP, Japan) that was placed 3 cm away from the edge of the indicator strains grown on the plate as described in the dual culture agar assay. The compounds in the filtrate were allowed to diffuse into the agar and the diameter of the clear-zone surrounding each cup was measured after incubation at 25°C for 2–5 days. The *in vitro* antioomycete activity of the crude extract was measured by the disk diffusion susceptibility test following the method described previously (1) with several modifications. The sterile paper disks loaded with extracts (50 μL/disk) ($\varphi$ = 8.0 mm; Advantec, Japan) were air-dried on a laminar hood for 30 min prior to placement on the petri dish. The disks were placed 3 cm away from the edge of the indicator strains as described in the cup plate diffusion method. Antioomycete activity of the purified metabolites from the highly active EPF extracts was verified by testing 50 μg and 100 μg of the compound (90–95% purity) obtained from our group's metabolite collection (Table S2) against *P. sojae* and *A. cochlioides*. No growth inhibition was observed in the control disks (loaded with 50 μL of only $CH_3CN$ or MeOH). The experiment was performed in duplicate.

**Extract preparation for antioomycete assay and high performance liquid chromatography analysis** Forty-seven strains selected for the second screening were cultivated in 30 mL of SMY+HP-20 for 21 days at 25°C without shaking. The whole culture broths including mycelia were extracted with 15 mL of either ethyl acetate (EtOAc) or *n*-butanol (BuOH), and the mixtures were centrifuged to separate the solvent layers from the aqueous layers. The solvent layers (1 mL) were evaporated *in vacuo*, the residues were re-dissolved in 200 μL of $CH_3CN$, and

**TABLE 1.** Antioomycete activity distribution of clavicipitoid entompathogenic fungi cultivated on PDA+ Sanagiko.

| No. | Species | Number of isolate displaying antioomycete activity[a] | | | | | | Total number of tested strain |
|---|---|---|---|---|---|---|---|---|
| | | − | −/+ | + | ++ | +++ | ++++ | |
| 1 | *Akanthomyces ampullifer* | | | | 1 | | | 1 |
| 2 | *Akanthomyces arachnophilus* | 1 | | | | | | 1 |
| 3 | *Akanthomyces cinereus* | | | | 1 | | | 1 |
| 4 | *Akanthomyces gracilis* | 1 | | | | | | 1 |
| 5 | *Akanthomyces novoguineensis* | 1 | | | 1 | | | 2 |
| 6 | *Akanthomyces* sp. | 1 | | 1 | | | | 2 |
| 7 | *Akanthomyces pistillariaeformis* | 3 | 1 | 3 | | | | 7 |
| 8 | *Aschersonia aleyrodis* | 2 | | | | | | 2 |
| 9 | *Beauveria bassiana* | 11 | 18 | 23 | 47 | 8 | | 107 |
| 10 | *Beauveria brongniartii* | | | 1 | 5 | | 1 | 7 |
| 11 | *Cordyceps indigotica* | | | | | | 2 | 2 |
| 12 | *Cordyceps militaris* | 2 | 1 | 3 | 1 | | | 7 |
| 13 | *Cordyceps prolifica* | 1 | | | 1 | 2 | | 4 |
| 14 | *Cordyceps ramosopulvinata* | | | 1 | | | | 1 |
| 15 | *Cordyceps* sp. | 5 | | 1 | 2 | | 1 | 9 |
| 16 | *Cordyceps takaomontana* | 3 | 1 | 1 | | 2 | | 7 |
| 17 | *Cordyceps tuberculata* | 1 | | | | | | 1 |
| 18 | *Elaphocordyceps ophioglassoides* | 1 | | | | | | 1 |
| 19 | *Elaphocordyceps paradoxa* | 1 | | | | | | 1 |
| 20 | *Gibellula leiopus* | 3 | | | | | | 3 |
| 21 | *Gibellula pulchra* | 1 | | | | | | 1 |
| 22 | *Hirsutella neovolkiana* | 1 | | | | | | 1 |
| 23 | *Hirsutella nutans* | | | | 2 | | | 2 |
| 24 | *Isaria cateniannulata* | 3 | 4 | 4 | 1 | 2 | | 14 |
| 25 | *Isaria farinosa* | 4 | | | 1 | 2 | | 7 |
| 26 | *Isaria fumosorosea* | 4 | 1 | 2 | | | | 7 |
| 27 | *Isaria* sp. | 8 | 1 | 2 | 2 | | 1 | 14 |
| 28 | *Isaria tenuipes* | 27 | 13 | 44 | 27 | 6 | 1 | 118 |
| 29 | *Lecanicillium lecanii* | 1 | | 2 | | | | 3 |
| 30 | *Metarhizium anisopliae* | 1 | | 7 | 39 | 12 | 4 | 65 |
| 31 | *Metarhizium flavoviridae* | | | | | | 1 | 1 |
| 32 | *Nomuraea rileyi* | | 1 | 2 | 1 | | | 4 |
| 33 | *Ophiocordyceps crinalis* | 1 | | | | | | 1 |
| 34 | *Ophiocordyceps stylophora* | 2 | | | | | | 2 |
| 35 | *Polycephalomyces* sp. | 2 | | | | | | 2 |
| 36 | *Torrubiella aurantia* | | 1 | | | | | 1 |
| 37 | *Torrubiella minutissima* | 2 | | | | 1 | | 3 |
| 38 | *Torrubiella superficialis* | | | | | | 1 | 1 |
| Total | | 94 | 42 | 97 | 132 | 35 | 12 | 412 |

