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IDENTIFICATION OF THREE HYDROXYFLAVAN PHYTOALEXINS FROM DAFFODIL BULBS

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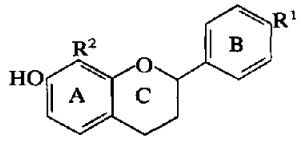
Key Word Index—*Narcissus pseudonarcissus*; Amaryllidaceae; daffodil bulbs; phytoalexins; hydroxyflavans; *Botrytis cinerea*.

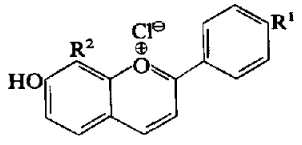
Abstract—Three phytoalexins were isolated from daffodil bulb scales inoculated with *Botrytis cinerea* and identified as 7-hydroxyflavan, 7,4′-dihydroxyflavan and 7,4′-dihydroxy-8-methylflavan. The structures of the phytoalexins were confirmed by total synthesis.

INTRODUCTION

Inoculation of *Narcissus pseudonarcissus* L. bulb scales with suspensions of conidia of *Botrytis cinerea* Pers. ex Fr. led to the formation of brown lesions confined to the tissue beneath inoculum droplets. Growth of *B. cinerea* was restricted within these limited lesions (O'Neill, T. M., unpublished results). Examination of Et_2O extracts of infected tissue by TLC plate bioassays with *Cladosporium herbarum* Pers. ex Link. revealed the presence within lesions of several antifungal compounds (phytoalexins) which were absent from fresh or frozen and thawed healthy tissues. Here we report the isolation of three of the phytoalexins, their identification as closely related hydroxyflavans (**1–3**) and their total syntheses.



1 $R^1 = R^2 = H$
2 $R^1 = OH$; $R^2 = H$
3 $R^1 = OH$; $R^2 = Me$



4 $R^1 = R^2 = H$
5 $R^1 = OH$; $R^2 = H$
6 $R^1 = OH$: $R^2 = Me$

RESULTS AND DISCUSSION

Three of the phytoalexins detected by TLC plate bioassays gave positive tests for phenols, being visualized on chromatograms by their yellow colour after spraying with diazotized *p*-nitroaniline [1]. Milligram quantities of these fungitoxic phenolics were extracted from bulb scales bearing limited lesions produced 5 days after inoculation with *B. cinerea*. The phytoalexins were isolated by gel filtration, TLC and HPLC, and identified as the hydroxyflavans (**1–3**) from the following evidence.

High resolution mass spectrometry gave the following molecular weights and formulae: (**1**) M^+ 226.0989, $C_{15}H_{14}O_2$; (**2**) M^+ 242.0946, $C_{15}H_{14}O_3$ and (**3**) M^+ 256.1098, $C_{16}H_{16}O_3$. Molecular ions were present as base peaks in the mass spectra of all three compounds. Compound **3** was most readily characterized as (−)-7,4′-dihydroxy-8-methylflavan, $[\alpha]_D^{25}$ −36.4° ($c = 0.33$, $CHCl_3$), by comparison of its physicochemical properties with those of the reported (−)-4′-hydroxy-7-methoxy-8-methylflavan [2]. Prominent fragment ions in the mass spectrum of **3** were at *m/e* 137 ($C_8H_9O_2$, 85%) and 120 (C_8H_8O, 96%) arising from rings A and B, respectively, by retro-Diels–Alder (RDA) cleavage. The UV spectrum of **3** exhibited maxima at 225, 279 and 283 sh nm (EtOH, log ε 4.27, 3.60, 3.59) differing only slightly from **2** λ_{max} 226, 284 and 290 sh nm (log ε 4.27, 3.67, 3.53) and **1** λ_{max} 211, 285 and 290 nm (log ε 4.41, 3.49, 3.43). The 90 MHz NMR spectrum of **3** in methanol-d_4 showed six aromatic protons, four as an A_2B_2 quartet centred at δ 6.80 and 7.26 and two as an AB quartet centred at 6.34 and 6.70. A singlet methyl signal at δ 2.01 (2.51 in pyridine-d_5) was assigned to the methyl substituent in ring A. In pyridine-d_5 solution the protons in the heterocyclic ring were observed as a one-proton quartet at δ 5.10 ($J = 8$ and 4 Hz) assigned to the C-2 proton, a two-proton multiplet at 2.1 (C-3 protons) and a two-proton multiplet at 2.8 (C-4 protons).

Compound **2** lacked the aromatic methyl substituent in ring A. Its NMR spectrum was very similar to **3** apart from changes associated with replacement of this methyl group by a proton. The mass spectrum of **2**

showed RDA fragments from ring A at *m/e* 123 (56%) and from ring B at *m/e* 120 (85%).

Compound **1** lacked the hydroxyl substituent in ring B but was otherwise identical to **2**. The difference was apparent in the aromatic region of the NMR spectrum and from the mass spectrum which gave a prominent RDA fragment at *m/e* 104 (46%), from ring B.

