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TWO PHYTOALEXINS FROM SUGARBEET (*BETA VULGARIS*) LEAVES

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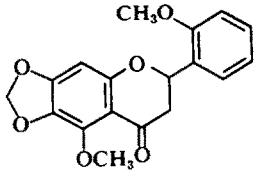
Abstract—Two phytoalexins have been isolated from the leaves of sugarbeet infected with *Cercospora beticola* and their structures have been shown to be 2',5-dimethoxy-6,7-methylenedioxyflavanone (**1**) and 2'-hydroxy-5-methoxy-6,7-methylenedioxyisoflavone (**2**).

Cercospora beticola is responsible for sugarbeet leaf spot disease. We now report the identification of two phytoalexins isolated from the leaves of sugarbeets infected with *C. beticola*. Phytoalexins are low molecular weight compounds formed *de novo* in higher plants following their infection by a fungal organism.[1]



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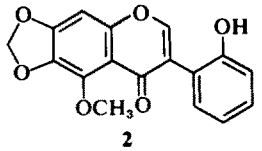

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The infected sugarbeet leaves were dehydrated and then extracted with dry acetone. Column and preparative plate chromatography allowed isolation of the pure components,[2] **1** and **2**.

Phytoalexins **1** and **2** were obtained as crystalline solids, m.p. 187–195° and 147–150°, respectively. High resolution mass spectrometry established the molecular formula of **1** as $C_{18}H_{16}O_6$ and of **2** as $C_{17}H_{12}O_6$. The appearance in the IR of a carbonyl absorption at 1670 cm^{-1} and in the UV of three maxima, and the presence in the NMR of two multiplets, one at 2·84 δ (2H) and the other at 5·68 δ (1H), indicated[3] that **1** had a flavanone structure. On the other hand, **2** had only two maxima in the UV. The multiplets found in the NMR of **1** did not appear in **2** but instead a singlet at 7·85 δ (1H) was present. The carbonyl absorption in the IR of **2** appeared at 1635 cm^{-1}. These data indicated[3] that **2** was an isoflavone.

The base peak in the mass spectrum of **1** was due to a fragment ion $C_9H_6O_5$, apparently from loss of a $C_9H_{10}O$ fragment. Phytoalexin **2** also yielded the same fragment ($C_9H_6O_5$) with loss of C_8H_6O. Both flavanones and isoflavones readily fragment *via* a retro Diels-Alder pathway which leaves the A ring intact, bearing the positive charge.[4] Two three-proton singlets were observed in the methoxy region of **1**, but only one appeared in the NMR of **2**. Also, the NMR of **1** showed a singlet at 5·93 δ (2H) typical of a methylenedioxy group and this was evident in the NMR of **2** also. Strong absorptions between 938–919 cm^{-1} in the IR of both compounds supported this assignment.[5] In addition, **2** showed a base shift in the UV typical of a phenol. Taken *in toto*, these data indicated part structures **3** and **4** for **1** and **2**, respectively.

More detailed analysis of the spectral data allowed us to postulate the placement of the substituents on the aromatic rings. The NMR of **1** showed a singlet at 4·07 δ (3H) typical of a methoxy group *peri* to a carbonyl function and *ortho* to an oxygenated substituent,[6] and this was evident in the NMR of **2** as well. This indicated the part structure **5** for the A rings of **1** and **2**.

Three other natural flavonoids have the same A ring arrangement: tlatlancuayin[7] **6** isolated from *Iresine celosioides*, irisolone[8] **7** isolated from *Iris nepalansis*, and meliternatin[9] **8** isolated from *Melicopa ternata*. By comparing the NMR chemical shifts of the aromatic proton and the protons of each substituent on the A ring, the A rings of **2** and **6**[7] were shown to be identical.

