Nematicidal Activity of Beauvericin Produced by the Fungus *Fusarium bulbicola*

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A nematicide, beauvericin (**1**), was isolated from cultures of the fungus *Fusarium bulbicola*, and its structure was identified by spectroscopic analysis. Compound **1** showed nematicidal activities against the pine wood nematode *Bursaphelenchus xylophilus* and the free-living nematode *Caenorhabditis elegans*.

Key words: Beauvericin, Nematicide, *Fusarium bulbicola*

Introduction

We have previously investigated fungal metabolites such as aspyrone (Kimura *et al.*, 1996), peniprequinolone (Kusano *et al.*, 2000), $\beta\gamma$-dehydrocurvularin (Kusano *et al.*, 2003), penipratynolene (Nakahara *et al.*, 2004), 5-hydroxymethyl-2-furoic acid (Kimura *et al.*, 2007), and fumiquinones A and B (Hayashi *et al.*, 2007) for their potential to act as nematicides against the pine wood nematode *Bursaphelenchus xylophilus* (Fukuda, 1997; Kuroda *et al.*, 1991), which causes pine wilt disease for the Japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Sieb. et Zucc.). Conventional control methods are currently based on the use of low-specific biocidal compounds acting as nerve poisons, like carbamates, and phosphorylated and halogenated organic compounds. Some of those compounds cause global environmental problems such as contamination of groundwater and destructive effects on the ozone layer (Gonzalez and Estevez-Braun, 1997). Since it was necessary to develop effective nematicides with low risk for humans and wildlife, we have focused our attention on new nematicides from fungal metabolites that are valuable natural sources for agrochemical development, and we found the presence of the regulators in the mycelia of *Fusarium bulbicola* (Fotso *et al.*, 2002). Our investigation on metabolites of this fungus has now led to the isolation of one active substance, beauvericin (**1**) (Hamill *et al.*, 1969). The present paper describes the production, isolation, structural determination, and nematicidal activities of **1**.

Material and Methods

General experimental procedures

The IR spectrum was recorded with a JASCO FT IR-7000 spectrometer and the ^{1}H and ^{13}C NMR spectra with a JEOL JNM-ECD 500 NMR spectrometer at 500 and 125 MHz, respectively. Chemical shifts are expressed in δ values with solvents as internal standards. HREIMS datum was obtained with a JEOL JMS-SX 102 mass spectrometer. Silica gel (Wako Pure Chemical Industries, Ltd., Osaka, Japan; 75–150 μm) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F254, 0.2 mm) were used for preparative TLC.

Fungal material and fermentation

Fusarium bulbicola was collected from soil in the city of Kitakyushu in April 1999, and authenticated by Dr. R. A. Samson of the Fungal Biodiversity Center at the Institute of the Royal Netherlands Academy of Arts and Sciences. A voucher specimen (No. S268) is deposited at

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the Laboratory of Food Science, Department of Nutritional Sciences, Faculty of Human Ecology, Yasuda Women's University, Hiroshima, Japan. One hundred twenty 500-ml Erlenmeyer flasks, each containing 250 ml of 3% sucrose, 1% malt extract, and 2% yeast extract, were individually inoculated with 1-cm^2 agar plug taken from a stock culture of the fungus maintained at 20 °C on potato dextrose agar. The fungus was statically grown at 24 °C for 28 d.

Extraction and isolation

The culture broth (30 l) was filtered to separate the mycelia, and the mycelial mats were dried at 25 °C for 1 week and then extracted three times with Me_2CO. The combined solvent was evaporated to dryness under reduced pressure. The resulting residue (3.7 g) was first fractionated by column chromatography on silica gel ($CHCl_3$/EtOAc). The fraction (0.37 g), obtained by elution with $CHCl_3$/EtOAc (9:1), was further purified by column chromatography on silica gel (*n*-hexane/EtOAc). The fraction (0.18 g), obtained by elution with *n*-hexane/EtOAc (7:3), was further purified by preparative TLC ($CHCl_3$/EtOAc/AcOH, 70:30:2, v/v/v) to obtain crude crystals of **1**. The crude crystals (83 mg) were recrystallized from EtOAc to afford 52 mg of **1**.

Beauvericin (**1**): IR (KBr): ν = 2970 (alkane), 1744 (ester), 1661 (amide), 1458, 1415, 1371, 1265, 1180, 700 cm^{-1}. – ^1H NMR (500 MHz, acetone-d_6): δ = 0.43 (d, J = 6.9 Hz, 3H, 8-H), 0.80 (d, J = 6.9 Hz, 3H, 9-H), 2.01 (m, 1H, 7-H), 2.97 (dd, J = 11.0, 14.5 Hz, 1H, 10-H), 2.98 (s, 3H, 4-CH_3), 3.34 (dd, J = 5.0, 14.5 Hz, 1H, 10-H), 4.92 (d, J = 8.5 Hz, 1H, 6-H), 5.44 (dd, J = 5.0, 11.0 Hz, 1H, 3-H), 7.24 (m, 5H, 12,13,14,15,16-H). – $^{13}C\{^1H\}$ NMR (125 MHz, acetone-d_6): δ = 17.51 (q, C-8), 18.27 (q, C-9), 29.69 (d, C-7), 32.37 (q, 4-CH_3), 34.75 (t, C-10), 57.40 (d, C-3), 75.46 (d, C-6), 126.73 (d, C-14), 128.52 (d, C-13,15), 128.90 (d, C-12,16), 136.66 (s, C-11), 169.29 (s, C-5), 169.92 (s, C-2). – HREIMS: m/z = 783.4095 [M^+], calcd. for $C_{45}H_{57}N_3O_9$; found 783.4044.