[a] Degree of antioomycete activity is expressed by the diameter of inhibition zone (d): No inhibition (−), very weak: $d$ <1 cm (−/+), weak: 1 ≤ $d$ <2 cm (+), moderate: 2 ≤ $d$ <3 cm (++), strong: 3 ≤ $d$ <4 cm (+++), very strong: $d$ ≥ 4 cm (++++).

**TABLE 2.** Metabolite analysis of highly active EPF extracts.

| | Strain[a] | Strain number | Activity of BuOH extract[a] | | Major metabolite(s) in BuOH extract[b] | Activity of EtOAc extract[a] | | Major metabolite(s) in EtOAc extract[b] |
|---|---|---|---|---|---|---|---|---|
| | | | PS | AC | | PS | AC | |
| 1 | *Isaria farinosa* | HF511 | 2.0 | 1.0 | Aranorosinol A<br>Paecilosetin | 2.0 | 2.0 | Aranorosinol A<br>Paecilosetin |
| 2 | *Isaria farinosa* | HF599 | 3.0 | 0 | Farinomalein | 4.0 | 0 | Farinomalein |
| 3 | *Isaria tenuipes* | HF340 | 2.0 | 0.8 | Beauvericin | 1.0 | 0 | Beauvericin |
| 4 | *Isaria tenuipes* | HF708 | 3.0 | 0.8 | Beauvericin | 0.8 | 0 | Beauvericin |
| 5 | *Isaria tenuipes* | HF822 | 2.0 | 0.8 | Beauvericin | 0.8 | 0 | Beauvericin |
| 6 | *Metarhizium anisopliae* | HF574 | 4.0 | 1.0 | Fungerin | 3.0 | 3.0 | Fungerin |
| 7 | *Metarhizium anisopliae* | HF608 | 1.0 | 1.0 | Helvolic acid | 2.0 | 1.0 | Helvolic acid |
| 8 | *Metarhizium anisopliae* | HF614 | 3.0 | 2.0 | Aurovertins | 0 | 0 | Aurovertins |
| 9 | *Metarhizium anisopliae* | HF616 | 3.0 | 2.0 | Aurovertins | 0 | 0 | Aurovertins |
| 10 | *Metarhizium anisopliae* | HF619 | 3.0 | 1.0 | Aurovertins | 3.0 | 0.8 | Aurovertins |
| 11 | *Metarhizium anisopliae* | HF622 | 2.0 | 1.0 | Helvolic acid | 2.0 | 1.0 | Helvolic acid |
| 12 | *Metarhizium anisopliae* | HF625 | 3.0 | 1.0 | Aurovertins | 0 | 0 | Aurovertins |
| 13 | *Metarhizium flavoviride* | HF698 | 4.0 | 3.0 | *N*-(Methyl-3-oxodec-6-enoyl)-2 pyrroline *N*-(methyl-3-oxodecanoyl)-2 pyrroline | 4.0 | 3.0 | *N*-(Methyl-3-oxodec-6-enoyl)-2 pyrroline *N*-(methyl-3-oxodecanoyl)-2 pyrroline |

[a] Antioomycete activity of EtOAc and BuOH extracts from liquid cultivation is expressed by the diameter of inhibition zone (cm) against *P. sojae* (PS) and *A. cochlioides* (AC) in antioomycete assay.

