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ISOLATION OF A NEW ISOFLAVAN PHYTOALEXIN FROM TWO *LOTUS* SPECIES

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Key Word Index *Lotus* spp.; Leguminosae; isoflavonoids; isoflavans; phytoalexins; antifungal activity; synthesis.

Abstract—A phytoalexin isolated from the fungus-inoculated leaflets of *Lotus angustissimus* and *L. edulis* has been characterised as 5,7-dimethoxy-2′,4′-dihydroxyisoflavan (lotisoflavan). The total synthesis of lotisoflavan is described.

INTRODUCTION

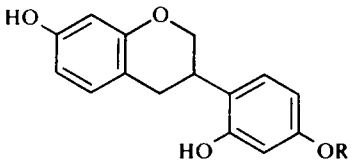
Isoflavan phytoalexins have been found to accumulate in the excised leaflets of various *Lotus* (Leguminosae–Papilionoideae: tribe Loteae) species following treatment with conidial suspensions of the fungal-elicitor, *Helminthosporium carbonum* [1–3]. These compounds include demethylvestitol (**1**) and vestitol (**2**) from *L. corniculatus* and *L. uliginosus* as well as the recently discovered fungitoxin 5-methoxyvestitol (**3**) from *L. hispidus* [2, 3]. Further investigation of the genus *Lotus* has now revealed that *H. carbonum*-inoculated leaflets of *L. angustissimus* (Section *Lotus*) and *L. edulis* (Section *Krokeria*) produce a new 5-oxygenated isoflavan which we have named lotisoflavan. This paper presents evidence to show that lotisoflavan is 5,7-dimethoxy-2′,4′-dihydroxyisoflavan (**4**).

RESULTS AND DISCUSSION

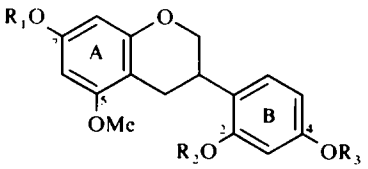
The MS of lotisoflavan (**4**)—isolated as a major component from the leaf diffusates of both *L. angustissimus* and *L. edulis*—displayed a typically isoflavan-like fragmentation pattern with M^+ 302 and major ions at *m/e* 167 (indicative of a dimethoxylated aromatic [A] ring) and 136/123 (dihydroxylated aromatic [B] ring) [4, 5]. The 5,7,2′,4′-oxygenation pattern of **4** was established by methylation (CH_2N_2) to give a non-phenolic product (M^+ 330) indistinguishable (UV, MS, TLC) from the 7,2′-di-*O*-methyl ether (**5**) of 5-methoxyvestitol (**3**). Together with the MS data, this result strongly suggested that lotisoflavan was 5,7-dimethoxy-2′,4′-dihydroxyisoflavan (**4**), a view fully confirmed by subsequent comparison (UV, MS, TLC) with synthetic material (see Experimental).

Fungus-induced diffusates from *L. angustissimus* were found to contain substantial quantities of lotisoflavan (61–64 μg/ml based on $\log \varepsilon = 3.63$ at 281 nm for **2** [1]) but only small amounts of two additional isoflavans, namely demethylvestitol **1** (trace to 3 μg/ml) and vestitol **2** (6–16 μg/ml). Isoflavan levels in tissues beneath the inoculum droplets were (a) demethylvestitol: trace only, as judged by TLC comparison with authentic material, (b) vestitol: 93–114 μg/g fr. wt and (c) lotisoflavan: 357–380 μg/g. Compounds **1**, **2** and **4** were not produced by leaflets treated with de-ionised H_2O. An intense inhibition zone corresponding to the position of lotisoflavan was obtained when extracts of *H. carbonum*-induced diffusates were tested (TLC plate bioassay) against *Cladosporium herbarum* [6].