Bioassay for nematicidal activity against Bursaphelenchus xylophilus

Nematicidal activities were measured in microwell plates with the pine wood nematode *Bursaphelenchus xylophilus*. *B. xylophilus* was cultured for about 2 weeks on a slant of *Botrytis cinerea* grown in potato-dextrose medium. Separation of the cultured nematodes and measurement of the nematicidal activity were carried out according to the method of Kusano *et al*. (2000).

Bioassay for nematicidal activity against Caenorhabditis elegans

Nematicidal activities were measured in microwell plates with the free-living nematode *Caenorhabditis elegans* according to the method of Kusano *et al.* (2000). Worms were cultivated on agar plates as described previously. For the assay, a suspension of adults and L4 larvae (more than 90%) from a 4-day-old culture was diluted with M9 buffer to a solution containing a definite number of nematodes (about 500 nematodes/ml). Test compounds and extracts were dissolved in 0.2 ml of 3% methanol. The nematode suspension (0.1 ml) thus obtained was added to 24-well plates with wells containing a definite amount of the test compound. After plates were kept at 18 °C for 2 d, the measurement of the nematicidal activity was carried out according to the method of Kusano *et al*. (2000).

Results and Discussion

The Me_2CO extract (3.7 g) from the mycelial mats of *F. bulbicola* was purified by silica gel column chromatography and preparative TLC to afford **1**.

Compound **1** was obtained as colourless plates. The molecular formula of **1** was established as $C_{45}H_{57}N_3O_9$ by HREIMS. The ^1H and ^{13}C NMR spectra, and PFG-HMQC experiments indicated the presence of three methyl, one methylene, three aliphatic methine, five aromatic methine, one aromatic quaternary and two carbonyl carbon atoms. The IR absorption band at 1744 cm^{-1} and a signal at δ 169.92 in the ^{13}C NMR spectrum indicated the presence of an ester carbonyl group. The IR absorption band at 1661 cm^{-1} and a signal at δ 169.29 in the ^{13}C NMR spectrum indicated the presence of an amide carbonyl group. The IR absorption band at 700 cm^{-1} and four sp^2 carbon atoms in the ^{13}C NMR spectrum indicated the presence of a monosubstituted benzene ring. Detailed analysis of PFG-HMBC experiments and the relative molecular mass (M^+ = 783) led to the structure of **1** (Fig. 1), a cyclic repeating sequence

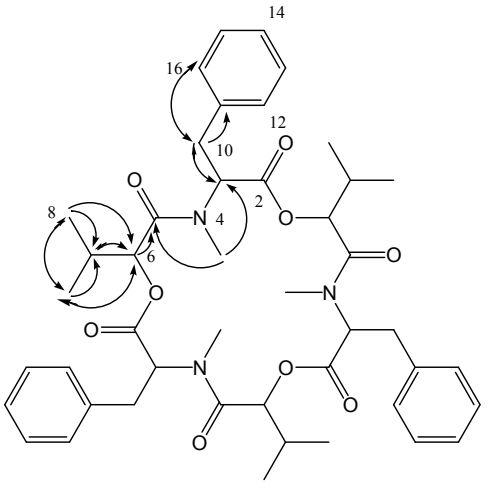

Fig. 1. Chemical structure of beauvericin (**1**).

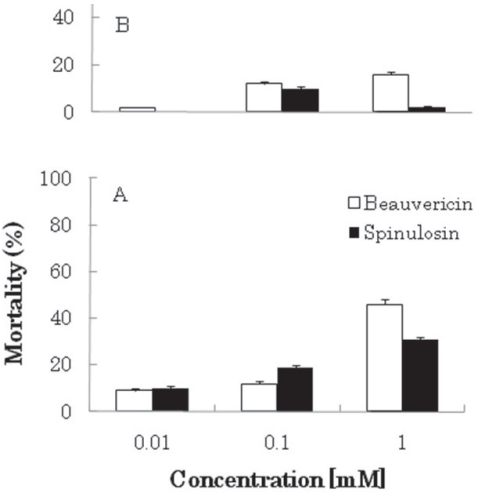

Fig. 2. Nematicidal activities of **1** against (A) *Bursaphelenchus xylophilus* and (B) *Caenorhabditis elegans*.

of three alternating molecules of *N*-methyl phenylalanine and 2-hydroxyisovaleric acid. From these results, **1** was identified as beauvericin (Fig. 1) by comparing the physicochemical properties with those reported (Hamill *et al*., 1969).

This is the first report on the nematicidal activities of **1**. Compound **1** is known to show cytotoxic effects in human acute lymphoblastic leukemia cells (Guey-Mei *et al*., 2004), inhibitory activities against acyl-CoA:cholesterol acyltransferase (Hasumi *et al.*, 1993), brine shrimp, and Gram-positive bacteria (Hamill *et al*., 1969).

The nematicidal activities of **1** were examined against *B. xylophilus* and *C. elegans* (Fig. 2). Spinulosin from *Aspergillus fumigatus* was used as positive control (Hayashi *et al*., 2007). Compound **1** had an effective nematicidal activity against *B. xylophilus* of 46% at a concentration of 1 mM, and weak nematicidal activities at the concentrations of 0.01 mM and 0.1 mM. On the other hand, **1** had weak nematicidal activities against the free-living nematode *C. elegans* at the concentrations of 0.1 mM and 1 mM.

Compound **1** showed effective nematicidal activity against *B. xylophilus* and weak nematicidal activity against *C. elegans*. The difference in nematicidal activities of **1** against the two test nematodes might be attributed to the chemical composition and the permeability to water of their culticles (Ellenby, 1946; Bird, 1958).

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