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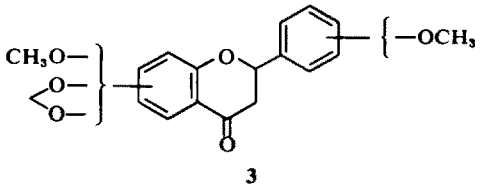

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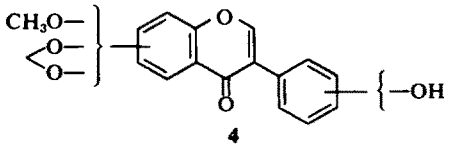

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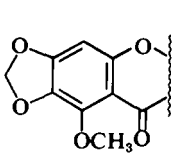

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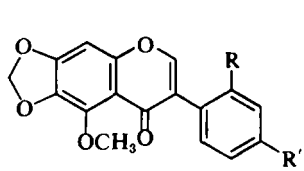
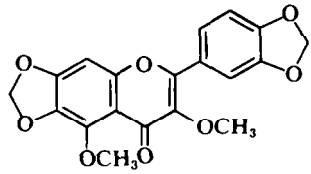


	R	R′
6	OCH_3	H
7	H	OH



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The position of the methoxy group in the **B** ring of **1** and the hydroxy group in the **B** ring of **2**, was readily determined by NMR. First-order analysis of the splitting patterns of the aromatic protons on the **B** rings was characteristic of *ortho*-disubstituted benzenes,[10] and not of *meta*- or *para*-disubstituted benzenes. Identical splitting patterns of the **B** ring aromatic protons were obtained in the NMR of **2** and **6**.[7] Thus, structures **1** (2′,5-dimethoxy-6,7-methylenedioxyflavanone) and **2** (2′-hydroxy-5-methoxy-6,7-methylenedioxyisoflavone) were assigned to the $C_{18}H_{16}O_6$ and $C_{17}H_{12}O_6$ metabolites, respectively.

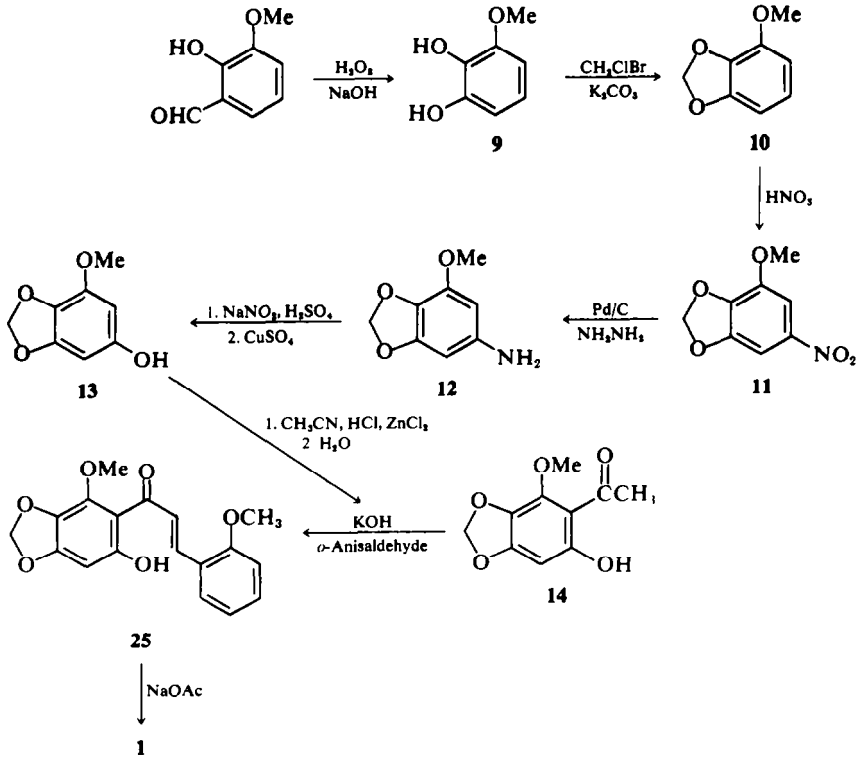
In order to assure the structure assignment, we synthesized the flavanone **1**. This was accomplished according to Scheme 1 with the details of the synthesis given in the Experimental Section.

