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A novel secondary metabolite from the *Eucalyptus* pathogen *Mycosphaerella cryptica*

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Summary

The most abundant metabolite of the eucalyptus leaf spot pathogen *Mycosphaerella cryptica* was extracted from agar cultures. The structure of the compound was elucidated by detailed studies of NMR and MS data and by comparison with derivatives. The compound is a previously undescribed diphenylether structurally related to pannaric acid found in lichens. Culture extracts from another species of *Mycosphaerella* isolated from the same environment yielded 5-hydroxymethylfuran-3-carboxylic acid, a furan acidic compound previously isolated from a basidiomycete fungus. Assays for bioactivity of these metabolites revealed no evidence for antimicrobial activity. Some phytotoxicity was seen on newly emerged leaves of *Eucalyptus globulus*, but not on juvenile or adult leaves, when treated with either metabolite.

1 Introduction

Mycosphaerella is a diverse genus that includes destructive pathogens of important native and plantation forest trees. One of the most prevalent *Eucalyptus* pathogens is *Mycosphaerella cryptica* (Cooke) Hansford [anamorph: *Kirramyces nubilosum* (Ganap. and Corbin) Andjic comb. nov.] (Andjic et al. 2007). *Mycosphaerella cryptica* causes necrotic lesions associated with premature defoliation and growth loss, and occurs on many *Eucalyptus* species in the *Eucalyptus* subgenera *Symphyomyrtus* and *Monocalyptus* (Park and Keane 1982).

Many plant pathogens produce toxins as part of their weaponry (Markham and Hille 2001), but whether *Mycosphaerella* spp. that are pathogenic to *Eucalyptus* produce bioactive metabolites is not known. *Mycosphaerella cryptica* produces a reddish pigment in agar and pigmentation has been noted in leaf lesions (Park and Keane 1982). These observations led us to the hypothesis that *M. cryptica* produces a bioactive compound that contributes to leaf damage. We aimed to test this hypothesis by isolating and characterizing the main secondary metabolites produced by *M. cryptica*, as well as from another unknown *Mycosphaerella* species isolated from the same environment as the *M. cryptica* isolate. The metabolites were tested for bioactivity towards micro-organisms and plants.

2 Materials and methods

Mycosphaerella spp. were from Scion, NZ Forest Research Institute Ltd, Rotorua, NZ: *M. cryptica* (NZFS no. 301C) and *Mycosphaerella* sp. (NZFS no. 108D) were both isolated from *Eucalyptus nitens* leaves in the Bay of Plenty, New Zealand, in the summer of

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1996/1997. Ribosomal ITS sequence of isolate 108D shows 98% nucleotide identity with *M. lateralis* (GenBank EU42176.1), but the isolate has not been formally characterized. Large scale cultures of each species were grown in 10 Roux flasks each with 100 ml of either MPGA (Malt extract 30; Peptone 3; Glucose 30; Agar 20 g l^{-1}, respectively, pH 6.4) or CSA (Corn steep Saccharose Agar: corn steep liquor Sigma-Aldrich C4648 10 ml l^{-1} plus glucose 90; saccharose 100; yeast extract 5; K_2HPO_4 2; agar 15 g l^{-1}, respectively, pH 6.4). Five flasks were incubated under light (1500 lux) and five in the dark at 24°C for 14 days.

The agar cultures were extracted twice with 100 ml ethylacetate/methanol (100 : 1). The solvent was dried on Na_2SO_4, filtered and evaporated under vacuum (Rotavapor® RII; Buchi Labortechnik AG, Flawil, Switzerland). Oily residues were defatted by hexane washing, then precipitates dried under vacuum for 6 h. The crude extracts were chromatographed on TLC (Silica Gel 60 F254; Merck KGaA, Darmstadt, Germany) in dimethylenechloride/methanol (15 : 1) plus formic acid 0.5% v/v. Pure metabolites were obtained by flash column chromatography on Silica gel (0.040–0.63 mm) eluted with dimethylenechloride/methanol at increasing polarity and then by preparative layer chromatography (PLC) on precoated Merck silica gel 60 F254 plates in hexane/ethylacetate (1 : 2). The mass spectra (MS) were measured with Finnigan Mat TSQ70 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and Bruker Esquire 3000 Plus instruments (Bruker Daltonics, Bremen, Germany), high resolution MS with a Bruker APEX-QZT ICR and NMR spectra with a Bruker AMX-600 spectrometer, at 600.13 MHz for ^{1}H and 150.92 MHz for ^{13}C.

