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An antifungal and plant growth promoting metabolite from a sterile dark ectotrophic fungus

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Abstract

A sterile dark ectotrophic fungus isolated from roots of an Australian native grass, *Neurachne alopecuroidea* produces compound **1** in liquid cultures. The structure of the metabolite was determined by spectroscopic and X-ray diffraction studies. The metabolite shows activity against phytopathogens and plant growth promoting activity, properties that are also expressed *in vivo* by the ectotrophic fungus.

Keywords: Ectotrophic fungus; *Neurachne alopecuroidea*; Antifungal; *Gaeumannomyces graminis* var. *tritici*; Plant growth promoter

1. Introduction

Take-all, the most important root disease of wheat worldwide, is caused by the fungus *Gaeumannomyces graminis* var. *tritici* (Ggt) (Freeman and Ward, 2004). A number of fungi with ectotrophic growth habits on roots, such as *Phialophora* spp., have been reported to confer cross-protection to cereals against Ggt (Deacon, 1981; Wong, 1981; Dewan and Sivasithamparam, 1988). Moreover, various sterile fungi isolated from roots and rhizosphere of grasses and cereals have been shown to be beneficial associates of some plant species (Dewan and Sivasithamparam, 1989; Newsham, 1999). In continuation of our work on the identification of fungi with potential as biocontrol agents, we have investigated the metabolites produced by a strain (SDEF 678) that occurs with high frequency in the roots of an Australian native grass, *Neurachne alopecuroidea*. This isolate inhibited growth of Ggt in dual plate antagonism tests and significantly increased the root growth of wheat seedlings grown in natural soil (Worth, 2002). We now report the isolation and structural determination of a metabolite produced in liquid cultures of SDEF 678 that exhibits antifungal and plant growth promoting activity.

2. Results and discussion

Extraction of liquid cultures of SDEF 678 with ethyl acetate yielded a dark brown oily residue. The extract was partitioned by flash chromatography (silica gel) with gradient elution into 15 fractions. One of the less polar fractions (F4) afforded a crystalline material that was homogeneous on tlc and which was assigned structure **1** on the following evidence. The HR-FABMS spectrum of **1** showed the $[M+H]^+$ ion at m/z 191.069283 corresponding to a molecular formula of $C_{11}H_{10}O_3$. The presence of a cyclohex-2,3-enone group was suggested from the ^{13}C NMR spectrum (δ_C 189.7, s; 126.7, d; 144.0, d) and the ^1H NMR spectrum (6.03, d, $J = 10.5$ Hz; 6.65, ddd, $J = 10.5$, 4.7, 2.6 Hz). The proton resonating at δ_H 6.65 showed coupling (4.7 Hz) to an oxymethine proton at δ_H

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4.64, which in turn was coupled to another oxymethine at δ_H 4.01. Signals for two olefinic protons, observed at δ_H 5.45 and 5.35, showed coupling to a vinylic methyl (δ_H 1.90), thus disclosing an isopropylene group. The connectivity between this group and the cyclohex-2,3-enone moiety involved acetylene carbons (δ_C 89.4, s, and 80.1, s). So far, six degrees of unsaturation have been assigned. The last one can be accommodated by assuming the presence of an epoxide ring at C5 and C6 of the cyclohexenone. Although support for the structure was obtained from the HMQC and HMBC spectra, no evidence was obtained to exclude an oxetan oxygen bridging C4 and C6. To resolve this ambiguity and to secure the relative stereochemistry, a single crystal X-ray diffraction study was undertaken. The results discussed below establish that the structure of the fungal metabolite is as shown in **1**.

The results of the single crystal X-ray study (Fig. 1; Table 1) are consistent in terms of stoichiometry, connectivity, and stereochemistry with that proposed for **1** above; the X-ray study is not definitive of the absolute configuration. The packing of the molecules in the lattice is dominated by the quasi-parallel disposition of planes and rods, and the hydroxyl group hydrogen-bonding intermolecularly to O(1) (O,H(40)···O(1) ($x - 1/2$, $1/2 - y$, $1 - z$) 2.907(3), 2.09(4) Å. The closest H···π interaction appears to be H(5)···C(10) ($11/2 - x$, $1 - y$, $z - 1/2$) 2.90(2) Å. Molecular geometry, although largely as expected, displays a few features of interest: the C_6 ring is significantly nonplanar, being folded about the C(1)···C(4) line (see Fig. 1 caption), the C(1–4)/C(1,4–6) interplanar dihedral angle being 15.8(2)°, with the C_2O ring making a dihedral of 78.2(2)° to C(1,4–6). C(6–11) is a closely planar sequence (χ^2 32), making a dihedral angle of 25.7(2)° to C(1,4–6), and 76.2(2)° to C(5,6)O. There is an interesting asymmetry in the C–O (epoxide) distances, C(5)–O(5) being appreciably the shorter. The usual diminutions of angles below 120° opposed to double bonds are found at C(1,9).

