Nematicidal Activity of 5-Hydroxymethyl-2-furoic Acid against Plant-Parasitic Nematodes

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A nematicide, 5-hydroxymethyl-2-furoic acid (**1**), was isolated from cultures of the fungus *Aspergillus* sp. and its structure was identified by spectroscopic analysis. Compound **1** showed effective nematicidal activities against the pine wood nematode *Bursaphelenchus xylophilus* and the free-living nematode *Caenorhabditis elegans* without inhibitory activity against plant growth, but **1** did not show any effective nematicidal activity against *Pratylenchus penetrans.*

Key words: 5-Hydroxymethyl-2-furoic Acid, Nematicide, *Aspergillus* sp.

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), and penipratynolene (Nakahara *et al.*, 2004) for their potential to act as nematicides against the root-lesion nematode *Pratylenchus penetrans*, which is a parasite of many crop plants and causes root necrosis (Pitcher *et al.*, 1963; Towshend, 1963). In addition, Japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Sieb. et Zucc.), the main species in Japan, have the highest susceptibility to pine wilt disease caused by the pine wood nematode *Bursaphelenchus xylophilus* (Fukuda, 1997; Kuroda *et al.*, 1991). Plant-parasitic nematodes cause crop losses that have been estimated to be 9% of the world's crop yield each year. Conventional control methods are currently based on the use of low-specific biocidal compounds acting as nerve poisons, like carbamates, halogenated organic compounds. Some of those compounds cause global environmental problems. Methyl bromide has a destructive effect on the ozone layer, and its production is being restricted (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 resources for agrochemical development, and we found the presence of the regulators in the culture filtrate of *Aspergillus* sp. Our investigation for metabolites of this fungus has now led to the isolation of one active substance, 5-hydroxymethyl-2-furoic acid (**1**). The present paper describes the production, isolation, structural determination, and biological activities of **1**.

Material and Methods

General experimental procedures

Melting point was determined using a Yanagimoto micromelting point apparatus and is uncorrected. The UV spectrum was recorded on a SHIMAZU UV-2200 spectrophotometer and the IR spectrum on a JASCO FT IR-7000 spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded 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., 75–150 μm) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.2 mm) were used for preparative TLC. 3-Nitropropionic acid (3-NPA), purchased from Sigma-Aldrich Chemical Company, USA, was employed as a positive control.

Fungal material and fermentation

Aspergillus sp. was collected from the soil in the city of Kitakyushu in April 2000. A voucher specimen (No. S328) is deposited at the laboratory of Bioorganic Chemistry, Department of Biological and Environmental Chemistry, Faculty of Agriculture, Tottori University. One hundred twenty 500-ml Erlenmeyer flasks, each containing 250 ml of malt extract medium supplemented with 3% polypeptone, 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 14 d.

Extraction and isolation

The culture broth (30 l) was filtered, and the filtrate was adjusted to pH 2.0 with 4 M HCl solution. The filtrate was successively extracted with EtOAc and concentrated *in vacuo*. The resulting residue (2.9 g) was first fractionated by column chromatography on silica gel (*n*-hexane/acetone). The fraction (1.08 g), obtained by elution with *n*-hexane/acetone 7:3, was further purified by column chromatography on silica gel (benzene/acetone). The fraction (0.72 g), obtained by elution with benzene/acetone 7:3, was further purified by column chromatography on silica gel (benzene/acetone). The fraction (0.14 g), obtained by elution with benzene/acetone 9:1, was purified by preparative TLC (*n*-hexane/EtOAc, 3:7, v/v). The fraction (33 mg) was further purified by preparative TLC ($CHCl_3$/MeOH, 9:1, v/v) to afford 17 mg of **1**.

5-Hydroxymethyl-2-furoic acid (**1**): M. p. 114–115 °C. – UV/vis (EtOH): λ_{max} (lg ε) = 252 (3.90), 292 nm (3.21). – IR (KBr): ν = 3292 (OH), 2962 (C=C), 2530 (COOH), 1657 (O–C=O), 1528 cm^{-1} (C=C). – ^{1}H NMR (500 MHz, acetone-d_6): δ = 4.60 (s, 2H, 5-CH_2OH), 6.48 (d, J = 3.4 Hz, 1H, 4-H), 7.16 (d, J = 3.4 Hz, 1H, 3-H). – ^{13}C{^{1}H} NMR (125 MHz, acetone-d_6): δ = 57.4 (t, 5-*C*H_2OH), 109.6 (d, C-4), 119.5 (d, C-3), 145 (s, C-2), 159.7 (s, C-5), 160.9 (s, 2-*C*OOH). – HREIMS: *m/z* (M^+): calcd. for $C_6H_6O_4$ 142.0266; found 142.0268.