Compound **1** showed weak anti-fungal properties against *C. beticola* and *Monilinia fructicola*. **2** was highly active against these two organisms. Detailed data on the biological activity of these phytoalexins will be published elsewhere.

EXPERIMENTAL

NMR spectra were recorded in $CDCl_3$ at 100 MHz and 220 MHz with TMS as internal reference. UV data are in EtOH solvent and IR spectra in KBr. All m.ps are corrected.

Isolation of phytoalexins **1** *and* **2**. Dried, finely ground, infected sugarbeet leaves were extracted with dry acetone.[1] The residue obtained on evaporation was chromatographed on a Sephadex LH 20 column with a 50:50 $CHCl_3$/MeOH solvent system. Further purification of the fractions containing **1** and **2** was by preparative silica-gel coated TLC plates. Phytoalexin **1** was obtained as a crystalline, white solid, m.p. 187–195° (decomp); λ_{max} 242 (ε 6840), 279 (ε 4790) and 335 nm (ε 2160); γ_{max} 1670, 1620 and 936 cm^{-1}; NMR: δ 7·56 (1H, d, J 8 Hz), 7·32 (1H, t, J 8 Hz), 7·02 (1H, t, J 8 Hz), 6·89 (1H, d, J 8 Hz), 6·27 (1H, s, C-8 H), 5·93 (2H, s, methylenedioxy), 5·68



(1H, m, C-2 H), 4·07 (3H, s, C-5 OMe), 3·82 (3H, s, C-2' OMe) and 2·84 (2H, m, C-3 H's); MS: *m/e* 328.0930 ($C_{18}H_{16}O_6$, 69%, M^+) and *m/e* 194.0217 ($C_9H_6O_5$, 100%, M^+—$C_9H_{10}O$). Phytoalexin **2** was obtained as a crystalline, white solid, m.p. 147–150°; λ_{max} 252 (ε 20,120), 285 (ε 11,920; sh) and 317 nm (ε 7,230); alkaline, λ_{max} 305 nm; γ_{max} 1635, 1580, 929 and 921 cm^{-1}; NMR: δ 7·85 (1H, s, C-2 H), 7·32 (2H, d, J 8 Hz), 6·93 (2H, t, J 8 Hz), 6·64 (1H, s, C-8 H), 6·08 (2H, s, methylenedioxy) and 4·09 (3H, s, C-5 OMe); MS: *m/e* 312.0707 ($C_{17}H_{12}O_6$, 100%, M^+) and *m/e* 194.0240 ($C_9H_6O_5$, 61%, M^+—C_8H_6O).

3-Methoxycatechol **9**. In a 2 l three-necked flask, fitted with a N_2 gas inlet tube, a dropping funnel, and a reflux condenser, were placed 182·4 g of 2-hydroxy-3-methoxybenzaldehyde and 600 ml of 2N NaOH. The mixture was stirred up for 30 min, then 852 ml of 6% H_2O_2 was added in portions of 50 ml. About 2 hr were required for the addition; the temperature was kept between 40° and 50°. After all the H_2O_2 was added, the dark-colored reaction mixture was allowed to cool to room temperature, and it was then saturated with sodium chloride, after which it was extracted four times with 250 ml portions of ether. The combined extracts were dried over anhydrous sodium sulfate. The ether was evaporated off, and the residue was distilled under reduced pressure. 3-Methoxycatechol (120 g; 70% yield) was collected at 135–40°/22 mm: cream solid; NMR: δ6·6 (3H, m, Ar–H's), 6·0 (2H, s, OH's) and 3·8 (3H, s, OCH_3); MS: *m/e* 140 (M^+) and *m/e* 125 (M^+—CH_3).