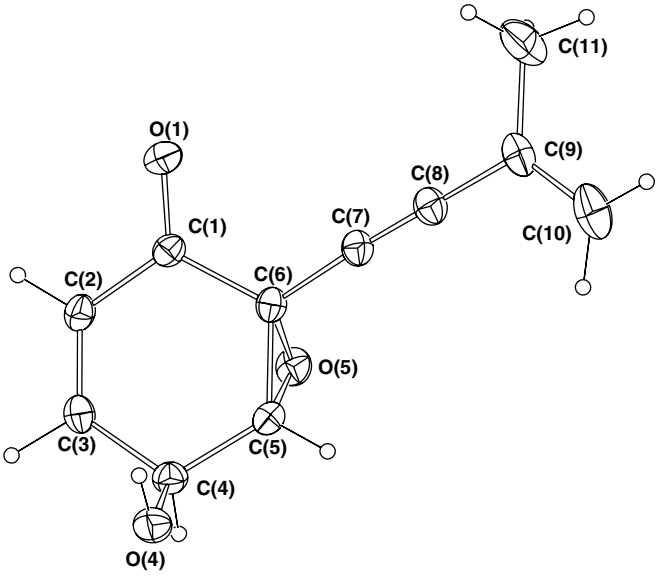


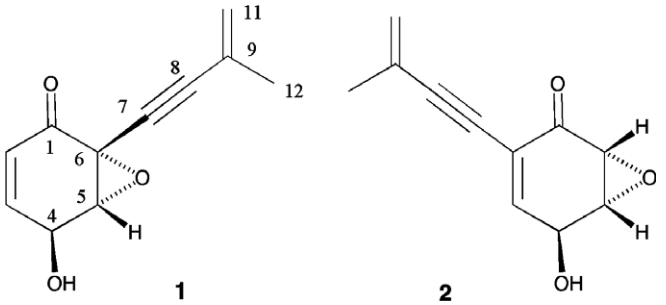
Fig. 1. Projection of **x**, normal to the six-membered 'plane' ($\chi^2 = 3491$; δC(1–6) 0.094(3), −0.077(4), −0.056(4), 0.132(4), −0.069(4), −0.046(3) Å; deviations of O(1,4,5) are 0.347(4), 1.396(4), −1.300(3) Å).

Table 1
Molecular non-hydrogen geometries

Atom	Parameter	Atom	Parameter
Distances (Å)			
C(1)–C(2)	1.456(4)	C(5)–O(5)	1.426(4)
C(1)–C(6)	1.510(4)	C(6)–O(5)	1.472(3)
C(1)–O(1)	1.223(3)	C(6)–C(7)	1.448(4)
C(2)–C(3)	1.324(3)	C(7)–C(8)	1.177(4)
C(3)–C(4)	1.502(5)	C(8)–C(9)	1.435(4)
C(4)–C(5)	1.499(4)	C(9)–C(10)	1.343(7)
C(4)–O(4)	1.423(4)	C(9)–C(11)	1.469(7)
C(5)–C(6)	1.480(4)		
Angles (°)			
C(2)–C(1)–C(6)	117.4(2)	C(1)–C(6)–O(5)	111.8(2)
C(2)–C(1)–O(1)	122.4(3)	C(5)–C(6)–O(5)	57.8(2)
C(6)–C(1)–O(1)	120.2(2)	C(5)–O(5)–C(6)	61.4(2)
C(1)–C(2)–C(3)	123.0(3)	C(1)–C(6)–C(7)	118.2(2)
C(2)–C(3)–C(4)	124.2(3)	C(5)–C(6)–C(7)	121.3(3)
C(3)–C(4)–C(5)	112.7(3)	O(5)–C(6)–C(7)	115.4(2)
C(3)–C(4)–O(4)	111.2(2)	C(6)–C(70–C(8)	176.9(3)
C(5)–C(4)–O(4)	111.4(3)	C(7)–C(8)–C(9)	179.6(4)
C(4)–C(5)–C(6)	122.3(3)	C(8)–C(9)–C(10)	118.7(4)
C(4)–C(5)–O(5)	116.1(2)	C(8)–C(9)–C(11)	117.2(4)
C(6)–C(5)–O(5)	60.9(2)	C(10)–C(9)–C(11)	124.1(4)