Our studies on the genus *Lotus* are continuing and have recently revealed that lotisoflavan (35–53 μg/ml) is also the principal leaf phytoalexin of *L. edulis*, a species closely related to both *L. angustissimus* and *L. hispidus* [7]. Apart from lotisoflavan, *L. edulis* diffusates contain vestitol (8–18 μg/ml) and 5-methoxyvestitol (7–12 μg/ml) but no detectable demethylvestitol. A fifth *Lotus* phytoalexin, sativan (7-hydroxy-2′,4′-dimethoxyisoflavan), is apparently not produced by either *L. angustissimus* or *L. edulis*.



1 R = H
2 R = Me



3 $R_1 = R_2 = H$; $R_3 = Me$
4 $R_1 = Me$; $R_2 = R_3 = H$
5 $R_1 = R_2 = R_3 = Me$

EXPERIMENTAL

Seeds of *Lotus angustissimus* L. and *L. edulis* L. were obtained from The Botanic Gardens of Caen (France) and Genova (Italy) respectively. Voucher specimens of these species have been deposited in the Botany Herbarium, University of Reading.

Isolation of compounds **1–4**. (a) Leaf diffusates. Extracts (EtOAc) were chromatographed (Si gel TLC; $CHCl_3$–MeOH, 50:1) [8] to give **1** (R_f 0.04), **2** + **3** (R_f 0.22) and **4** (R_f 0.15). After elution (EtOH), isoflavans **1** and **2** + **3** were separated and/or purified as described elsewhere [2, 3]. Lotisoflavan (**4**) was additionally chromatographed in *n*-pentane–Et_2O–HOAc (PEA), 75:25:3, × 3. Compounds **1–3** were identified by direct comparison (UV/TLC) with authentic material. (b) Leaf tissues (*L. angustissimus* only). Extracts (EtOH) of excised, inoculated leaf tissues [2] were chromatographed ($CHCl_3$–MeOH, 10:1) and zones at R_f 0.65 (**2**), 0.57 (**4**) and 0.32 (**1**) removed and eluted. These eluates were reduced to dryness (*in vacuo*, 40°) and the presence of **1** confirmed by TLC comparison (as a spot in 2 solvent systems) with demethylvestitol previously isolated from *L. uliginosus* [2]. Pure samples of **2** and **4** were obtained after further TLC in PEA, 75:25:3, × 3 (**2**) or 75:25:6 (**4**, R_f 0.33).

5,7-Dimethoxy-2′,4′-dihydroxyisoflavan **4** (*lotisoflavan*). Diazotised *p*-nitroaniline, orange; Gibbs reagent, purple/blue. λ_{max}^{EtOH} nm 212 (100%), 230 sh (47%), 281 (11%), 287 sh (8%); $\lambda_{max}^{EtOH+NaOH}$ nm 213, 243 sh, 280 sh, 294. MS *m/e* (rel. int.): 303 (3), 302 (M^+; 20), 179 (5), 168 (12), 167 (100), 166 (6), 151 (3), 138 (7), 137 (9), 136 (12), 135 (6), 123 (6). DiMe ether **5** (CH_2N_2) (R_f 0.49, $CHCl_3$–CCl_4, 1:1) UV and MS as lit. [3]. Diacetate (Py–Ac_2O) (R_f 0.70, $CHCl_3$) λ_{max}^{EtOH} nm 214, 230 sh, 267, 271. MS *m/e* (rel. int.): 387 (5), 386 (M^+; 20), 344 (6), 343 (9), 302 (2), 301 (6), 179 (10), 178 (10), 168 (10), 167 (90), 166 (100), 138 (26), 137 (12), 136 (32), 135 (10), 123 (24), 107 (11).