[b] The metabolites were identified by comparison with authentic standards using HPLC-DAD-HRMS analysis.

the extracts (50 μL) were subjected to bioassay as described previously. For high performance liquid chromatography (HPLC) analysis, extracts (1 mL) were evaporated *in vacuo*, re-dissolved in 1 mL of dimethyl sulfoxide (DMSO) and filtrated with a 0.2-μm polytetrafluoroethylene (PTFE) filter (Advantec, Japan).

**HPLC analysis** HPLC with diode array detection was carried out on a Varian Microsorb C18 column (4.6 × 100 mm) using an Agilent HP1100 system equipped with a photo diode array detector (DAD). The analysis was done at a flow-rate of 1.2 mL/min with a stepwise gradient of $CH_3CN$-0.15% $KH_2PO_4$ (pH 3.5) (15%–85% v/v): 0–3 min, 15%; 3–6 min, 15–40%; 6–12 min, 40%; 12–19 min, 40–55%; 19–22 min, 55–85%; 22–29 min, 85%; 29–32 min, 85 to 15%. The UV spectra were collected by DAD from 200 to 600 nm. The compounds in the active extracts were tentatively identified by comparing the retention times and UV–Vis spectra of the peaks with an internal reference standard database and previous data from our group. The compounds were further characterized and identified by comparison of their UV spectra, high-resolution mass spectra and NMR literature data with those of authentic standards. HPLC with diode array detection and high-resolution mass spectrometric detection (HPLC-DAD-HRMS) was carried out using an Imtakt Cadenza CD-C18 column (2.5 × 150 mm) on an Agilent 1200 system equipped with a photo diode array detector (DAD) and coupled to a Bruker micrOTOFQ-II under a positive ESI mode.

## RESULTS AND DISCUSSION

**Screening of clavicipitoid EPF** With the aim of evaluating the potential of EPF as an alternative source of antioomycete compounds, 412 strains of EPF isolated from various sites in Japan were investigated. Preliminary screening by dual culture assay showed that 67.0% (276 strains) exhibited significant inhibitory activity (diameter of inhibition zone (d) ≥1 cm) against at least one of the two phytopathogens *P. sojae* and *A. cochlioides* (Table 1). These active strains were distributed in 25 species of 9 genera out of the 38 species of 14 genera tested, suggesting that the ability to produce antioomycete compounds is widely present among clavicipitoid EPF. The ability of EPF to produce a series of antimicrobial compounds have been reviewed recently (20) and it was noted that these antimicrobials might play an important role in competing for nutrients with other microorganisms, particularly in the soil habitat. Fungal strains were classified into

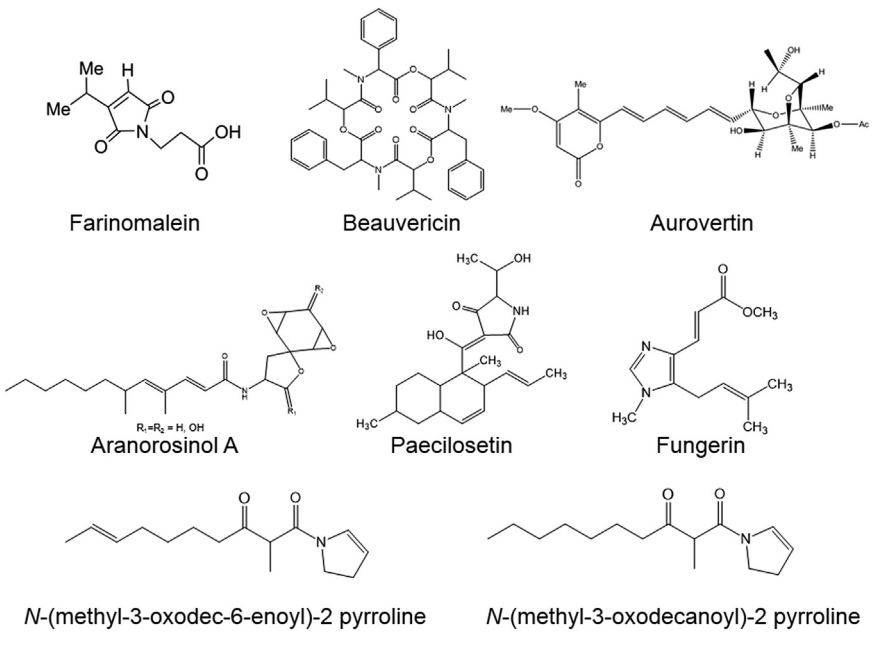


FIG. 1. Antioomycete metabolites from entomopathogenic fungi (EPF).

six groups based on the strength of their activity towards the plant pathogenic oomycetes as shown in Table S1. The assessment of EPF revealed that almost half of the active strains (47.5%) exhibited moderate activity ($2 \leq d < 3$ cm) against at least one indicator strain in the initial screening, whereas 17.0% of the strains (47 strains) showed strong ($3 \leq d < 4$ cm) or very strong ($d \geq 4$ cm) antioomycete activity. These 47 highly active strains were selected as candidates for further study with a focus on the identification of antioomycete compounds.