Bioassay for nematicidal activity against Pratylenchus penetrans

Nematicidal activities were measured in microwell plates with the root-lesion nematode *Pratylenchus penetrans* according to the method of Kusano *et al.* (2000). *P. penetrans* was cultured for about 2 weeks on a slant of alfalfa grown in Krusberg medium. The cultured nematodes were separated from the callus by the Baermann funnel technique and counted under a microscope. An aqueous suspension containing a definite number of nematodes (about 500 nematodes/ml) was prepared by dilution. Test compounds and extracts were dissolved in methanol and added to the nematode suspension (up to 3% volume of the suspension). The nematode suspension thus prepared was transferred to 24-well plates with wells containing a definite amount of the test compound. While plates were kept at 24 °C for 14 d, nematodes in the wells were counted under a microscope once in 2 d for a total of 6 measurements from 4 d after treatment. The nematicidal activity is expressed as follows: mortality (%) = [(B – A)/B] × 100, where A is the number of nematodes alive after being treated with the test compound, and B is the number of nematodes alive in the control wells (3% methanol in distilled water).

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 the 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).

Bioassay for the growth of lettuce, carrot, and raddish seedlings

Lettuce (*Lactuca sativa* cv. Kingcisco), carrot (*Daucus carota* cv. Benikoshigosun), and raddish (*Raphanus sativus* Linn. cv. Frenchbreakfast) seeds were purchased from Takii Nursery and sown in a Petri dish (150 × 25 mm) lined with a filter paper containing deionized water. After 1 d under continuous light (100 μE/m^2 s) at 24 °C, seedlings were selected for uniformity (radicles; 2 mm) and transferred into a mini-Petri dish (35 × 15 mm) lined with filter paper containing 1 ml of deionized water and a defined amount of the test compound. The Petri dish was kept at 24 °C for 4 d under continuous light (100 μE/m^2 s). The length of the hypocotyls and roots treated with the compound was measured and the mean value of the length was compared with an untreated control (Kusano *et al.*, 1998). Triplicate experiments were conducted.

Bioassay for the growth of rice and barnyard millet seedlings

Rice (*Oryza sativa* L. cv. Nihonbare) and barnyard millet (*Echinochloa esculenta* cv. Shirohie) seeds were sterilized with 75% ethanol for 30 s, immersed in sodium hypochlorite solution (antiformin) for 2 h, rinsed under running water for 3 h, and transferred to a Petri dish (150 × 25 mm) containing deionized water. After 3 d under continuous light (100 μE/m^2 s) at 30 °C, seven seedlings were selected for uniformity (radicles; 2 mm) and transferred into a test tube (35 × 15 mm) containing 1 ml of deionized water and a defined amount of the test compound. The test tube was sealed with a polyethylene film and incubated for 7 d under continuous light (100 μE/m^2 s) at 30 °C. The total length and the lengths of the second leaf sheath and primary root after treatment with the compound were measured and the mean lengths were compared with an untreated control (Kusano *et al.*, 1998). Triplicate experiments were conducted.

Results and Discussion

The EtOAc-soluble acidic fraction (2.9 g) from the filtrate of *Penicillium* sp. was purified by silica gel column chromatography and preparative TLC to afford **1**.

Compound **1** was obtained as colorless needles. The molecular formula of **1** was established as $C_6H_6O_4$ by HREIMS. The ^{1}H and ^{13}C NMR spectra, and PFG-HMQC experiments indicated the presence of one O-substituted aliphatic carbon atom, two aromatic methine carbon atoms, two O-substituted aromatic quarternary carbon atoms, one carbonyl group. The IR absorption band at 3292 cm^{-1} indicated the presence of a hydroxy group. The IR absorption band at 2962 cm^{-1} and a signal at δ 160.9 in the ^{13}C NMR spectrum indicated the presence of a carboxy group. Detailed

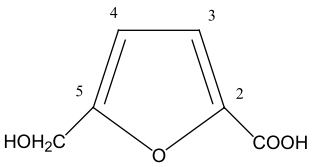

Fig. 1. Structure of 5-hydroxymethyl-2-furoic acid (**1**).

