PERGAMON

Phytochemistry 62 (2003) 779–782

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Dihydroisocoumarins and a tetralone from *Cytospora eucalypticola*

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Received 7 August 2002; received in revised form 24 October 2002

Abstract

Two dihydroisocoumarins, 3,5-dimethyl-8-hydroxy-7-methoxy-3,4-dihydroisocoumarin and 3,5-dimethyl-8-methoxy-3,4-dihydroisocoumarin were isolated from a culture filtrate of *Cytospora eucalypticola*, together with three known dihydroisocoumarins and a tetralone derivative. Their structures were determined by spectroscopic methods. These isocoumarins are mildly antifungal, and antibacterial towards gram positive bacteria. A known compound, 5-hydroxymethylmellein, showed mild antifeedant activity towards *Spodoptera littoralis*.

Keywords: *Cytospora eucalypticola*; Isocoumarins; Polyketides; Anti-microbial activity; Anti-insect activity

1. Introduction

During a systematic screening of fungi in the United Kingdom for anti-microbial and anti-insect activities, *Cytospora eucalypticola* van der Westhinger (Coelomycete) strain SS8 was found to produce anti-fungal and anti-bacterial metabolites and to release them into the growth medium. Prior to this study this species had not been investigated chemically, although metabolites, mainly of polyketide origin, have been isolated from some *Cytospora* species (Brady et al., 2000a, b; Gurusiddaiah and Ronald, 1981; Hanson et al., 1994 and references therein; Lee et al., 1996; Ronald and Gurusiddaiah, 1980; Stevens-Miles et al., 1996). Activity-guided fractionation of the EtOAc extract of the culture filtrate of *C. eucalypticola* yielded five dihydroisocoumarins, two of which are new, and a tetralone. Their isolation, structure determination and biological activity are described in this paper.

2. Results and discussion

Fractionation of a culture filtrate of *Cytospora eucalypticola* strain SS8 by solvent extraction, Si gel CC and semi-prep. HPLC yielded **1–6** as colourless crystalline substances. The UV spectra of **1–5** were typical of the isocoumarin class of compounds (Hill, 1986). The molecular formula of **1** was determined to be $C_{12}H_{14}O_4$ by high-resolution MS. Resonances for one carbonyl, six aromatic, one methoxyl, one oxygenated methine, one methylene, and two methyl carbons were present in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum contained resonances for a hydrogen-bonded OH group (δ 11.20, 1H, *s*), a single aromatic proton (δ 6.90, 1H, *s*), a methoxyl group (δ 3.89, 3H, *s*), an aromatic methyl group (δ 2.21, 3H, *s*) and a $CH_2CH(CH_3)OR$ fragment. Analysis of HSQC and HMBC data confirmed that the compound was a 3,4-dihydroisocoumarin derivative. Long-range correlations from the exchangeable hydroxyl proton at δ 11.20 (8-OH) (hydrogen bonded to the carbonyl at C-1) were observed to the quaternary carbon bearing the methoxyl group at δ_C 146.9 (C-7) and also to δ_C 151.0 (C-8) and 108.4 (C-8a). These confirmed that the methoxyl substituent was adjacent to the hydrogen-bonded OH group on the aromatic ring. NOE connectivities detected in 1D selective NOE experiments between the aromatic proton at δ 6.90 (H-6) and the protons of the aromatic methoxyl (7-OCH_3) and methyl (5-CH_3) groups confirmed the relative positions of these groups. Other important correlations (HMBC) observed from δ 2.90 and 2.68 (4-CH_2) to δ_C 124.3 (C-5) allowed the structural relationship between the aromatic ring and aliphatic $CH_2CH(CH_3)OR$ fragment to be

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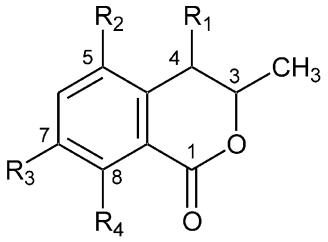
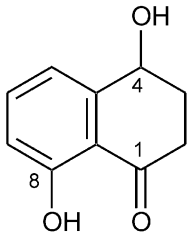
PII: S0031-9422(02)00606-4



1: $R_1 = H$, $R_2 = CH_3$, $R_3 = OCH_3$, $R_4 = OH$
2: $R_1 = R_3 = H$, $R_2 = CH_3$, $R_4 = OCH_3$
3: $R_1 = R_3 = H$, $R_2 = CH_3$, $R_4 = OH$
4: $R_1 = R_3 = H$, $R_2 = CH_2OH$, $R_4 = OH$
5: $R_1 = R_4 = OH$, $R_2 = CH_3$, $R_3 = H$



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correctly established. Compound **1** was therefore identified as 3,5-dimethyl-8-hydroxy-7-methoxy-3,4-dihydroisocoumarin, a new natural product.

