PERGAMON

Phytochemistry 52 (1999) 1399–1401

PHYTOCHEMISTRY

3-*o*-Methylfunicone, a fungitoxic metabolite produced by the fungus *Penicillium pinophilum*

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Received 17 February 1999; received in revised form 17 May 1999; accepted 18 May 1999

Abstract

The production of 3-*o*-methylfunicone by *Penicillium pinophilum* is reported. The compound is (*E*)-3-methoxy-2-propenyl-5-(2′-carbomethoxy-4′-6′-dimethoxybenzoyl)-4-pyrone. It completely inhibited growth of *Rhizoctonia solani* and other species of phytopathogenic fungi cultured on water agar at a concentration of 0.1 mg ml^{-1}.

Keywords: *Penicillium pinophilum*; Fungitoxic metabolite; 3-*o*-methylfunicone

1. Introduction

In southern Italy *Penicillium pinophilum* Hedgcock is one of the most frequently encountered antagonists of *Rhizoctonia solani* Kühn, a fungal pathogen of tobacco (*Nicotiana tabacum* L.). Mechanisms of suppression of growth of the pathogen by *P. pinophilum* have been brought back to mycoparasitism and cell lysis (Alagesaboopathi, 1994), and the production of lytic enzymes, such as cellulase, β-glucosidase, xylanase and endoglucanase, by the species has been documented (Bhat & Wood, 1989; Brown, Collin & Wood, 1987). However, unlike other mycoparasites, neither penetration nor coiling of *R. solani* hyphae were observed on dual cultures in petri dishes. Therefore assays were carried out in the laboratory to evaluate if other biochemical factors, such as fungitoxic metabolites, play a role in the expression of antagonism.

In this respect we describe the isolation of 3-*o*-methylfunicone (**1**) from both culture filtrate and mycelium of *P. pinophilum*. 3-*o*-Methylfunicone is the methylated derivative of funicone, a metabolite produced by the fungus *Penicillium funiculosum* Thom (Merlini, Nasini & Selva, 1970). The latter species is included in the same taxonomic complex as *P. pinophilum*, with which it has been occasionally confused (Van Reenen-Hoekstra, Frisvad, Samson & Stolk, 1990).

2. Results and discussion

3-*o*-Methylfunicone (**1**) was isolated from both culture filtrate and fresh mycelium of 21-day-old stationary cultures of *P. pinophilum*. Its molecular formula was determined by EI mass spectroscopy ($[M]^+$ m/z 388). The presence of an aromatic ring in the molecule was suggested by an IR band at 1606 cm^{-1} while three bands at 1714, 1683 and 1637 cm^{-1} provided evidence for carbonyl groups. The IR, ^1H NMR and ^{13}C NMR spectra of **1** showed partial structures: the signals at δ 3.72 (3H, s), 3.78 (3H, s), 3.8 (3H, s), 3.86 (3H, s) were respectively attributed to methoxyl groups. The two ethylenic protons (δ 6.52 and 6.62) were in *E* configuration because of the mutual coupling constant (J = 15.0 Hz). Again the ethylenic proton at δ 6.62 was linked to the methyl group at δ 1.94 with a coupling constant of 5.0 Hz, which allowed us to suggest a

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0031-9422/99/$ - see front matter
PII: S0031-9422(99)00320-9

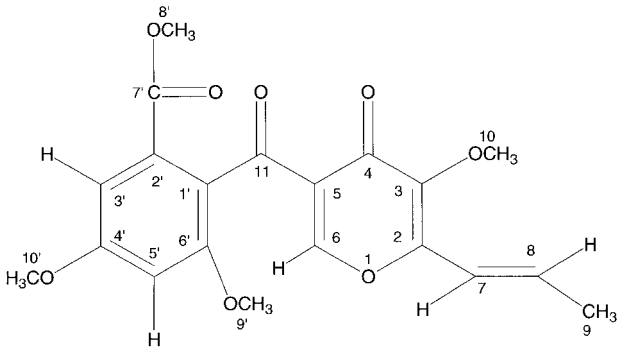
propenyl tail. The aromatic protons (δ 7.08 and 6.63) had a meta coupling constant (J = 2.0 Hz).