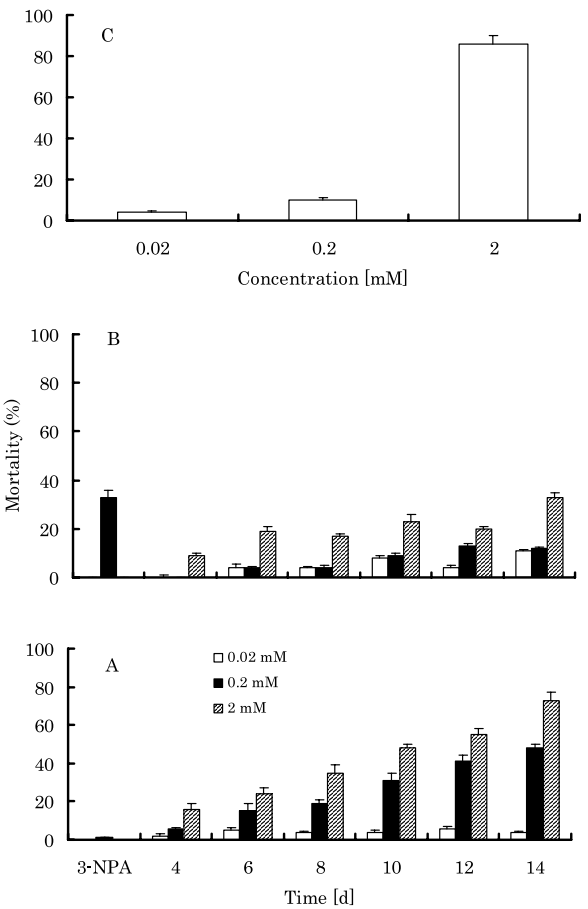

Fig. 2. Nematicidal activities of **1** against *Bursaphelenchus xylophilus* (A), *Pratylenchus penetrans* (B), and *Caenorhabditis elegans* (C).

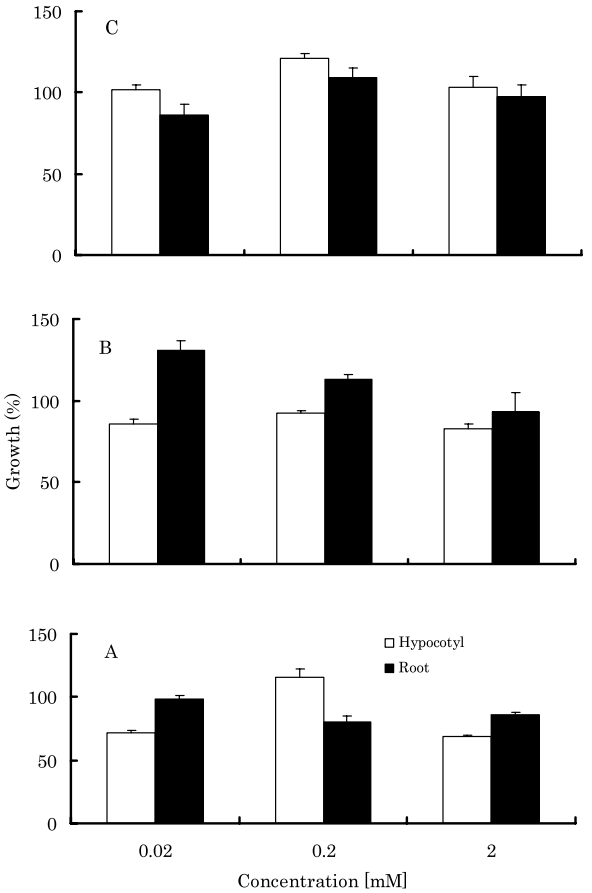

Fig. 3. Plant growth activities of **1** against lettuce (A), carrot (B), and raddish (C) seedlings.

analysis of PFG-HMBC experiments led to the structure of **1** (Fig. 1). From those results, **1** was identified as 5-hydroxymethyl-2-furoic acid (Fig. 1) by comparing the physicochemical properties with those reported (Munekata and Tamura, 1981).