In the second screening, the 47 selected strains were cultivated in liquid medium under static conditions (without shaking), because extraction of the antioomycete compounds from solid culture is laborious and time-consuming. In addition, liquid cultivation enables easier scaling-up for the isolation of the active compounds. Although PDA was used for solid cultivation, liquid cultivation was conducted in SMY medium, because when cultivated on PDB, none of the strains showed any antioomycete activity against either indicator strain. Twenty-three out of the 47 tested strains (14 strains of *Metarhizium anisopliae*, 3 strains of *Isaria tenuipes*, 2 strains each of *Cordyceps indigotica* and *Isaria farinosa,* and 1 strain each of *Beauveria bassiana* and *Metarhizium flavoviride*) displayed significant antioomycete activity (diameter of inhibition zone $\geq$1 cm) in the SMY culture supernatant (Table S2). Among these, we focused on compounds extractable either by BuOH or EtOAc showing strong activity (diameter of inhibition zone $\geq$2 cm) for identification of the antioomycete compounds that are listed in Table 2. Neither extracts of *B. bassiana* nor those of *C. indigotica* showed antioomycete activity. A preliminary experiment showed that the active principle(s) is present in the flow-through and might include highly polar compounds such as exopolysaccharides, which are commonly found in EPF (21).

**Isolation and identification of active compounds** EPF extracts which showed strong antioomycete activity were subjected to HPLC-DAD analysis. Compounds in the extracts responsible for the antioomycete activity were estimated by comparing the physicochemical data (HPLC retention time and UV−Vis spectrum) with those of the compounds registered in our in-house library, a collection produced in the course of our continuous search for new metabolites. The compounds exhibiting the activity were identified further by HRMS and/or NMR after purification. The antioomycete activity of all compounds was finally confirmed by testing the authentic standards against *P. sojae* and *A. cochlioides*. Typically, eight compounds [farinomalein, beauvericin, aurovertin D, paecilosetin, aranorosinol A, fungerin, *N*-(methyl-3-oxodec-6-enoyl)-2-pyrroline (F2A) and *N*-(methyl-3-oxodecanoyl)-2-pyrroline (F2B)] were identified as the primary antioomycete compounds from EPF (Table 2 and Fig. 1).

Two antibiotics, paecilosetin and aranorosinol A from *I. farinosa* HF511 were previously isolated in our laboratory with structure identification by NMR and HRMS analyses (Fig. 2). In this study, these two compounds were found to be the major metabolites in the culture extract of HF511 (Fig. 1) and were confirmed to possess antioomycete activities against both *P. sojae* and *A. cochlioides*, as shown in Table 2. To our knowledge, this is the first report on the antioomycete activity of the two compounds. Paecilosetin is the analog of equisetin (polyketide-non-ribosomal peptide (PK-NRP) hybrid) and was previously isolated from a New Zealand isolate of *I. farinosa* (22). However, there has been no report on the isolation of aranorosinol A from any insect pathogenic fungi (23). In addition to strain HF511, we have isolated a novel antioomycete compound bearing a maleimide ring (named farinomalein) from another strain of *I. farinosa* (HF599). The details on the isolation and structure elucidation of this compound have been previously described (19). Farinomalein showed potent and specific activity against *P. sojae* and was confirmed to be the sole active principle of HF599 extract. Therefore, the principle of antioomycete components from *I. farinosa* are paecilocetin, aranorosinol A or farinomalein.