A literature search revealed that a compound with the same planar structure was claimed to have been isolated from a fungus (DSM 3650) that infected corn roots (Wriede et al., 1988). Unfortunately, the ^{1}H NMR parameters quoted for this compound: 1.0 (3H, s); 4.05 (1H, s); 4.5 (1H, m); 5.4–5.46 (1H, d); 5.95–6.05 (2H, m); 6.7–6.8 (1H, m), appear to contain typographical errors and/or errors of assignment. In the absence of any other valid points of comparison, the structure of the metabolite from strain DSM 3650 remains uncertain.

It is interesting to note that **2**, a regioisomer of **1**, has been isolated as a phytotoxic metabolite of *Pestalotiopsis theae* (Nagata et al., 1992). The relative configuration of the two metabolites is the same.



The inhibitory activity of 1 on the growth of five phytopathogens was measured in a plate assay. The growth of *Phytophthora cinnamomi* and Ggt was totally inhibited at 0.98 μg/ml, *Rhizoctonia solani* at 7.81 μg/ml, *Pythium irregulare* and *Alternaria alternata* at 15.63 μg/ml. Interestingly, **1** also showed *in vitro* growth promotion of barley seed-

lings. At concentrations of 0.1 and 1.0 μg/ml, stem length increased by 8.2% and 24.7%, respectively, compared to the control.

From fractionation of the original extract, two other fractions were obtained. Although the components could not be obtained in a pure form, their NMR spectra clearly indicated them to be permutations of cyclohexene-1,4-diols. Whereas these diols showed little or no inhibition of the growth of the phytopathogens tested, they significantly increased (22–27%) the *in vitro* growth of barley seedlings at concentrations of 0.1 and 1.0 μg/ml, respectively.

3. Experimental

3.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM 500 operating at 500 (^{1}H) and 125 (^{13}C) MHz, using residual and deuterated solvent peaks as reference standards. MS spectra were obtained with a VG Autospec mass spectrometer. Flash chromatography was carried out using Merck silica gel 60 GF254 and for TLC analysis, precoated silica gel plates (Merck Kieselgel 60 GF_{254}, 0.25 mm) were used. The optical rotation was measured using a Perkin–Elmer 141 Polarimeter and a microcell of one decimetre path length at ambient temperature.

3.2. Fungal material

The fungal strain was isolated from the roots of the Australian native grass, *Neurachne alopecuroidea*. Roots collected from intact soil cores at Hyden, Western Australia, were rinsed under the tap, cut into-5 mm pieces and washed for 6 h in a root washing apparatus. The root pieces were blotted dry, surface sterilised (1.2% NaOCl) and plated onto 1/10 strength potato dextrose agar (PDA) containing streptomycin (50 μg/ml). The fungus was picked from the root pieces and subcultured onto 1/2 PDA and streptomycin until pure. It was characterised as a fungus by microscopic examination of orthomorphological characteristics. The fungus was maintained on 1/5 PDA slopes at 4 °C. It has been deposited in the Soil Science and Plant Nutrition collection at the University of Western Australia, as WUF 883. Because of lack of sporulation by the fungus, classical taxonomic methods were not applicable for classification. Currently, we are determining the affinities using molecular methods.

3.3. Preparation of liquid cultures

An inoculum of SDEF strain 678, removed from the margin of a 7-day-old culture growing on potato dextrose agar, was inoculated into each of four 3 L flasks containing potato dextrose broth (1 L). The still cultures were left standing for 4 months at 23 °C ±2 (room temperature).