This is the first report on the nematicidal and plant growth activities of **1**. Compound **1** is known to show cytotoxic activity against SV40-transformed cells *in vitro* and slightly effective antitumor activity against Sarcoma 180 *in vitro* (Munekata and Tamura, 1981).

The nematicidal activities of **1** were examined against *P. penetrans*, *B. xylophilus*, and *C. elegans*. Compound **1** had a weak nematicidal activity against *P. penetrans* of 8% at a concentration of 2 mM after 4 days of incubation, but **1** had no nematicidal activity at the concentrations of 0.02 mM and 0.2 mM. Compound **1** had weak nematicidal activities of 11%, 12%, and 33% at the concentrations of 0.02 mM, 0.2 mM, and 2 mM after 14 days of incubation, respectively. Compound **1** showed nematicidal activity similar to that of 2 mM 3-nitropropionic acid (Fig. 2). Compound **1** had nematicidal activity against *B. xylophilus* of 2%, 6%, and 16% at the concentrations of 0.02 mM, 0.2 mM, and 2 mM after 4 days of incubation, respectively. Compound **1** also caused 4%, 48%, and 73% mortality at the same three concentrations after 14 days of incubation, respectively. Compound **1** promoted nematicidal activity against *B. xylophilus* in proportion to its exposure time from 4 days to 14 days (Fig. 2). Compound **1** had nematicidal activities against *C. elegans* of 4%, 10%, and 86% at the same three concentrations after 2 days incubation, respectively (Fig. 2).

Phytotoxic activities of **1** were examined against lettuce, carrot, raddish, rice, and barnyard millet seedlings. Compound **1** had no inhibitory activities against hypocotyl elongation and root growth of lettuce seedlings at a concentration of 0.02 mM, but it showed weak inhibitory activities against root growth at the concentrations of 0.2 mM and 2 mM (Fig. 3A). It promoted root growth of carrot seedlings at a concentration of 0.02 mM, but **1** did not show any promotive activity against the root

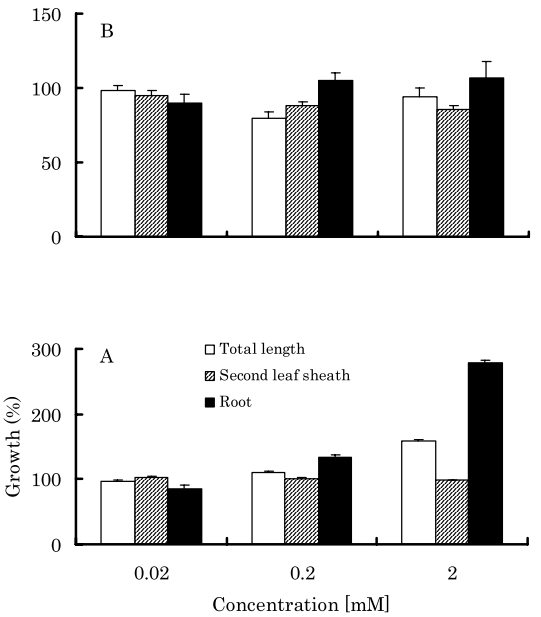

Fig. 4. Plant growth activities of **1** against rice (A) and barnyard millet (B) seedlings.

growth at the concentrations of 0.2 mM and 2 mM, respectively (Fig. 3B). Compound **1** had no inhibitory activities against the hypocotyl elongation and root growth of raddish seedlings (Fig. 3C). Compound **1** promoted total length and root growth of rice seedlings at the concentrations of 0.2 mM and 2 mM, and total length at 2 mM (Fig. 4A). Compound **1** had no inhibitory activity against the growth of barnyard millet seedlings (Fig. 4B).

Compound **1** showed effective nematicidal activities against *B. xylophilus* and *C. elegans* without inhibitory activity against plant growth, but **1** did not show any effective nematicidal activity against *P. penetrans*. The difference in nematicidal activities of **1** against the three test nematodes might be attributed to the chemical composition and the permeability to water of their cuticles (Ellenby, 1946; Bird, 1958).

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