The cyclohexadepsipeptide beauvericin was previously isolated in our laboratory from the strain *I. tenuipes* HF633 and the structure

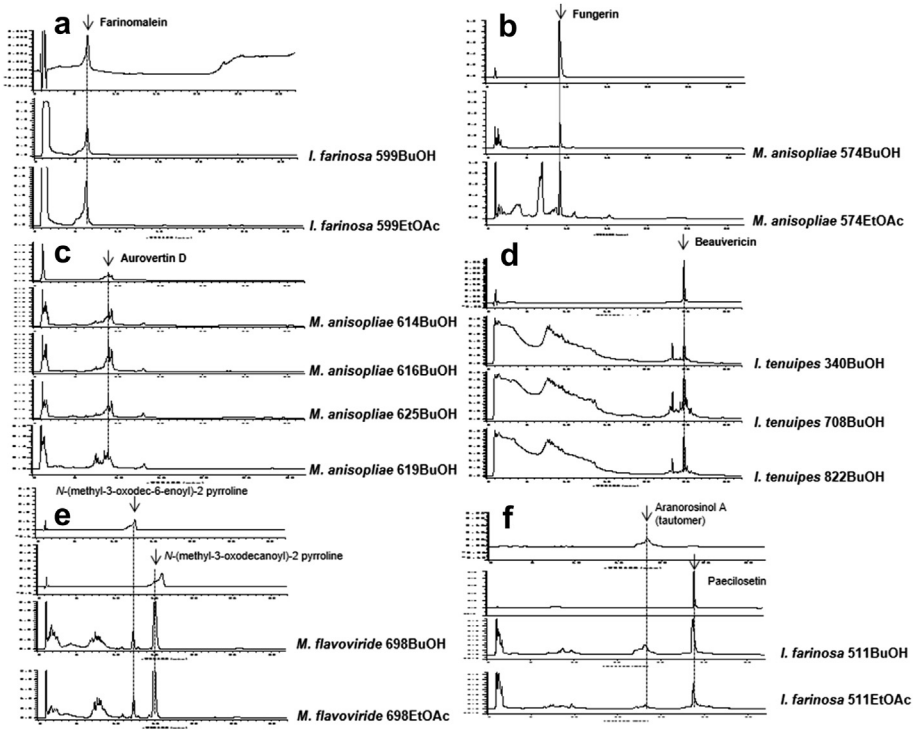


FIG. 2. Chemical structures, HPLC chromatogram, and UV−Vis spectra of antioomycete metabolites produced by EPF extracts. (a) Farinomalein from *I. farinosa* HF599. (b) Fungerin from *M. anisopliae* HF574. (c) Aurovertin D from *M. anisopliae* HF614, HF616, HF619, and HF625. (d) Beauvericin from *I. tenuipes* HF340, HF740, HF822. (e) *N*-(Methyl-3-oxodec-6-enoyl)-2 pyrroline, and *N*-(methyl-3-oxodecanoyl)-2 pyrroline from *M. flavoviridae* HF698. (e) Aranorosinol A and paecilosetin from *I. farinosa* HF511.

**TABLE 3.** Antioomycete assay of metabolites detected in EPF extracts.

| No. | Compound | *P. sojae* (50 μg/disk) | *P. sojae* (100 μg/disk) | *A. cochlioides* (50 μg/disk) | *A. cochlioides* (100 μg/disk) |
|---|---|---|---|---|---|
| 1 | Farinomalein | ++ | +++ | + | + |
| 2 | Beauvericin | −/+ | −/+ | ++ | +++ |
| 3 | Aurovertin D | + | ++ | −/+ | + |
| 4 | Helvolic acid | - | - | - | - |
| 5 | Aranorosinol A | ++ | ++ | + | ++ |
| 6 | Fungerin | −/+ | + | −/+ | + |
| 7 | *N*-(Methyl-3-oxodec-6-enoyl)-2 pyrroline | −/+ | −/+ | −/+ | ++ |
| 8 | *N*-(Methyl-3-oxodecanoyl)-2 pyrroline | ++ | +++ | + | ++ |
| 9 | Paecilosetin | ++ | +++ | +++ | +++ |

Degree of antioomycete activity is expressed by the diameter of inhibition zone (d): No inhibition (−), very weak: $d < 1$ cm (−/+), weak: $1 \leq d < 2$ cm (+), moderate: $2 \leq d < 3$ cm (++), strong: $3 \leq d < 4$ cm (+++), very strong: $d \geq 4$ cm (++++).

