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PSORALEN AND OTHER LINEAR FURANOCOUMARINS AS PHYTOALEXINS IN *GLEHNIA LITTORALIS**

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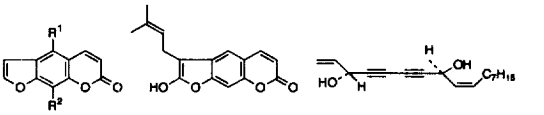
Key Word Index—*Glehnia littoralis*; Umbelliferae; phytoalexins; stress metabolite; furanocoumarin; psoralen; xanthotoxin; bergapten; demethylsuberosin; crude drug.

Abstract—Inoculation of *Glehnia littoralis* root slices with *Pseudomonas cichorii* induced the production of four linear furanocoumarin phytoalexins, psoralen, xanthotoxin, bergapten and demethylsuberosin, of which the former three have been reported as constituents of the crude drug Glehnia root. A time-course study on the respective *P. cichorri*-inoculated, UV-irradiated and non-irradiated root slices showed greatly increasing concentrations of the furanocoumarins after stress treatment. Psoralen, xanthotoxin and bergapten in the crude drug were considered, at least in part, to be stress metabolites produced during processing. © 1997 Elsevier Science Ltd

INTRODUCTION

Induction and accumulation of phytoalexins, antimicrobial compounds synthesized by plants after their exposure to microorganisms, is considered to be one of the most important defence mechanisms of plants [2, 3]. During the course of our studies on phytoalexins from vegetables, we have found several new types of compounds, for example, a series of sulphur-containing indolic compounds from cruciferous vegetables [4] and a sesquiterpenoid with a troponoid structure from lettuce [5]. As an extension of the studies, medicinal plants were screened for phytoalexin production since these plants have been a rich source of bioactive compounds. TLC bioassay of an acetone extract from *Glehnia littoralis* root slices inoculated with *Pseudomonas cichorii* showed the presence of inhibition spots due to newly induced antifungal compounds.

Glehnia littoralis is a perennial herb growing on seashore of the northern Pacific countries including Japan. Glehnia root, the root and rhizome of this plant, is described in the Chinese and Japanese Pharmacopoeia and has been traditionally used as a diaphoretic, an antipyretic and an analgesic. Antipyretic [6] and analgesic [7] activities in rabbits have been reported from alcoholic extract from the roots.



1: R^1=R^2=H
2: R^1=H, R^2=OMe
3: R^1=OMe, R^2=H
5: R^1=H, R^2=OCH_2CH=$C(Me)_2$
6: R^1=OCH_2CH=$C(Me)_2$, R^2=H
8: R^1=geranyloxy, R^2=H

4 7

The present paper describes the identification of the induced antifungal compounds **1**–**4** in *G. littoralis* as psoralen, xanthotoxin, bergapten and demethylsuberosin, respectively. The presence of these furanocoumarins in Glehnia root is discussed.

RESULTS AND DISCUSSION

Sliced roots of *G. littoralis* were aged, inoculated with the bacterium *P. cichorii*, incubated at 25° for 3 days and then extracted with acetone. Successive bioassay-directed chromatographic separation of the extract gave six antifungal furanocoumarins (**1**–**6**) and falcarindiol (**7**). Falcarindiol has been isolated recently from *G. littoralis* as a potent allelochemical [8]. The furanocoumarins were identified as psoralen (**1**), xanthotoxin (**2**), bergapten (**3**), demethylsuberosin (**4**), imperatorin (**5**) and isoimperatorin (**6**) on the basis of spectral data and comparison with those reported [9, 10]. Since compounds **1**–**4** were barely detectable in healthy roots and their contents increased markedly as a result of the inoculation, these are regarded as phytoalexins of *G. littoralis*. In contrast, compounds

* Part 24 in the series 'Studies on Stress Metabolites'. For Part 23, see ref. [1].

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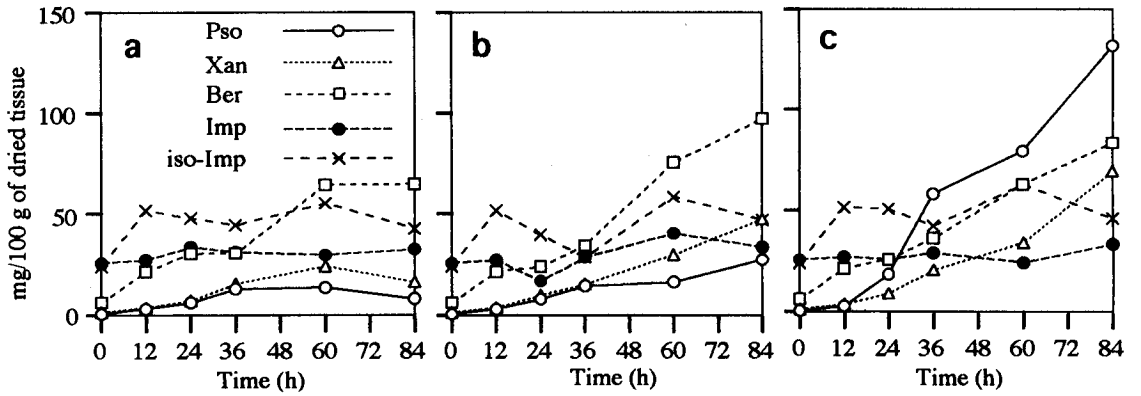

Fig. 1. Time-course study of furanocoumarin contents (mg 100 g^{-1} of dried tissue) in root tissues of *Glehnia littoralis* incubated at 25°: only slicing (a); UV-irradiated at 12 hr after slicing (b); inoculated with *Pseudomonas cichorii* 12 hr after slicing (c). Abbreviations: Pso, psoralen; Xan, xanthotoxin; Ber, bergapten; Imp, imperatorin; iso-Imp; isoimperatorin.