2,3-Methylenedioxyanisole **10**. In a l.l. flask equipped with a reflux condenser, were placed 120 g of 3-methoxycatechol, 177 g of chlorobromomethane, 200 ml of ethyleneglycol and 128 g of anhydrous potassium carbonate. The mixture was refluxed, under constant stirring, for 48 hr. 500 ml of distilled water was then added and the water was distilled off. Extraction of the distillate with ether yielded 65 g (50% yield) of 2,3-methylenedioxyanisole: white solid, m.p. 42–44° (Lit.[11] 41·5°).

2,3-Methylenedioxy-5-nitroanisole **11**. 2,3-Methylenedioxyanisole (25 g) was added very slowly to 190 ml of cold (ice bath), stirred, concentrated nitric acid. About 10 min after the addition was complete, the mixture was poured into 6 l of ice-water. The product was filtered and washed with water. Recrystallization of the product from 800 ml of 95% EtOH, gave 23 g (70% yield) of 2,3-methylenedioxy-5-nitroanisole: yellow solid, m.p. 144–46° (Lit.[12] 145–46°).

5-Amino-2,3-methylenedioxyanisole **12**. 2,3 Methylenedioxy-5-nitroanisole (23 g) was placed in a 1 l round-bottom flask equipped with a reflux condenser and stirrer, and 400 ml of 95% EtOH was added. The solution was warmed until the solid partially dissolved and then 1 g of 10% Pd/C (pre-moistened with EtOH) was added. Finally, 20 ml of hydrazine (95% +) was poured very slowly down the reflux condenser, and the mixture was refluxed for 30 min. The solution (warm) was filtered over Celite, and the 95% EtOH and excess hydrazine were distilled off. The remaining solvent was evaporated off under a water-aspirator vacuum to yield 19·4 g (97% yield) of 5-amino-2, 3-methylenedioxyanisole: tan solid, m.p. 81–84° (Lit.[12] 78–80°).

3-Methyoxy-4,5-methylenedioxyphenol **13**. A suspension of 13 g of 5-amino-2, 3-methylenedioxyanisole in 400 ml of water was cooled to 0° in an ice-bath. The mixture was acidified with 66 ml of 5 N H_2SO_4, and a solution of 5·8 g of sodium nitrite in 50 ml of water was added slowly to the stirred mixture. The unreacted nitrous acid was decomposed by the addition of 300 mg of urea. A 2 l, 3-necked flask equipped with a dropping funnel and a reflux condenser was set up. Into this flask, 800 g of copper sulfate and 800 ml of water were added. The copper sulfate solution was brought to boiling and then the diazonium sulfate solution was added slowly to the boiling solution. Ten minutes after the addition was complete, the solution was cooled and extracted with several portions of ether (8 × 500 ml portions). The ether was dried with sodium sulfate and then evaporated off to yield a dark, oily residue. Distillation of the crude product at 130°/0·3 mm gave 4·6 g (35% yield) of 3-methoxy-4, 5-methylenedioxyphenol; yellow-brown solid, m.p. 87–90° (Lit.[12] 89–91°).

6'-Hydroxy-2'-methoxy-3',4'-methylenedioxyacetophenone **14**. 3-Methoxy-4,5-methylenedioxyphenol (1·5 g) and acetonitrile (1·2 g) were placed in a 100 ml, 2-necked flask containing 60 ml of dry ether, and then anhydrous zinc chloride (2·7 g) was added. The flask was equipped with a gas inlet tube, a stirrer and a cold finger filled with dry ice. The stirred mixture was saturated with dry HCl gas in an ice-bath and allowed to stand for 48 hr in the refrigerator. Then, the ether layer was decanted from the oily residue and the oily residue was placed into 100 ml of water and heated to boiling for 1 hr. The aqueous solution was placed in a liquid–liquid extractor and continuously extracted with $CHCl_3$ overnight. The residue obtained upon evaporation was dissolved in a minimum amount of 1:1 benzene/$CHCl_3$ and then placed on a silica gel (60–200 mesh) column (25 × 5 cm). The column was eluted first with benzene (500 ml), and then $CHCl_3$ (1 l). A yellow band was eluted in $CHCl_3$. Upon evaporation of the $CHCl_3$, 580 mg (32% yield) of 6'-hydroxy-2'-methoxy-3', 4'-methylenedioxyacetophenone was obtained; yellow solid, m.p. 109–112°; NMR: δ 13·77 (1H, s, OH ortho to a carbonyl group), 6·21 (1H, s, Ar–H), 5·95 (2H, s, methylenedioxy), 4·10 (3H, s, OCH_3) and 2·61 (3H, s, CH_3); λ_{max} 243 (ε 11,140), 284 (ε 11,870) and 350 nm (ε 4000) alkaline, λ_{max} 246, 280 and 365 nm; MS: *m/e* 210 (60%, M^+), *m/e* 195 (100%, M^+—CH_3) and *m/e* 180 (48%, M^+—CH_3—CH_3). Calc. for $C_{10}H_{10}O_5$: C, 57·14; H, 4·76. Found: C, 57·79; H, 4·91%.