IR band at 1640 cm^{-1} and a signal at δ 172.4 (s) in ^{13}C NMR were in a good agreement for a γ-pyrone structure in the molecule. So, the singlet at δ_H 8.49, which appeared in δ_C at 159.1, was attributable to the methine proton in the α-position in the γ-pyrone ring.

Finally, by comparison with data reported in the literature for isofunicone (^1H NMR and ^{13}C NMR) (Kimura, Yoshinari, Shimada & Hamasaki, 1995) and funicone (^1H NMR) (Merlini et al., 1970), we were able to conclude that our molecule was a 3-*o*-methyl derivative of funicone. Since there were only some minor differences around C3 of the pyrone ring, we infer without further spectral investigations that the structure of **1** is (*E*)-3-methoxy-2-propenyl-5-(2′-carbomethoxy-4′-6′-dimethoxybenzoyl)-4-pyrone.

Another compound recovered together with **1**, identified as 2-methyl-4-hydroxy-methylbenzoate, was found to be inactive in biological assays.

Biological assays carried out with **1** evidenced complete inhibition of growth of two *R. solani* isolates grown on 2% water agar (WA) in 4-cm petri dishes at a concentration of 0.1 mg ml^{-1}, while growth was observed at lower concentrations and appeared to be less and less inhibited as concentration decreased by reason of 1:10 on end dilutions. Inhibition at the same extent was observed towards other soil-borne phytopathogenic fungi grown in the same conditions, such as *Fusarium solani* (Mart.) Appel et Wollenw., *Cylindrocladium scoparium* Morgan and *Alternaria alternata* (Fries) Keissl., which demonstrates that compound **1** has a broad antifungal activity.



3. Experimental

3.1. General

IR: $CHCl_3$, Biorad FTS 155 apparatus; NMR: AM 400 and DRX 500 MHz (^1H) and 125 MHz (^{13}C) Brooker Spectrometers with Aspecta 3000 computer and $CDCl_3$ and TMS as int. standard; EIMS: 70 eV, MS Hewlett Packard 5989B equipped with H.P. MS-G gas cromatograph 5890 series II plus and H.P. ultra Vega 1280 Vectra computer, WCOT capillary column packed with 1% SE-30; UV: Varian DMS-90 MeOH.

3.2. Isolation of Penicillium pinophilum and production of culture filtrates

P. pinophilum isolate LT4 was recovered by plating a soil suspension on developed *R. solani* colonies grown on potato–dextrose agar (PDA) amended with 200 ppm streptomycine sulphate in 9-cm petri dishes. Mycoparasitic behaviour was evident by the ability to overgrow the *R. solani* colonies. The isolate was transferred to new PDA dishes for classification and storage of pure cultures in the mycological collection of the Tobacco Experimental Institute, Scafati. Mycelial plugs from actively growing cultures were used to inoculate 150 ml potato–dextrose broth (PDB) in 500 ml Erlenmayer flasks. Stationary cultures were kept in the dark at 26 ± 2°C for 21 days.

3.3. Biological assays

Inhibition of growth of two *R. solani* isolates (RT23 and RT20) belonging to different anastomosis groups (AG 2 and AG 4 respectively), which had been recovered from diseased tobacco plants (Nicoletti & Lahoz, 1995), was evaluated on WA. Since **1** was poorly water-soluble, it was dissolved in ethanol at the concentration of 8 mg/ml before testing. A set of 3 on end dilutions in ethanol (1:10, 1:100, 1:1000) was prepared, and 0.1 ml of each dilution were added to 7.9 ml solidifying WA in 4-cm Petri dishes. Control plates received 0.1 ml ethanol in the same amount of WA. Diameter of the colonies was measured after 5-day growth in the dark at 26 ± 2°C; the assay was repeated twice. The other species of above-mentioned phytopathogenic fungi were tested under the same experimental conditions.