3.4. Extraction and isolation of metabolites

The cultures were filtered under vacuum through filter paper, the filtrate was acidified to pH 4 with 5 N HCl and extracted with an equal volume of ethyl acetate. The organic layer was concentrated *in vacuo* and a portion (808 mg) of the residue (1.35 g) was fractionated by flash chromatography on silica gel (100 g) eluting with a gradient of light petroleum/ethyl acetate (1:1 to 0:1). Fifteen fractions were collected and of these fraction 4 (97.5 mg), fraction 8 (49.5 mg) and fraction 9 (235 mg) contained the bulk of material recovered. Fraction 4 could be recrystallised from ether/light petroleum to give 5-hydroxy-1-(3-methyl-3-buten-1-ynyl)-7-oxabicyclo[4.1.0]-hept-3-en-2-one (**1**) as needles, m.p. 97–98 °C; $[\alpha]_D$ +327.7° ($CHCl_3$; c 0.5). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.90 (3H, dd, $J_{10,11a} = 1.1$ Hz, $J_{10,11b} = 1.6$ Hz, H_3-10), 4.01 (1H, dd, $J_{3,5} = 2.6$ Hz, $J_{4,5} = 1.3$ Hz. H-5); 4.64 (1H, dd, $J_{2,4} = 1.3$ Hz, $J_{3,4} = 4.7$ Hz, H-4); 5.35 (1H, m, H-11b); 5.45, (1H, m, H-11a); 6.03 (dd, $J_{2,3} = 10.5$ Hz, $J_{2,4} = 1.3$ Hz, H-2); 6.65 (1H, ddd, $J_{2,3} = 10.5$ Hz, $J_{3,4} = 4.7$ Hz, $J_{3,5} = 2.6$ Hz, H-3); ^{13}C NMR δ 23.2 (q, C9), 53.3 (s, C6), 63.3 (d, C4), 66.2 (d, C5), 80.1 (s, C8), 80.1 (s, C7), 125.1 (t, C11), 125.6 (s, C10), 126.7 (d, C2), 144.0 (d, C3), 189.7 (s, C1). HR-FABMS *m/z* 191.069283 (Calc. for $C_{11}H_{11}O_3$ $[M+H]^+$).

3.5. Structure determinations

A full sphere of CCD area detector diffractometer data was measured (Bruker AXS instrument, ω-scans, monochromatic Mo Kα radiation, $\lambda = 0.7107_3$ Å, $2\theta_{max} = 52.5°$; *T ca.* 298 K) yielding 5818 reflections, merging to 1194 independent after 'empirical'/multiscan 'absorption correction' ($R_{int} = 0.050$; 'T'$_{min/max} = 0.93$), 1012 with $F > 4\sigma(F)$ being considered 'observed' and used in the full matrix least squares refinement on F^2, refining anisotropic displacement parameter for C, O, and $(x, y, z, U_{iso})_H$ throughout. Conventional residuals at convergence were $R = 0.040$, $R_w = 0.064$ (reflection weights: $(\sigma^2(F^2) + 0.06\ F^2)^{-1}$). Neutral atom scattering factors were employed within the Xtal 3.7 program system (Hall et al., 2001). Pertinent results are given below and in CCDC deposition 609342, and the figure which shows C, O atoms with 20% probability amplitude ellipsoids, hydrogen atoms having arbitrary radii of 0.1 Å.

3.5.1. Crystal/refinement data

$C_{11}H_{10}O_3$, $M = 190.2$. Orthorhombic, space group $P2_12_12_1$ (D_2^4, No. 19), $a = 6.436(2)$, $b = 8.330(2)$, $c = 18.481(2)$ Å, $V = 990.8$ Å^3. D_c ($Z = 4$) $= 1.27_5$ g/cm^3. $\mu_{Mo} = 0.093$ mm^{-1}; specimen: $0.54 \times 0.52 \times 0.50$ mm.

3.6. Minimum inhibitory concentration (MIC) test

The method used was essentially that described by Li and Strobel (2001). Potato dextrose broth (200 μl) amended with various concentrations (0.9–1000 μg/ml) of the test samples was introduced into wells of a 96-well microtitration plate. Agar plugs (3 mm diameter) obtained from a rapidly growing culture of the pathogen to be observed (*Phytophtora cinnamomi*, *Pythium irregulare*, *Rhizoctonia solani*, *Alternaria alternata*, or *Gaeumannomyces graminis* var *tritici*) were inoculated and the plates were incubated at 25 °C for 4 d. Each well was examined and the concentration of the test sample in a well showing no growth next to a well having some growth was taken as the MIC value.

3.7. Plant growth promotion bioassay

The method described by Gillespie-Sasse et al. (1991) was used. Briefly, uniformly germinated seedlings of barley (*Hordeum vulgare*) with seminal roots 5–8 mm long and green coleoptiles were placed on cotton wool and inserted into sterilised glass tubes (300 mm long; 6 mm i.d.). A 1 ml sample of solution containing various amounts of test sample (10–0.001 μg/ml) was introduced into the tube so that the seedling roots came into contact with the solution. The seedlings were kept at 25/20 °C, day/night, and the growth of first and second leaves were measured with a root length scanner (Win/MAC Rhizo program, USA) for up to 15 days. Each treatment consisted of five replicates and was repeated.

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