In addition to showing fungitoxic activity in TLC plate bioassays, samples of the compounds purified by HPLC were also shown to be active in tests against germinated spores of *B. cinerea* in liquid culture; ED_{50} values against germ tube growth of 22, 65 and 32 μg/ml being recorded for **1–3**, respectively.

Since only small quantities of the pure natural compounds were available, synthetic racemates of **1–3** were prepared for structural confirmation. Flavylium salts (**4–6**) were prepared by Robinson condensation of the appropriate hydroxybenzaldehydes with the corresponding acetophenones. Catalytic hydrogenation of the flavylium salts (**4–6**) gave the racemic flavans (**1–3**). The chromatographic and spectral properties of the natural and synthetic compounds were identical. The fungitoxicity of the synthetic racemic derivatives was confirmed by TLC plate bioassays with *C. herbarum*.

Flavans unsubstituted in the pyran ring are not common natural products, having been reported only as minor constituents in the roots of *Dianella revoluta* R. Br. and *Stypandra grandis* C. T. White (Xanthorrhoeceae) [2] and in *Xanthorrhea* resins [3]. Although several fungitoxic flavonoids (including some isoflavans) have been described as phytoalexins [4, 5], this is the first report of flavans possessing antifungal activity and accumulating in response to fungal infection. Phytoalexins have been characterized from only three other monocotyledonous species, *Orchis militaris* L. [6] *Loroglossum hircinum* (L.) Rich. [7] and *Oryza sativa* L. [8].

EXPERIMENTAL

Preparation of bulb scales tissue. Fleshy scales dissected from daffodil bulbs, cv Golden Harvest, and with their adaxial epidermis removed, were placed stripped-surface uppermost on moist tissue paper in plastic sandwich boxes. Droplets (20 μl) of a suspension of *B. cinerea* conidia (10^5/ml) in sterile distilled H_2O were pipetted onto the stripped surface. After incubation in closed boxes in the dark at 18° for 5 days, tissue was scraped from inoculum sites with a scalpel blade and stored at −20°. In order to examine for the presence of preformed antifungal compounds released after tissue damage, stripped scales were frozen at −20° and then allowed to thaw before extraction without inoculation. About 5 g of tissue inoculated with *B. cinerea* or H_2O, and frozen/thawed scales were collected for the detection of fungitoxic compounds.

Bioassays. TLC plate bioassays with *C. herbarum* and assays against sporelings of *B. cinerea* were carried out as previously described [9] except that for the latter the flavans were added to nutrient solution in DMSO (final concn 2% v/v) giving a range of phytoalexin concns from 5 to 500 μg/ml. DMSO was also added to phytoalexin free controls.

Extraction of phytoalexins. Excised tissue was homogenized (Sorval omnimixer) in redistilled Et_2O (at least 10 ml Et_2O/g.fr.wt tissue) and left to soak at 4° for 3 hr. The ethereal supernatant was collected and the homogenate washed twice with Et_2O. Bulked Et_2O extracts were dried, filtered, and evapd *in vacuo* at 30°. Phytoalexins were detected by TLC plate bioassays on chromatograms of extracts from 0.1–1.0 g fr. wt of tissue streaked over 1.5 cm origins on pre-coated TLC plates (Merck Si gel 5715) and developed in a range of solvents [9].

Isolation of phytoalexins. The Et_2O extract from 60 g fr. wt infected tissue was applied to a 70 × 2.5 cm column of Sephadex LH20 in MeOH. Following elution with MeOH at 2 ml/min, flavans **1, 2** and **3** were recovered in 20 ml fractions Nos. 25–27, 29–31 and 28–31, respectively. The phytoalexins were further purified from selected fractions by PLC on pre-coated plates (as above) developed in Et_2O–petrol (bp 40–60°), 2:1 and recognized as quenching bands under UV radiation (254 nm) at R_f 0.31–0.40 (**1**), 0.24–0.31 (**2**) and 0.47–0.56 (**3**). After elution with MeOH and Et_2O yields of *ca* 6 mg of each phytoalexin were recovered. Further purification was by HPLC on an ODS Hypersil (5 μm) column (20 × 0.8 cm) with UV detection [10]. Aliquots of 2 mg of the phytoalexins in 30 μl MeOH were injected and isocratic elution was with 35% MeOH in 5% HCOOH at 5 ml/min. Retention times for **1–3** were 64–72, 16–20 and 22–30 min.