Bioactivity against bacteria (*Bacillus megaterium*, *Escherichia coli*) or fungi (*Aspergillus nidulans*, *Lophodermium conigenum*, *Cyclaneusma minus*, *Eutypa lata*, *Fusarium graminearum*, *Trichoderma harzianum* and *Trichoderma viride*) was tested using agar diffusion assays with 10 or 20 μg of metabolite, in acetone, on 6 mm diameter filter paper discs.

Metabolite sensitivity of juvenile and adult leaves of *Eucalyptus nitens* or juvenile leaves of *Eucalyptus globulus*, was tested in damp chambers. On the adaxial surface of each leaf, three or four needle puncture wounds were made on each side of the mid vein. Wounds on one side were injected with 0.2, 1 or 20 μg of metabolite/wound, each dissolved in 2–4 μl acetone. Wounds on the other side were acetone-only controls. At least six replicate leaves were assayed for each metabolite at each concentration, in either dark (continual) or light (12 h dark: 12 h light with 72 W black light and 36 W white light tubes). Leaves were incubated at 22°C day: 14–16°C night and monitored for up to 16 days. Tests were carried out in March (Southern Autumn), May (end of Autumn), August (end of winter) and November (Spring) 2007. Young newly emerged leaves of *E. globulus* (leaves 1–4 from the branch tip) were tested in two trials in spring. The leaves were retained on branches immersed in water to prevent drying out, placed in a large damp-chamber and incubated as above. Metabolites were suspended in water instead of acetone, because acetone and other organic solvents (methanol, ethyl acetate, chloroform or ethanol) caused damage to the young leaf tissue. Inoculations were made as above, except with either 1 or 20 μg metabolite/wound and with fewer wounds per leaf due to their small size.

3 Results and discussion

Crude extracts were obtained from *Mycosphaerella* cultures on two agar media and in two growth conditions (light and dark). The medium affected the quantity of metabolites produced, with *Mycosphaerella* sp. isolate 108D producing fivefold more metabolites on CSA medium than on MPGA. In contrast, *M. cryptica* was unable to grow on CSA, possibly because of the high sugar concentration, but this was not investigated. TLC

1a $R^1=R^2=H$

1b $R^1=Me$, $R^2=H$

1c $R^1=R^2=Me$

2

3

Fig. 1. Molecular formulae of compound **1a** isolated from *Mycosphaerella cryptica,* and derivatives, compound **2** (pannaric acid, previously isolated from lichens) and compound **3** isolated from *Mycospharella* sp. isolate 108D.

profiles of metabolites obtained under the different conditions were the same within each species, which allowed crude extracts to be pooled for further analysis.

Mycosphaerella cryptica produced the new metabolite **1a** as the only main compound, which is a yellow solid and represented the 30% of the crude extract. The spectrometric data[1] of **1a** were compatible with $C_{16}H_{14}O_7$, a diphenylether metabolite having a molecular weight of 318 and the molecular formula **1a**, corresponding to 3-methyl-5-hydroxy-1-(3′-hydroxy-4′-carboxy-5′methyl-phenoxy)-benzoic acid. The compound **1a** is isolated as a natural product for the first time, because previously it was only known as an intermediate in the biosynthesis of pannaric acid **2** formed by a phenol coupling between the position C6–C6′. Pannaric acid **2** and other depsides have been isolated from species in the lichen genera *Psoroma*, *Lepraria* and *Leproloma* (Elix and Parker 1987). To confirm the structure, the compound **1a** was treated with a solution of diazomethane, and the dimethylester derivative **1b** was obtained[2]. To confirm the presence of other hydroxyl groups, **1b** was further treated extensively with diazomethane and the tetramethylderivative **1c**[3] was afforded. Their structures were confirmed by an extensive examination of the ^{1}H and ^{13}C NMR spectra and NOEs values, which

[1]cmp 185–187°C. HREIMS [M^+], *m/z*, found 318.0724, calcd. for $C_{16}H_{14}O_7$ 318.0739; ESIMS *m/z* 341 [M + Na]$^+$, 319 [M + 1]$^+$ and 300 [M − 18]$^+$; ^{1}H NMR (DMSO-d_6) : 8.20 (1H, s, OH-3′), 6.42 (1H, *br* d, J = 2.2 Hz, H-4), 6.16 (1H, *br* d, J = 2.2 Hz, H-6), 6.03 (1H, *br* d, J = 2.5 Hz, H-6′), 5.99 (1H, *br* d, J = 2.5 Hz, H-2′), 4.00 (3H, *br* signal, 5-,7-,7′-OH), 2.49 (3 H, *br* s, H_3-8′) and 2.24 (3 H, *br* s, H_3-8). ^{13}C NMR (DMSO-d_6): 172.2 (C-7′), 168.2 (C-7), 166.2 (C-3′), 158.6 (C-5), 158.1(C-1′), 154.2 (C-1), 142.7 (C-5′), 137.6 (C-3), 118.8 (C-2), 113.9 (C-4′), 112.5 (C-4), 109.6 (C-6′), 103.8 (C-6), 103.2 (C-2′), 23.2 (C-8′) and 19.5 (C-8).

