Rapid Paper

A β -Hydroxychalcone and Flavonoids from Alfalfa Callus Stimulated by a Fungal Naphthoquinone, PO-1

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A fungal naphthoquinone, PO-1, was rapidly absorbed by alfalfa callus, and a prominent accumulation of 4',7-dihydroxyflavone, 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-1,3-propanedione and 6-hydroxy-4',7-dimethoxyisoflavone was seen in the PO-1-treated callus. This 1,3-propanedione (β -hydroxychalcone) showed pronounced antifungal activity.

A fungal metabolite, PO-1, was isolated as a stimulating factor for the secondary metabolite production of alfalfa callus in our laboratory¹⁾. Our previous study showed that alfalfa callus stimulated with this compound induced several metabolites which possessed pronounced antifungal activities. This paper deals with the isolation and identification of the newly accumulated metabolites in the PO-1-treated callus together with their biological activities. Their accumulation mechanisms are also discussed.

Induction of new metabolites in alfalfa callus fed on PO-1

The callus induced from alfalfa cotyledon with 2,4-D was transferred into MS medium containing PO-1 at a concentration of 100 μ g/ml, and then shaken for 48 hr on a rotary shaker. The callus rapidly absorbed PO-1, and the dark yellow color of the medium disappeared 24 hr after transplantation. The brownish-colored callus was harvested at 48 hr and soaked in acetone. The aqueous extract, afforded after removing the acetone *in vacuo*, was partitioned with ethyl acetate. The organic phase showed antifungal activity against *Pyricularia oryzae*.

Isolation and structures of the new metabolites

The organic phase was analyzed on silica gel F₂₅₄ TLC (benzene–acetone, 7:3) in comparison of the extract of the callus grown without PO-1. A strong UV absorbing spot at *Rf* 0.6 was detected only in the treated callus. After spraying with aluminum chloride, intense fluorescent spots emerged at *Rf* 0.7, 0.6 and 0.3 under UV light (360 nm). However, the two spots at *Rf* 0.7 and 0.3 were only detected as very faint fluorescent spots in the control. The concentrate obtained after partition was subjected to silica gel column chromatography (benzene–ethyl acetate). Compound 1 with *Rf* 0.3 was obtained in a pale yellow crystalline form from the 70% ethyl acetate eluate. This compound possessed strong fluorescence. The UV [$\lambda_{\max}$ (MeOH) nm: 253, 312, 328] and the IR [$\nu_{\max}$ (KBr) cm^{-1} : 3450, 1640~1620, 1395,

1260] spectra showed the characteristic features of flavones. The MS analysis, EI-MS: m/z 254 (M^+), as well as the $^1\text{H-NMR}$ analysis [δ_{H} (acetone- d_6): 7.94 (1H, d, $J=8.6$ Hz), 7.91 (2H, dd, $J=2.1, 6.7$ Hz), 7.02 (1H, d, $J=2.3$ Hz), 7.02 (2H, dd, $J=2.1, 6.7$ Hz), 6.95 (1H, dd, $J=2.3, 8.6$ Hz), 6.58 (1H, s)] indicated that **1** was 4',7-dihydroxyflavone, which had been isolated from leaves of alfalfa inoculated with *Pseudopeziza medicaginis*.^{2,3)}

A compound with R_f 0.6 was obtained in an amorphous crystalline form from the 50% ethyl acetate eluate. This compound (**2**) was characterized by a UV-absorbing spot on silica gel F_{254} TLC, and was not detected in the callus grown without PO-1. The UV [λ_{max} (EtOH) nm: 272, 382, 395] spectrum showed the characteristic features of chalcones. The MS of this compound [EI-MS: m/z 272 (M^+) and its acetate [m/z (rel. int.) 440 (M^+ , 5), 340 (60), 298 (70), 256 (80), 137 (80), 120 (100)] suggested the presence of four hydroxyls. Methylation with diazomethane gave the same product [EI-MS m/z (rel. int.): 282 (M^+ , 100), 254 (7), 238 (10), 149 (90)], 4',7-dimethoxyflavone, as obtained from methylating **1**, indicating that **2** could be 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-1,3-propanedione. Its spectral data were identical to those of a 1,3-diphenylpropanedione, lico-dione, which had been found in *Glycyrrhiza* callus.^{4,5)} Thus, **2** was identified as licodione. However, the presence of two singlets with odd proton ratios (0.7H at δ 7.00, and 0.5 H at δ 4.64) suggested that this compound would occur in tautomeric forms (**2**, **2'**) and exist predominantly in the enol form to an extent of about 75%, judging from the integration ratios

of these two singlets.