Compound **2** had a molecular formula of $C_{12}H_{14}O_3$ by high-resolution MS and was identified as a 3,4-dihydroisocoumarin derivative by comparison of its ^1H NMR spectrum with that of **1**. In particular, a close similarity between the chemical shift values and coupling constants for the protons of the $CH_2CH(CH_3)OR$ fragment of **1** and **2** was noted. One of the 4-CH_2 protons at δ 2.87 gave a NOE connectivity to the protons of the aromatic methyl group at δ 2.23 (3H, *s*) thus locating the latter at C-5. Further NOE connectivities were detected between the aromatic proton at δ 7.31 (1H, *d*, $J = 8.6$ Hz) and 5-CH_3, and between the remaining *ortho*-coupled aromatic proton at δ 6.83 (1H, *d*, $J = 8.6$ Hz) and the protons of the aromatic methoxyl group (δ 3.92, 3H, *s*). This indicated that the methoxyl group was located at C-8. As expected, no downfield-shifted resonance corresponding to the exchangeable 8-OH group of **1** was present in the ^1H NMR spectrum of **2**. Compound **2** was therefore identified as 3,5-dimethyl-8-methoxy-3,4-dihydroisocoumarin. This has only been reported previously as a synthetic product formed by methylation of 3,5-dimethyl-8-hydroxy-3,4-dihydroisocoumarin (5-methylmellein, **3**) (de Alvarenga et al., 1978).

The structures of **3–6** were determined by NMR spectroscopy to be the known compounds, 3,5-dimethyl-8-hydroxy-3,4-dihydroisocoumarin (5-methylmellein, **3**), 8-hydroxy-5-hydroxymethyl-3-methyl-3,4-dihydroisocoumarin (5-hydroxymethylmellein, **4**), 4,8-dihydroxy-3,5-dimethyl-3,4-dihydroisocoumarin (4-hydroxy-5-methylmellein, **5**) and 4,8-dihydroxy-1-tetralone (isosclerone, **6**). ^1H and ^{13}C NMR resonance assignments obtained for **3** and **4** were in good agreement with published values (Okuno et al., 1986) and those for **5** were identical to (3*R*, 4*R*)-4,8-dihydroxy-3,5-dimethyl-3,4-dihydroisocoumarin (Okuno et al., 1986). ^{13}C NMR resonance assignments for **6** have not been given previously and are included in Section 3.5 for reference. This compound was isolated originally from two other ascomycetes, *Scytalidium* sp. (Findlay and Kwan, 1973) and *Sclerotinia sclerotiorum* (Morita and Aoki, 1974).

The new dihydroisocoumarin **1** was the most active of the 4 compounds tested against the fungi and bacteria (Table 1). The lack of antifungal activity of the tetralone (**6**) had previously been noted by Findlay and Kwan (1973). However, this compound exhibited mild antibacterial activity towards *Bacillus subtilis*. In the antifeedant assay against larvae of *Spodoptera littoralis* compound **4** showed activity at 100 ppm [feeding index (mean ± S.E.M.) 42.1 ± 6.42; $P < 0.05$], whereas compound **3** did not influence feeding (feeding index, -30.1 ± 20.14; $P > 0.05$). This suggests that the substituent at C-5 can influence the behavioural response of the larvae. Neither compound caused any larval mortality when applied topically to diet which suggests that although compound **4** decreased feeding over an 18-h bioassay the larvae could adapt to the compound and feed. No mortality was recorded over a 7-day bioassay period.

Isocoumarins are known to possess various biological activities, and the frequent occurrence of phytotoxins of this type in plant pathogenic fungi has been noted before (Hill, 1986). It is of particular ecological interest therefore that the phytotoxins **3–5** (Okuno et al., 1986) have also been found in *C. eucalypticola*, a pathogen of *Eucalyptus* spp.