was confirmed by HRMS and NMR measurement (data not shown). In this study, this antibiotic was detected in the extracts of three *I. tenuipes* strains (HF340, HF708, and HF822). A good correlation was found between the amount of beauvericin and the antioomycete activity of the *I. tenuipes* extracts (Table 2 and Fig. 1) in the early screening and it was later confirmed that beauvericin is one of the compounds responsible for the antioomycete activity, as can be seen in Table 3. The stronger activity observed in the BuOH extracts of HF340, HF708 and HF822 when compared to the EtOAc extracts seems to be due to the presence of another compound in the *n*-BuOH extracts. This compound is identified as a beauvericin analog based on the UV absorption and HR-MS data. Beauvericin has been reported to have a diverse array of biological activities *in vitro* and has been established to be a virulence factor of the entomopathogenic fungus *B. bassiana* (24). The identification of beauvericin from 4 strains of *I. tenuipes* in this study also supported a recent report that beauvericin could serve as a chemotaxonomic marker of the *I. tenuipes* complex (25).

We identified three different metabolites from seven active extracts of *M. anisopliae*: fungerin from HF574, helvolic acid from HF608 and HF622, and an array of aurovertins from HF614, HF616, HF619, and HF625 (Table 2 and Fig. 1). Fungerin, an alkaloidal compound first isolated from *Fusarium* sp., is a metabolite of *M. anisopliae* and has been shown to exhibit antifungal and insecticidal activities (26,27). In the present study, although the crude extract of HF574 exhibited strong antioomycete activity against both indicator strains (Table 2), purified fungerin showed weak activity against both (Table 3), suggesting that, in addition to fungerin, there is another antioomycete compound in the extract of HF574. We have previously reported the isolation of helvolic acid and its new derivative from *M. anisopliae* HF293 during the screening of new antibacterial compounds (28). In this study, helvolic acid was also detected in a considerable quantity in both EtOAc and BuOH extracts of HF608 and HF622. However, the antioomycete activity was most likely attributed to another unidentified compound(s) present in the extracts of HF608 and HF622, because purified helvolic acid did not cause any inhibition on either indicator strain even at a concentration of 100 μg/disk (Table 3). With respect to aurovertins, aurovertin D was detected as the major metabolite of strain HF614, HF616, and HF625. The amount of aurovertin D detected by HPLC-DAD analysis corresponded well with the antioomycete activity of the extracts. We tested the antioomycete activity of aurovertin D, the major aurovertin produced in *M. anisopliae*, and it showed weak to moderate antioomycete activity against *P. sojae* and *A. cochlioides*. As can be seen in Table 2 and Fig. 1, a high level of aurovertin D, together with lower levels of aurovertins E, G, and H, was detected in the BuOH and EtOAc extracts of HF614, HF616 and HF625. The extracts of HF619 contain different types of aurovertins, as indicated by the HPLC-DAD-HRMS data. In a separate study, we have reported the isolation and structure identification of a series of aurovertin derivatives, including the newly reported aurovertins F–H (29).

In the early screening against oomycetes, *Metarhizium flavoviridae* HF698 showed strong activity against both pathogens. Our group previously screened this strain for the production of antibacterial, antiyeast and antifungal compounds, which resulted in the isolation of two highly active antibiotics, *N*-(methyl-3-oxodec-6-enoyl)-2-pyrroline and *N*-(methyl-3-oxodecanoyl)-2-pyrroline (Fig. 1). These compounds were first isolated from *Penicillium brevicompactum* and are known to possess strong fungicidal and insecticidal activities (30–32). It is interesting to note that these insecticidals have never been reported from any insect pathogenic fungi. In this study, it was found that these compounds also showed highly potent activities against *P. sojae* and *A. cochlioides*, and therefore it is concluded that they are the compounds responsible for the antioomycete activity of this strain (Table 3).

In this study, we systematically screened clavicipitoid EPF for antioomycete activities and identified 8 bioactive compounds that have not previously been shown to exhibit antioomycete activities. Our experience in dealing with metabolites of EPF accelerated the identification of antioomycete metabolites from EPF. The results from this work indicate a potential use of the highly active strains of clavicipitoid EPF as biocontrol agents for plant diseases caused by *P. sojae* and *A. cochlioides* in addition to their current use against agriculturally important pests. Moreover, the isolation of the new compound farinomalein from EPF further raised the prospect of using these insect fungal pathogens as sources for the discovery of new bioactive compounds.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2013.10.014.

## ACKNOWLEDGMENTS

We thank Ms. Ayako Tomio for technical assistance during the HPLC-DAD-HRMS analysis. This study was supported in part by a scholarship from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan to S. P. and by a grant for a joint program in the field of biotechnology under the Japan Society for the Promotion of Science (JSPS), the National Research Council of Thailand (NRCT) and the National Science and Technology Development Agency (NSTDA) of Thailand to S. K., H. K. and T. N.