2,2'-Dimethoxy-6'-hydroxy-3',4'-methylenedioxychalcone **15**. 6'-Hydroxy-2'-methoxy-3',4'-methylenedioxyacetophenone (100 mg), *ortho*-anisladehyde (150 mg), 10 g of potassium hydroxide, 15 ml of MeOH and 15 ml of water were placed in a 50 ml round-bottom flask, equipped with a reflux condenser, and refluxed for 24 hr. At that time, the mixture was poured into a 500 ml beaker containing 300 ml of water. Dry ice was added to the contents in the beaker until a yellow precipitate formed. The solid was filtered off, washed with water, dissolved in acetone and refiltered. The acetone was evaporated and the residue was placed on a preparative silica gel TLC plate (Brinkmann, F-254). The plate was developed with a 90:10 benzene/acetone solvent system. A bright orange band at R_f 0·75–0·90 was scraped off of the plate and eluted with $CHCl_3$. Upon evaporation of the solvent, the residue was sublimed at 80° under a water-aspirator vacuum. The starting ketone was removed from the chalcone by this method. The residue which did not sublime, yielded 60 mg (39% yield) of 2,2'-dimethoxy-6'-hydroxy-3',4'-methylenedioxychalcone: orange crystals, m.p. 168–171°; NMR: δ 14·26 (1H, s, OH *ortho* to carbonyl group), 8·30–6·96 [6H, m, (C-3, 4, 5 and 6 H's + Hα + Hβ)] 6·30 (1H, s, C-5' H) 6·00 (2H, s, methylenedioxy), 4·10 (3H, s, C-2', OMe) and 3·96 (3H, s, C-2 OMe); λ_{max} 240 (ε

12,460), 312 (ε 16,400), and 358 nm (ε 20,000); alkaline. λ_{max} 288, 330 and 430 nm: MS: *m/e* 328 (38%, M^+) and *m/e* 194 (100%, $C_9H_6O_5$); Calc. for $C_{18}H_{18}O_6$, $\frac{1}{2}H_2O$: C, 64·14; H, 5·11; Found: C, 64·32; H, 5·33.

2',5-*Dimethoxy*-6,7-*methylenedioxyflavanone* **L** 2,2'-Dimethoxy-6'-hydroxy-3',4'-methylenedioxychalcone (20 mg) was dissolved in a MeOH (15 ml) and water (5 ml) solution. Sodium acetate (500 mg) was added, and the mixture was refluxed for 48 hr. The solution was extracted continuously with $CHCl_3$ (after dilution with 50 ml of water for 1 day). The residue obtained upon evaporation of the $CHCl_3$ was placed on a preparative silica gel TLC plate (Brinkmann F-254) and developed with 90:10 benzene/acetone solvent. A band at R_f 0·40–0·60, which fluoresced blue under longwave UV light, was scraped off of the plate and eluted with $CHCl_3$. Upon evaporation of the $CHCl_3$, 10 mg of a very light, yellow solid was obtained, which had R_f value, m.p., and NMR and UV spectra identical with isolated **1**.

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