Compound **3** with R_f 0.7 showed quite similar UV adsorption bands [λ_{max} (EtOH) nm: 234, 258, 309] to that of the isoflavone, texasin 7-*O*- β -D-glucoside⁶⁾ (**6**), but not to afrormosin⁷⁾ (**4**). A characteristic 1H singlet appearing downfield (δ 7.91) was ascribed to H-2 of the isoflavone skeleton. The MS, EI-MS: m/z 298 (M^+), and its acetate, m/z 340 (M^+), suggested that one hydroxyl was present in this compound. In the $^1\text{H-NMR}$ spectrum (acetone- d_6), two characteristic 3H singlets at δ 3.82 and 3.99 were rationally assigned to aromatic methoxyls at C-4' and C-7, respectively, by analyzing the splitting patterns as well as by NOE experiments. The chemical shifts of the A_2X_2 -split signals (δ 6.95, 2H, d, $J=8.7$ Hz; δ 7.48, 2H, d, $J=8.7$ Hz) suggested that one of the methoxyls was assigned at C-4'. Although the coupling constant between the two aromatic broad singlets at δ 6.96 and 7.63 was not clearly measured, these signals were shown to be coupled by the related COSY experiment. Therefore, two 1H broad singlets at δ 7.63 and 6.96 were assigned to H-5 and H-8, respectively. The other downfield 1H singlet (δ 7.91) was assignable to H-2. An NOE was seen between a methyl at δ 3.99 and an aromatic proton (H-8) at δ 6.96 (NOE increment: +ca. 15%). As expected, the $^1\text{H-NMR}$ spectrum of **3** was almost identical to that of the aglycone portion of texasin 7-*O*- β -D-glucoside (δ 7.84: H-2; 7.63: H-5; 6.93: H-8) but not to that of afrormosin (**4**, δ 7.95: H-2; 7.75: H-5; 7.30: H-8). Therefore, the structure of this new isoflavone named alfalone was elucidated as 6-hydroxy-4',7-dimethoxyisoflavone, which was identified for the first time as a natural product.⁸⁾

Antifungal activity of the PO-1-induced flavonoids and their accumulation mechanism

Compounds **1**, **2** and **3** were subjected to spore germination tests with *Pyricularia oryzae* and *Cladosporium herbarum*. Compounds **1** and **2** completely inhibited *Pyricularia* spore germination at a concentration of 20 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$, respectively. Compound **3** did not

FIG. 1. Naphthoquinone-uptake Response of Alfalfa Callus.

Figures show the quinone contents left in the culture medium after 48 hr of incubation.

show any inhibitory activity toward these fungi at concentrations lower than 50 $\mu\text{g}/\text{ml}$.

PO-1 was quickly taken up into alfalfa callus, which consequently turned to dark yellow. In order to determine whether or not this unique property was common to quinones, several commercially available naphthoquinones were tested (Fig. 1). Neither the quinones without a side chain nor shikonin (7),^{9,10} a naturally occurring naphthoquinone bearing many structural similarities to PO-1, were taken up by alfalfa callus. Among these quinones, only PO-1 had such a unique property. The dark yellow color of the treated callus almost faded within several days after PO-1 was administered, suggesting that the absorbed PO-1 (about 200 μg of PO-1/g fresh weight of callus as estimated through the uptake experiment) was quickly degraded or converted into a colorless compound(s). In the meantime, the original biosynthetic pathways responsible for secondary metabolites might have been interfered with by the absorbed PO-1. A relatively high PO-1 concentration (100 $\mu\text{g}/\text{ml}$) was required to accumulate the three flavonoids. The accumulation of **1**, **2** and **3** was confirmed during a certain period, 48 hr to several days after treatment, where the callus maintained its dark yellow color and its growth was suppressed. This suggests that PO-1 acted as a metabolic inhibitor toward a certain flavonoid biosynthesis pathway rather than as an elicitor. Therefore, compounds