Synthesis of 7,4'-dihydroxy-8-methylflavan (**3**). 2,4-Dihydroxy-3-methylbenzaldehyde **7** was synthesized by formylation of 1,6-dihydroxytoluene with dimethylformamide and phosphoryl chloride [11]. The product, recrystallized from H_2O, had mp 150–153°, MS M^+ 152 (base peak), NMR ($CDCl_3$) δ 9.65 CHO. 4-Hydroxyacetophenone (7.16 g) and **7** (8.00 g) were dissolved at 1° in EtOAc (85 ml) saturated with HCl gas and kept for 3 days at 1°. The mixture was then poured into dry Et_2O (300 ml) and the crystalline flavylium salt (**6**) was collected (4.3 g). NMR (TFA-*d*): δ 8.95 and 8.14 AB quartet, ring C protons; 8.35 and 7.29 A_2B_2 quartet, ring B protons; 7.96 and 7.60 AB quartet, ring A protons; 2.71 singlet Me group. Flavylium salt (**6**) (0.2 g) dissolved in HOAc (15 ml) was hydrogenated over reduced PtO_2 catalyst (50 mg) allowing a hydrogen uptake equivalent to 2 mol. After work-up followed by column chromatography (SiO_2 gel, $CHCl_3$) and PLC (Et_2O–petrol, 2:1), the product **3** (79 mg) was obtained as a colourless glass.

Synthesis of 7,4'-dihydroxyflavan (**2**). 4-Hydroxyacetophenone (1.36 g) and 2,4-dihydroxybenzaldehyde (1.38 g) were dissolved at 1° in EtOAc (24 ml) saturated with HCl gas. After 3 days at 1°, the flavylium salt **5** was collected (1.1 g). NMR (DMSO-d_6): δ 9.20 and 8.44 AB quartet, ring C protons; 8.43 and 7.16 A_2B_2 quartet, ring B protons; 7.50 *dd* J = 8.5, 2.5 Hz, 7.65 *d* J = 2.5 Hz and 8.20 *d* J = 8.5 Hz, ring A protons. Catalytic hydrogenation of **5** as described above gave **2** (179 mg).

Synthesis of 7-hydroxyflavan (**1**). Acetophenone (1.20 g) and 2,4-dihydroxybenzaldehyde (1.38 g) were condensed in HCl saturated EtOAc at 1°. The flavylium salt **4** was collected (2.05 g) after 3 days. NMR (TFA-*d*): δ 9.14 and 8.41 AB quartet, ring C protons; 7.64 *dd* J = 8.5, 2.5 Hz, 7.71 *d* J = 2.5 Hz and 8.19 *d* J = 8.5 Hz, ring A protons; 7.8 and 8.37 *m*'s 5H ring B protons. Catalytic hydrogenation of **4** (505 mg) as described above gave (±)-7-hydroxyflavan (363 mg) as an oil.

The purified compounds had the following properties:

7,4'-Dihydroxy-8-methylflavan, mp 132–135°; MS *m/e* (rel. int.): 257 (16) 256 (M^+, 100), 150 (26), 149 (10) 137 (85), 133 (13), 120 (96), 119 (10), 107 (25). NMR (Py-d_5): δ 7.53 and 7.24 4H A_2B_2 quartet ring B protons; 6.96 and 6.84 2H AB quartet ring A protons; 5.10 1H *dd* J = 8, 4 Hz

C-2 proton; 4.90 2H broad —OH; 2.8 2H *m* C-4 protons; 2.51 3H *s* Me; 2.1 2H *m* C-3 protons. NMR (MeOH-d_4): δ 7.26 and 6.80 4H A_2B_2 quartet ring B protons; 6.70 and 6.34 2H AB quartet ring A protons; 4.95 1H *dd* $J = 8$, 4 Hz C-2 proton; 2.7 2H *m* C-4 protons; 2.01 3H *s* Me; 2.0 2H *m* C-3 protons.

7,4′-Dihydroxyflavan, mp 197–198°; MS *m/e* (rel. int.): 243 (17), 242 (100), 136 (23), 135 (11), 133 (15), 123 (56), 121 (14), 120 (85), 119 (15), 107 (30). NMR ($CHCl_3$-*d*–MeOH-d_4): δ 7.24 and 6.83 4H A_2B_2 quartet ring B protons; 6.35 *d* $J = 2.5$ Hz, 6.39 *dd* $J = 8.5$ and 2.5 Hz and 6.89 *d* $J = 8.5$ Hz, ring A protons; 4.91 1H *dd* $J = 8$, 4 Hz C-2 proton; 2.8 2H *m* C-4 protons; 2.1 2H *m* C-3 protons.

7-Hydroxyflavan, MS *m/e* (rel. int.): 227 (17), 226 (100), 135 (23), 123 (13), 122 (10), 117 (16), 104 (56), 103 (13), 91 (26). NMR ($CHCl_3$-*d*): δ 7.37 5H *s* ring B protons; 6.40 *m* 2H and 6.94 *d* $J = 8.5$ Hz, ring A protons; 5.05 1H *dd* $J = 8$, 4 Hz C-2 proton; 2.7 2H *m* C-4 protons; 2.1 2H *m* C-3 protons.

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