Synthesis of (±)*-lotisoflavan*. 2-Hydroxy-4,6-dimethoxyacetophenone (0.4 g) and 2,4-dibenzyloxybenzaldehyde (0.65 g) were dissolved in warm EtOH (15 ml) and then stirred overnight (room temp.) with KOH (2 g) in H_2O (2 ml). After filtering, the filtrate was diluted (H_2O), shaken with EtOAc (× 2) and the combined extracts washed (H_2O) before being evapd. The residue was crystallized from MeOH to give 2,4-dibenzyloxy-2′-hydroxy-4′,6′-dimethoxychalcone (0.52 g), mp 105–106°. ^{1}H NMR (60 MHz; $CDCl_3$; TMS): δ 3.68, 3.82 (2 × 3H, singlets, OMe), 5.07, 5.14 (2 × 2H, singlets, OCH_2Ph), 5.88, 6.08 (2 × 1H, doublets, J = 2 Hz, H-3′, -5′), 6.60 (1H, *dd*, J = 9, 2 Hz, H-5), 6.60 (1H, *d*, J = 2 Hz, H-3), 7.40 (10H, *s*, Ph), 7.58 (1H, *d*, J = 9 Hz, H-6), 7.92 (1H, *d*, J = 16 Hz, H-α), 8.17 (1H, *d*, J = 16 Hz, H-β), 14.6 (1H, s, OH). MS *m/e* (rel. int.): 496 (M^+; 1), 406 (1), 405 (4), 389 (1), 200 (3), 181 (10), 180 (2), 154 (3), 152 (1), 125 (2), 108 (8), 107 (6), 92 (8), 91 (100). The above chalcone (0.45 g) was acetylated [Py (10 ml)·Ac_2O (1 ml), room temp., overnight] and the reaction mixture poured into ice-H_2O. The product was collected by filtration, dissolved in EtOAc and the resulting soln washed (dil. HCl followed by H_2O) and then evapd to dryness. This acetate (without further purification) was dissolved in MeOH (50 ml) and stirred overnight (room temp.) with $Tl(NO_3)_3 \cdot 3H_2O$ (0.45 g). Solid KOH (0.5 g) was then added and stirring continued for a further 1 hr. After neutralization (conc HCl), the mixture was treated with 10% HCl (10 ml) and heated under reflux for 1.5 hr. At this point, the reaction mixture was concd, diluted (H_2O) and finally extracted with EtOAc (× 2). The extracts were filtered, reduced to dryness and the residue crystallized from MeOH to give 5,7-dimethoxy-2′,4′-dibenzyloxyisoflavone (0.37 g), mp 178–180°. ^{1}H NMR (60 MHz; $CDCl_3$; TMS): δ 3.84, 3.90 (2 × 3H, singlets, OMe), 5.00, 5.03 (2 × 2H, singlets, OCH_2Ph), 6.33 (1H, *d*, J = 2 Hz, H-6), 6.38 (1H, *d*, J = 2 Hz, H-8), 6.57 (1H, *dd*, J = 8, 2 Hz, H-5′), 6.68 (1H, *d*, J = 2 Hz, H-3′), 7.20–7.40 (11H, *m*, 2 × Ph, H-6′), 7.73 (1H, *s*, H-2). MS *m/e* (rel. int.): 495 (2), 494 (M^+; 7), 404 (3), 403 (11), 376 (1), 375 (6), 181 (2), 137 (1), 92 (8), 91 (100). This isoflavone (0.2 g) in glacial HOAc (20 ml) was hydrogenated (room temp., overnight) using 10% Pd/C (50 mg) as the catalyst. Work up and Si gel TLC (*n*-hexane–Me_2CO, 2:1) gave (±)-5,7-dimethoxy-2′,4′-dihydroxyisoflavan (lotisoflavan) which was recrystallized from EtOH–H_2O (65 mg), mp 135–137°. ^{1}H NMR (60 MHz; $CDCl_3$ + DMSO-d_6; TMS): δ 2.70 (2H, *m*, H-4), 3.10–3.50 (1H, *m*, H-3), 3.75, 3.78 (2 × 3H, singlets, OMe), 3.92 (1H, *t*, J = 9 Hz, H-2ax), 4.28 (1H, *dd*, J = 9, 3 Hz, H-2eq), 6.03 (2H, *s*, H-6, -8), 6.27 (1H, *dd*, J = 8, 2 Hz, H-5′), 6.42 (1H, *d*, J = 2 Hz, H-3′), 6.86 (1H, *d*, J = 8 Hz, H-6′), 8.78, 8.96 (2 × 1H, singlets, OH). MS and UV data as given for the natural product. Synthetic and *Lotus*-derived lotisoflavan were chromatographically indistinguishable in 5 TLC solvent systems.

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