FIG. 2. Cyclization Mechanism of Licodione (2).

which were not detectable under normal culture conditions, due to their small content and/or their high turnover speed, could have been accumulated in the treated callus to a TLC-detectable level. This may be consistent with the finding that **1** and **3** were accumulated in the treated callus by about 20 times as much as in the callus grown without PO-1. As already mentioned, **2** (licodione) was only found in the treated callus, and not in the untreated callus. Licodione was easily cyclized to give 4',7-dihydroxyflavone (**1**) under relatively mild acidic conditions, suggesting that this labile compound should act as an important key intermediate in flavone biosynthesis (Fig. 2).

Some quinones are known as effective inhibitors toward the electron transport system¹¹, and some may also interfere with the detoxication system of oxygen radicals responsible for cell viability.¹² PO-1 suppressed the proliferation rate of the callus fed on PO-1 within the first several days after feeding,¹ and consequently the overall metabolism of the callus would slow down. Such a stress condition brought about by PO-1 would be favorable to secondary metabolite production, since secondary metabolites tend to accumulate in the callus of the stationary growth phase and not in that of the logarithmic growth phase.^{10,13} For secondary metabolite production in a tissue culture system, quinone derivatives which can be rapidly taken up by the callus could be useful.

Neither tomato nor tobacco callus absorbed PO-1. However, *Ageratum conyzoides* callus (*Compositae*) also rapidly absorbed this quinone. The ability to take up PO-1 was dependent upon callus origins. An appreciable amount of PO-1 was detected in the methanolic extract of PO-1-fed callus. However, the dark brownish portion of the callus was not extractable either with polar or nonpolar solvents. It has not been clarified that mechanism was involved in the rapid uptake of PO-1 into alfalfa callus as well as *Ageratum* callus and which compounds PO-1 was converted into. It would be quite interesting to determine the membrane function that is responsible for the selective absorption of quinones.

PO-1 has been applied to a variety of calli and their secondary metabolites have been examined.

EXPERIMENTAL

¹H-NMR spectra were recorded with a Varian VXR-500 instrument. Mass spectra were recorded with a JEOL JMS-D300, and UV spectra were recorded on a Shimadzu UV3000 spectrophotometer.

1) *Extraction and isolation of flavonoids.* Clamps of an alfalfa callus (100 g) in the logarithmic growth phase were transferred to a 2000 ml Erlenmeyer flask containing 500 ml of MS medium and PO-1 (100 µg/ml), which was then shaken on a rotary shaker for 48 hr at 25°C in the dark. The harvested callus was rinsed with water and soaked in 200 ml of acetone-methanol (1:2). The aqueous concentrate afforded after removing the solvent *in vacuo* was partitioned with ethyl acetate. The organic phase was concentrated to give a brownish residue (155 mg). This showed inhibitory activity against *Pyricularia oryzae* spore germination at a concentration of 100 µg/ml. Chromatographic separation (silica gel, ethyl acetate/benzene) afforded new metabolites in the fractions (30, 50 and 70% ethyl acetate/benzene) which were subjected to pre-TLC to give **3** (3.8 mg), **2** (2.1 mg) and **1** (1.2 mg), respectively.