## References

1. **Deora, A., Hashidoko, Y., Islam, M. Y., and Tahara, S.:** Antagonistic rhizoplane bacteria induce diverse morphological alterations in Peronosporomycete hyphae during in vitro interaction, Europ. J. Plant Pathol., **112**, 311–322 (2005).
2. **Kaitany, R. C., Hart, L. P., and Safir, G. R.:** Virulence composition of *Phytophthora sojae* in Michigan, Plant Dis., **85**, 1103–1106 (2001).
3. **West, P., Appiah, A. A., and Gow, A. R. N.:** Advances in research on oomycete root pathogens, Physiol. Mol. Plant Pathol., **62**, 99–113 (2003).
4. **Madoui, M. A., Gaulin, E., Mathe, C., Clemente, H. S., Couloux, A., Wincker, P., and Dumas, B.:** AphanoDB: a genomic resource for *Aphanomyces* pathogens, BMC Genomics, **8**, 471 (2007).
5. **Erwin, D. C. and Ribeiro, O. K.:** Phytophthora diseases worldwide. The American Phytopathological Society, St. Paul, MN (1996).
6. **Kuhajek, J. M., Jeffers, S. N., Slattery, M., and Wedge, D. E.:** A rapid microbioassay for discovery of novel fungicides for *Phytophthora* spp., Phytopathology, **93**, 46–53 (2003).
7. **Butt, T. M. and Copping, L. G.:** Fungal biological control agents, Pestic. Outlook, **11**, 186–191 (2000).
8. **Sugimoto, T., Watanabe, K., Furiki, M., Walker, D. R., Yoshida, S., Aino, M., Kanto, T., and Irie, K.:** The effect of potassium nitrate on the reduction of Phytophthora stem rot disease of soybeans, the growth rate and zoospore release of *Phytophthora sojae*, J. Phytopathol., **157**, 380–388 (2009).