[2]*Compound 1b*: 50 mg **1a** dissolved in CH_2Cl_2–MeOH (9 : 1) was treated with a solution of CH_2N_2 for 10 min, and evaporation of the solution gave **1b**, oil, ESIMS, *m/z* 369 [M + Na]$^+$ and 347 [M + 1]$^+$; ^{1}H NMR (acetone-d_6): 11.42 (1H, s, OH-3′), 8.45 (1H, s, OH-5), 6.56 and 6.41 (2H, *br* d, J = 2.4 Hz, H-4 and -6′), 6.40 and 6.26 (2H, *br* d, J = 2.4 Hz, H-6 and 2′), 3.95 (6H, s, 7- and 7′-OMe), 2.50 (3H, *br* s, H_3-8′) and 2.27 (3H, *br* s, H_3-8).

[3]*Compound 1c*: a solution of compound **1b** (30 mg), in CH_2Cl_2–MeOH (9 : 1) was treated repeatedly with a solution of CH_2N_2 in dry CH_2Cl_2 until only one spot appeared on TLC plates; PLC of the residue on CH_2Cl_2–MeOH (15 : 1) gave compound **1c** (25 mg) as an oil: ESIMS *m/z* 397 [M + Na]$^+$ and 375 [M + 1]$^+$. For ^{1}H and ^{13}C NMR spectra, see Table S1. The NMR data showed that the two aromatic rings share the same substitution; two *meta*-protons, one methyl, one methoxyl and one carbomethoxy groups and the last oxygen atom must connect the two rings. The NOEs values and the ^{13}C NMR data allowed the structure of compound **1c** to be established. In the A ring, the OMe group is *ortho* to the H-4 and H-6 protons, while in the B ring, the OMe is *ortho* positioned to only one proton. In both rings, the Me is next to one proton and one carboxymethyl group respectively. It follows that the ethereal oxygen is bonded to C-1 in ring A and to C-1′ in ring B.

demonstrated that the respective substituents on rings A or B did not present a symmetrical system (Fig. 1).

The main metabolite produced by *Mycosphaerella* sp. isolate 108D represented 25% of the total crude extract. The pure polar compound **3**, $C_6H_6O_4$ has furanic and acidic characteristics and a molecular weight of 164. The ^{1}H NMR and MS of **3** are congruent with the structure of 5-hydroxymethylfuran-3-carboxylic acid (Fig. 1). Metabolite **3** has previously been isolated as a natural metabolite from the basidiomycete *Polyporus ciliatus* (Cabrera et al. 2002), but its biological activity is unknown.

The metabolites were tested for bioactivity against micro-organisms and eucalyptus leaves. Neither of the metabolites showed any detectable growth inhibition of two bacterial or seven fungal species tested (results not shown). Phytotoxicity assays were carried out at different times of year on two species of *Eucalyptus* that are susceptible to Mycosphaerella leaf spot disease, and with juvenile, mature and newly emerged leaves. Juvenile and adult *E. nitens* and *E. globulus* leaves were not sensitive to either metabolite (up to 20 μg metabolite/wound). On the other hand, newly emerged leaves of *E. globulus* were acutely sensitive when injected with 20 μg of either metabolite/wound, showing dark necrotic regions of irregular shape (in either light or dark). Lower concentrations of metabolites ($\leq$ 1 μg per wound) caused no damage to the leaf tissue. The newly emerged leaves were also acutely sensitive to acetone and other organic solvents; hence on these leaves the metabolites were suspended in water. Because of this broad sensitivity of the newly emerged leaves, it seems unlikely that compound **1a** is a pathogenicity factor required for leaf infection or colonization by *M. cryptica.* Nevertheless, both compounds represented a major part of the fungal crude extracts and a role in virulence is not ruled out. The types and biological activities of other components produced in culture by these *Mycosphaerella* species await further investigation.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. NMR data of compound **1c**.

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