Compound **1**. EI-MS *m/z* (rel. int.): 254 (*M*⁺, 100), 111 (75), 104 (78), 90 (81); UV $\lambda_{\max}$ (MeOH) nm (ϵ): 253 sh (15,000), 312 sh (30,000), 328 (33,000); IR $\nu_{\max}$ (KBr) *cm*⁻¹: 3450, 1640~1620, 1395, 1260; NMR δ_{H} (500 MHz, acetone-*d*₆): 7.94 (1H, d, *J*=8.6 Hz), 7.91 (2H, dd, *J*=2.1, 6.7 Hz), 7.02 (1H, d, *J*=2.3 Hz), 7.02 (2H, dd, *J*=2.1, 6.7 Hz), 6.95 (1H, dd, *J*=2.3, 8.6 Hz), 6.58 (1H, s). 4',7-Dimethyl **1** (4',7-dimethylflavone), EI-MS *m/z* (rel. int): 282 (*M*⁺, 100), 239 (25), 149 (92), 132 (64); UV $\lambda_{\max}$

(MeOH) nm (ϵ): 256 (17,000), 312 (19,500), 324 (21,000); IR $\nu_{\max}$ (KBr) *cm*⁻¹: 3420, 1630, 1610, 1380, 1256. HPLC: *R*_t=8.0; column packing, Inertsil ODS-5 (Gasukuro Kogyo Inc.); flow rate, 0.5 ml/min; mobile phase, 80% MeOH; detection, UV 254 nm. Silica gel TLC (Kieselgel 60 F₂₅₄): *R*_f=0.4 (benzene-EtOAc, 4:3).

Compound **2**. EI-MS *m/z* (rel. int.): 272 (*M*⁺, 35), 256 (30), 137 (60), 92 (100); UV $\lambda_{\max}$ (MeOH) nm (ϵ): 272 (11,600), 382 (4000), 395 (3600); IR $\nu_{\max}$ (KBr) *cm*⁻¹: 3380, 1630, 1610~1585, 836, 786; NMR δ_{H} (500 MHz, acetone-*d*₆): 8.00 (1H, d, *J*=8.7 Hz), 7.91 (2H, d, *J*=8.8 Hz), 7.01 (2H, d, *J*=8.8 Hz), 7.00 (0.7 H, s), 6.45 (1H, dd, *J*=2.4, 8.7 Hz), 6.32 (1H, d, *J*=2.4 Hz), 4.64 (0.5 H, s).

Compound **3**. EI-MS *m/z* (rel. int.): 298 (*M*⁺, 7), 268 (100), 253 (18), 132 (97); UV $\lambda_{\max}$ (EtOH) nm (ϵ): 234 (14,000), 258 (26,000), 309 (8000); IR $\nu_{\max}$ (KBr) *cm*⁻¹: 3160, 1635, 1590, 1290, 1260; NMR δ_{H} (500 MHz, acetone-*d*₆): 7.91 (1H, s), 7.63 (1H, br. s), 7.48 (2H, d, *J*=8.7 Hz), 6.96 (1H, br. s), 6.95 (2H, d, *J*=8.7 Hz), 3.99 (3H, s), 3.82 (3H, s).

2) *Antifungal tests.* After a 24 hr incubation at 30°C, the antifungal activities were evaluated toward *Pyricularia oryzae* and *Cladosporium herbarum*. The spore germination test was carried out in small test tubes containing 5.0, 4.0, 3.0, 2.0, 1.0 and 0.5 µg of samples/100 µl of 2% sucrose at 30°C for 24 hr. Germination was examined under an ordinary microscope.

3) *Feeding experiment with commercially available quinones.* Synthetic quinones (1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone and 5-hydroxy-1,4-naphthoquinone) as well as shikonin were purchased from Wako Chemicals (Osaka). Clamps of callus (1 g) were introduced into the MS medium (5 ml) containing each quinone at a concentration of 100 µg/ml and then rotated on a roller tube apparatus at 25°C for 48 hr. After incubating, the medium was filtered, and the UV absorbance of the filtrate was measured at the UV absorption maximum (naphthoquinone, 246 nm; 2-hydroxynaphthoquinone, 276 nm; 5-hydroxynaphthoquinone, 250 nm; shikonin, 280 nm; PO-1, 263 nm) of each quinone. None of the quinones were absorbed by alfalfa callus. 5-Hydroxy-1,4-naphthoquinone showed a phytotoxic effect at this concentration and the callus surface turned to a brownish color. However, the original color of the medium due to the quinone did not fade.

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