3. Experimental

3.1. General experimental procedures

The HPLC system consisted of a Waters 600 pump with a 717 autosampler and a 996 photodiode-array detector, with a Waters Radial-Pak C_8 cartridge (8 mm i.d. × 100 mm, 4 μm) column. NMR spectra were recorded at 30 °C in $CDCl_3$ or CD_3OD on a Bruker 400 MHz instrument. Standard pulse sequences and parameters were used for the experiments and an

Table 1
Antifungal and antibacterial activities of compounds **1**, **3**, **4** and **6**

Test organisms	MIC[a] (μg/ml)					
	1	**3**	Nystatin[b]	**4**	**6**	Chloramphenicol[b]
Aspergillus niger	50	100	12.5	100	>400	–
Cladosporium herbarum	50	100	12.5	100	>400	–
Bacillus subtilis	25	200	–	200	100	3.13
Pseudomonas syringae	100	>400	–	>400	>400	3.13

[a] MIC, minimum inhibitory concentration.
[b] Positive controls.

internal reference (TMS) was used for chemical shift calibration. High resolution ESI–MS (positive mode) were obtained on a Bruker Apex II instrument with an internal calibrant.

3.2. Fermentation and isolation

The fungus was originally isolated from the bark of *Eucalyptus perriniana* cv. Spinning Gum (Myrtaceae) growing at the Royal Botanic Gardens, Kew (accession number 1972-4176). This isolate was identified by E. Punithalingam at Kew, and accessioned (KC1636). The fungus was maintained on malt extract–agar medium (Oxoid). For the metabolite production, malt extract (2%)-glucose (2%) medium (400 ml) in 2-l conical flasks was inoculated with the seed culture (3 days old, 5 ml each) in the same medium. Four flasks were shaken orbitally at 150 rpm at 25 °C for 18 days under fluorescent light (2×40 W) with a 12:12 h light–dark photoperiod. The culture filtrate was extracted with EtOAc (3×500 ml) and the combined organic phase was dried over Na_2SO_4 and concd. The residue (278 mg) was chromatographed over a Si gel column (Merck, 40–63 μm, 2 cm i.d.×14 cm) eluted stepwise with mixtures of hexane and EtOAc. Fractions eluted with 20% EtOAc gave **3** as colourless needles (6.0 mg). Fractions eluted with 40–60% EtOAc (20.8 mg) were found to be active against *Cladosporium herbarum* and further chromatographed by HPLC using 25–100% MeOH in water, linear gradient over 30 min, flow rate 2 ml/min. The compounds were eluted at t_R(min): 10.1 (**6**, 1.3 mg), 10.8 (**4**, 5.4 mg), 12.6 (**5**, 1.3 mg), 14.3 (**2**, 1.4 mg), 16.0 (**1**, 1.0 mg) and 17.9 min. (**3**, 1.8 mg).

3.3. 3,5-Dimethyl-8-hydroxy-7-methoxy-3,4-dihydroisocoumarin (*1*)

Colourless needles; UV (EtOH) λ_{max} (nm) (log ε): 257 (3.5), 338 (3.2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.20 (1H, *s*, 5-OH), 6.90 (1H, *s*, H-6), 4.67 (1H, *m*, H-3), 3.89 (3H, *s*, 7-OCH_3), 2.90 (1H, *dd*, J=16.4, 3.4 Hz, 4-CH_{2A}), 2.68 (1H, *dd*, J=16.4, 11.4 Hz, 4-CH_{2B}), 2.21 (3H, *s*, 5-CH_3), 1.54 (3H, *d*, J=6.2 Hz, 3-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.6 (C-1), 151.0 (C-8), 146.9 (C-7), 127.7 (C-4a), 124.3 (C-5), 119.9 (C-6), 108.4 (C-8a), 76.0 (C-3), 56.4 (7-O$\underline{C}H_3$), 31.5 (C-4), 20.9 (3-$\underline{C}H_3$), 18.3 (5-$\underline{C}H_3$); HRESIMS m/z: 223.0963 $[M+H]^+$ (calc. for $C_{12}H_{15}O_4$, 223.0965).