9. **Lee, S. Y., Nakajima, I., Ihara, F., Kinoshita, H., and Nihira, T.:** Cultivation of entomopathogenic fungi for the search of antibacterial compounds, Mycopathologia, **160**, 321–325 (2005).
10. **Timmer, W. L., Graham, H. J., and Zitko, E. S.:** Metalaxyl-resistant isolates of *Phytophthora nicotianae*: occurrence, sensitivity, and competitive parasitic ability on citrus, Plant. Dis., **82**, 254–261 (1998).
11. **Deahl, L. K., DeMuth, P. S., Pelter, G., and Ormrod, J. D.:** First report of resistance of *Phytophthora infestans* to metalaxyl in Eastern Washington and Southwestern British Columbia, Plant. Dis., **77**, 429 (1993).
12. **Lee, J. Y., Sherman, D. H., and Hwang, B. K.:** *In vitro* antimicrobial and *in vivo* antioomycete activities of the novel antibiotic thiobutacin, Pest. Manag. Sci., **64**, 172–177 (2008).
13. **Jan Scholte, E., Knols, B. G., Samson, R. A., and Takken, W.:** Entomopathogenic fungi for mosquito control, J. Insect Sci., **4**, 1–24 (2004).
14. **Thomas, M. and Read, A.:** Can fungal biopesticides control malaria? Nat. Rev. Microbiol., **5**, 377–383 (2007).
15. **Zimmermann, G.:** Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*, Biocontrol Sci. Technol., **17**, 553–596 (2007).
16. **Ihara, F., Yaginuma, K., Kobayashi, N., Mishiro, K., and Sato, T.:** Screening of entomopathogenic fungi against the brown-winged green bug, *Plautia stali* Scott (Hemiptera: Pentatomidae), Appl. Entomol. Zool., **38**, 495–500 (2001).
17. **Isaka, M., Kittakoop, P., Kirtikara, K., Hywel-Jones, N. L., and Thebtaranoth, Y.:** Bioactive substances from insect pathogenic fungi, Acc. Chem. Res., **38**, 813–823 (2005).
18. **Schmidt, K., Li, Z., Schubert, B., Huang, B., Stoyanova, S., and Hamburger, M.:** Screening of entomopathogenic Deuteromycetes for activities on targets involved in degenerative diseases of the central nervous system, J. Ethnopharmacol., **89**, 251–260 (2003).
19. **Putri, S. P., Kinoshita, H., Ihara, F., Igarashi, Y., and Nihira, T.:** Farinomalein, a maleimide-bearing compound from the entomopathogenic fungus *Paecilomyces farinosus*, J. Nat. Prod., **72**, 1544–1546 (2009).
20. **Molnar, I., Gibson, D. M., and Krasnoff, S. B.:** Secondary metabolites from entomopathogenic Hypocrealean fungi, Nat. Prod. Rep., **27**, 1241–1275 (2010).
21. **Lang, G., Blunt, J. W., Cummings, N. J., Cole, A. L., and Munro, M. H.:** Paecilosetin, a new bioactive fungal metabolite from a New Zealand isolate of *Paecilomyces farinosus*, J. Nat. Prod., **68**, 810–811 (2005).
22. **Roy, K., Vijayakumar, E. K. S., Mukhopdhyay, T., Chatterjee, S., Bhat, R. G., Blumbach, J., and Ganguli, B. N.:** Aranorosinol A and aranorosinol B, two new metabolites from *Pseudoarachniotus roseus*: production, isolation, structure elucidation and biological properties, J. Antibiot., **45**, 1592–1598 (1992).
23. **Phoon, C. W., Somanadhan, B., Heng, S. C. H., Ngo, A., Ng, S. B., Butler, M. S., Buss, A. D., and Sim, M. M.:** Isolation and total synthesis of gymnastatin N, a POLO-like kinase 1 active constituent from the fungus *Arachniotus punctatus*, Tetrahedron, **60**, 1619–11628 (2004).
24. **Xu, Y., Orozco, R., Wijeratne, E. M. K., Gunatilaka, A. A. L., Stock, S. P., and Molnár:** Biosynthesis of the cyclooligomer depsipeptide beauvericin, a virulence factor of the entomopathogenic fungus *Beauveria bassiana*, Chem. Biol., **15**, 898–907 (2008).
25. **Luangsa-Ard, J. J., Berkaew, P., Ridkaew, R., Hywel-Jones, N. L., and Isaka, M.:** A beauvericin hot spot in the genus Isaria, Mycol. Res., **113**, 1389–1395 (2009).
26. **Kato, Y., Koshino, H., Uzawa, J., and Anzai, K.:** Fungerin, a new antifungal alkaloid from *Fusarium* sp., Biosci. Biotechnol. Biochem., **60**, 2081–2083 (1996).
27. **Uchida, R., Imasato, R., Yamaguchi, Y., Masuma, R., Shiomi, K., Tomoda, H., and Omura, S.:** New insecticidal antibiotics, hydroxyfungerins A and B produced by *Metarhizium* sp. FKI-1079, J. Antibiot., **58**, 804–809 (2005).
28. **Lee, S. Y., Kinoshita, H., Ihara, F., Igarashi, Y., and Nihira, T.:** Identification of novel derivative of helvolic acid from *Metarhizium anisopliae* grown in medium with insect component, J. Biosci. Bioeng., **105**, 476–480 (2008).
29. **Azumi, M., Kinoshita, H., Nihira, T., Ihara, F., Fujita, T., and Igarashi, Y.:** Aurovertins F–H from the entomopathogenic fungus *Metarhizium anisopliae*, J. Nat. Prod., **71**, 278–280 (2008).
30. **Moya, P., Cantín, A., Castillo, M. A., Primo, J., Miranda, M. A., and Primo-Yúfera, E.:** Isolation, structural assignment and synthesis of N-(2-methyl-3-oxodecanoyl)-2-pyrroline, a new natural product from *Penicillium brevicompactum* with in vivo anti-juvenile hormone activity, J. Org. Chem., **63**, 8530–8535 (1998).
31. **Moya, P., Cantín, A., Miranda, M. A., Primo, J., and Primo-Yúfera, E.:** Synthesis and biological activities of new analogues of the active fungal metaboliotes *N*-(2-methyl-3-oxodecanoyl)-2-pyrroline and *N*-(2-methyl-3-oxodec-8-enoyl)-2-pyrroline, J. Agric. Food Chem., **47**, 3866–3871 (1999).
32. **Cantín, A., Moya, P., Miranda, M. A., Primo, J., and Primo-Yúfera, E.:** Synthesis and biological evaluation of new analogues of the active fungal metabolites *N*-(2-methyl-3-oxodecanoyl)-2-pyrroline and *N*-(2-methyl-3-oxodec-8-enoyl)-2-pyrroline (II), J. Agric. Food Chem., **48**, 3682–3688 (2000).