3.4. Extraction and isolation of *1*

Fungal cultures were filtered through 0.45 μm sterile Whatman filters. 1.5 l culture filtrate was lyophilized until reduction to 150 ml. The concentrated culture filtrate was extracted with chloroform; the extract was vacuum dryed in a rotovapor at 40–45°C and about 120 mg residue was obtained containing substantially compound **1** and 2-methyl-4-hydroxy-methylbenzoate. It was fractionated by silica-gel TLC 0.5 mm thickness, F 254, eluting with $CHCl_3$-MeOH (98:2); two bands were evidenced with R_f 0.7 (**1**) and R_f 0.2 (2-methyl-4-hydroxy-methylbenzoate). After scraping the UV visible bands, we obtained 22 mg of **1** and 5 mg of 2-

methyl-4-hydroxy-methylbenzoate. We also extracted fresh mycelium (6 g dry wt 110°, 8 h) with a mixture of Me_2CO–MeOH (1:1). The extract was taken to dryness, recovered with water (150 ml) and treated many times with $CHCl_3$, obtaining about 200 mg residue. By means of silica-gel TLC cromatography four compounds were evidenced: **1**, ergosterol, a fatty acid and 2-methyl-4-hydroxy-methylbenzoate. The residue was fractionated by silica gel CC (Merck 70-230 Mesh ASTM) with a $CHCl_3$–MeOH mixture to give **1** (8 mg) and 2-methyl-4-hydroxy-methylbenzoate (14 mg).

3.5. 3-o-Methylfunicone (*1*)

$C_{20}H_{20}O_8$ (M, 388). UV λ_{max} (MeOH) nm: 293, 249; IR ν_{max} ($CHCl_3$) cm^{-1}, 2934, 2849, 1714, 1683, 1637, 1606, 1444; ^1H NMR (400 and 500 MHz, $CDCl_3$) δ 1.94 (3H, dd, J = 6 Hz, J = 1.17 Hz, H-9), 3.72 (3H, s, H-8′), 3.78 (3H, s, H-9′), 3.8 (3H, s, H-10′), 3.86 (3H, s, H-10), 6.52 (1H, br d, J = 15 Hz, H-7), 6.62 (1H, m, H-8), 6.63 (1H, d, J = 2 Hz, H-5′), 7.08 (1H, d, J = 2 Hz, H-3′), 8.49 (1H, s, H-6); ^{13}C NMR (125 MHz, $CDCl_3$): δ 18.9 (q, C-9), 52.3 (q, C-8′), 53.6 (q, C-10′), 56.0 (q, C-10), 60.6 (q, C-9′), 103.0 (d, C-5′), 105.3 (d, C-3′), 118.5 (d, C-8), 126.1 (s, C-5), 126.2 (s, C-1′), 129.8 (s, C-2′), 134.8 (d, C-7), 144.2 (s, C-6′), 154.6 (s, C-2), 157.4 (s, C-3), 159.1 (d, C-6), 160.8 (s, C-4′), 166.4 (s, C-7′), 172.4 (s, C-4), 191.8 (s, C-11); GCEIMS, 70 eV m/z (rel. int.): 388 $[M]^+$ (54), 357 $[M–OMe]^+$ (42), 329 $[M–COOMe]^+$ (54), 223 $[M–165]^+$ (49), 192 $[M–196]^+$ (100), 63 $[M–CH{=}CH\text{-}CO]^+$ (40).

Acknowledgements

We thank Professor F. Marziano, Department of Arboriculture, Botany and Plant Pathology, Faculty of Agriculture, University of Naples, Italy, for classification of isolate LT4. Thanks are also due to Mrs. P. Salzano and Mr. R. Turco for their skilful support.

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