3.4. 3,5-Dimethyl-8-methoxy-3,4-dihydroisocoumarin (*2*)

Colourless needles; UV (EtOH) λ_{max} (nm) (log ε): 245 (3.5), 314 (3.4); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.31 (1H, *d*, J=8.6 Hz, H-6), 6.83 (1H, *d*, J=8.6 Hz, H-7), 4.50 (1H, *m*, H-3), 3.92 (3H, *s*, 8-OCH_3), 2.87 (1H, *dd*, J=16.4, 2.9 Hz, 4-CH_{2A}), 2.68 (1H, *dd*, J=16.4, 11.4 Hz, 4-CH_{2B}), 2.23 (3H, *s*, 5-CH_3), 1.50 (3H, *d*, J=6.3 Hz, 3-CH_3); HRESIMS m/z: 207.1015 $[M+H]^+$ (calc. for $C_{12}H_{15}O_3$, 207.1016).

3.5. 4,8-Dihydroxy-1-tetralone (isosclerone) (*6*)

Colourless solid; UV (EtOH) λ_{max} (nm) (log ε): 259 (3.5), 333 (3.1); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.50 (1H, *dd*, J=8.3, 7.6 Hz, H-6), 7.07 (1H, *dm*, J=7.6 Hz, H-5), 6.84 (1H, *dm*, J=8.3 Hz, H-7), 4.84 (1H, *dd*, J=8.0, 3.8 Hz, H-4), 2.90 (1H, *ddd*, J=17.8, 7.6, 4.7 Hz, 2-CH_{2A}), 2.66 (1H, *ddd*, J=17.8, 9.1, 4.8 Hz, 2-CH_{2B}), 2.30 (1H, *m*, 3-CH_{2A}), 2.10 (1H, *m*, 3-CH_{2B}); ^{13}C NMR (CD_3OD, 100 MHz) δ 206.3 (C-1), 163.7 (C-8), 148.6 (C-4a), 137.9 (C-6), 118.8 (C-5), 117.6 (C-7), 116.5 (C-8a), 68.3 (C-4), 36.1 (C-2), 32.6 (C-3).

3.6. Antifungal assay

The antifungal activity was traced by TLC-autobiography (Homans and Fuchs, 1970) during the isolation, using the conidia of *Cladosporium herbarum* (IMI300461) suspended in malt-extract (2%) solution. The activity of isolated compounds was tested against *C. herbarum* and *Aspergillus niger* (IMI149007) using a 1/2-microdilution method in 96-well plates in the final concentration range of 400–3.13 μg/ml, in malt extract (2%) solution. Nystatin was used as a positive control

and tested in the same concentration range as the compounds. The test was performed in four replicates per concentration per test fungus. The seeded plates were incubated in the dark at 25 °C for 72 h and the concentrations that gave complete growth inhibition in all four replicates were considered as the active concentration.

3.7. Antibacterial assay

The isolated compounds were tested against *Bacillus subtilis* (IMI347329) and *Pseudomonas syringae* (IMI347448) in a medium containing peptone (1%), NaCl (0.5%) and yeast extract (0.3%), and the test compounds (400–3.13 μg/ml), in four replicates as above. Chloramphenicol was used as the positive control. Plates were incubated overnight in the dark at 37 °C on a rotary shaker (120 rpm). Following this 20 μl of *p*-iodonitrotetrazolium violet soln (500 μg/ml in 20% EtOH) were added and incubated for a further 30 min in the same condition. The colour development was observed visually, and the active concentration determined as above.

3.8. Antifeedant assay

Compounds **3** and **4** were tested for antifeedant activity against final stadium larvae of *Spodoptera littoralis* in a choice bioassay using glass-fibre discs (Simmonds et al., 1990). The amount eaten of the control (C) and compound-treated (T) discs after an 18 h period was used to calculate the feeding index $[(C-T)/(C+T)]\%$. Data were analysed using the Wilcoxon matched-pairs test. Each compound was tested against 10 larvae.

3.9. Toxicity

Larvae 8–12 h into the second stadium were placed on their rearing diet (1.5 ml/well) in wells of a Bioserve bioassay tray (C-D International Inc., Italy; Simmonds and Stevenson, 2001). The diet had been treated topically with 100 μl of a 100 ppm solution of either compound **3** or **4**. Untreated diet was used as a control. Larval mortality was recorded after 72 h and 7 days. Each compound was tested against 25 larvae.

Acknowledgements

We thank S. Sivakumaran for isolation of the fungus and E. Punithalingam for identification. The microbial test organisms used in the study were a generous gift from CABI Bioscience, Egham, Surrey, UK. Research with *Spodoptera littoralis* was covered by a DEFRA license.

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