5 and **6** were present in almost the same amounts as in the healthy root tissue and are regarded as normal constituents. Furanocoumarins **1**–**3** have been reported previously as phytoalexins in celery (*Apium graveolens*) [11], parsnip (*Pastinaca sativa*) [12], parsley (*Petroselinum hortense*) [13], carrot (*Daucus carota*) [14] and are known as potent photosensitizers. They form light-induced mono- or di-adducts with the pyrimidine base of DNA when activated by near ultraviolet light (300–380 nm), causing lethal, mutagenic and clastogenic effects [15]. Compound **4** was isolated as a phytoalexin for the first time.

Interestingly, furanocoumarins **1**–**3** have been described as constituents of the crude drug Glehnia root [9], while these were scarcely detected in the fresh roots or rhizomes of *G. littoralis*. Therefore, the present isolation of **1**–**3** as phytoalexins raised a question whether the compounds in the crude drug are normal secondary metabolites. The furanocoumarin contents in the crude drug showed considerable variation depending on the method of storage and/or processing of the raw material [16]. Slow drying seemed to increase the amounts of **1**–**3** in the crude drug [16].

A time-course study on the contents of the furanocoumarins in *P. cichorii*-inoculated, UV-irradiated or non-irradiated *G. littoralis* root slices gave further information. Furanocoumarin contents over a 3 day period were followed by HPLC (Fig. 1): *P. cichorii* was shown to be a better phytoalexin elicitor than UV irradiation. The concentrations of **1**–**3** increased markedly after each stress treatment followed by incubation, while those of **5** and **6** were essentially unaltered. Contents of **4** were not evaluated due to the presence of interfering peaks. Compounds **1**–**3** were also induced in untreated root slices [Fig. 1(a)], indicating facile induction of these furanocoumarins. Therefore, the reported furanocoumarins **1**–**3** in the crude drug Glehnia root [9] seemed to be, at least in part, stress metabolites induced by injury or by slow drying during the processing of raw material. The present findings further suggest that some reported constituents of other crude drugs could be stress metabolites produced during processing.

Oyanagi and co-workers reported that furanocoumarin contents in the root of *G. littoralis* varied considerably depending on its geographical origins [17]. They collected fruits of plants growing at different places and cultivated their seedlings in a field. Since HPLC profiles of linear furanocoumarins in the root of the cultivated plants were the same as those of the plants growing in the wild in their respective original habitats, they suggested that *G. littoralis* plants in different habitats vary genetically [18]. They classified the species in Japan into two types, namely, northern and southern types [17, 18]. The former contains **5** and **6** as major and **1**–**3** as minor constituents. On the other hand, bergamottin (**8**) was the major compound in the latter. In the southern type, **1**–**3** occurred in trace or very small amounts [17, 18]. Since our results were obtained using *G. littoralis* of the northern type cultivated in Hokkaido, we next examined phytoalexin induction in the southern type using wild *G. littoralis* collected in Shimane Prefecture. HPLC analysis of an extract from root slices exposed to *P. cichorii* verified the production of **1**–**3** (Fig. 2), indi-

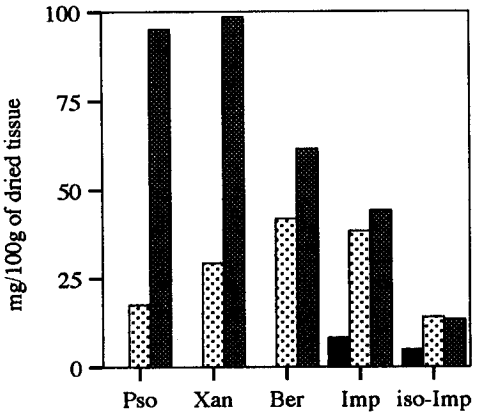

Fig. 2. Furanocoumarin contents in roots of southern type *Glehnia littoralis*. ■: sliced and freeze-dried immediately. ⊡: sliced and incubated for 84 hr. ■: inoculated with *Pseudomonas cichorii* after 12 hr of ageing and incubated for an additional 72 hr.

cating that both types of *G. littoralis* have the ability to produce these furanocoumarins as phytoalexins. Variation of furanocoumarin contents in the southern type of *G. littoralis* during storage and processing will be reported elsewhere [19].

EXPERIMENTAL

Plant material. Glehnia littoralis of the northern type was cultivated in Nayoro, Hokkaido, Japan, and donated by Mr Furuki. Wild *G. littoralis* of the southern type was collected in Gotsu, Shimane Prefecture, Japan, by Mr Ohtani. Both types are common plants in Japan. Plants used in this study are maintained at the medicinal plants garden of Hokkaido Institute of Public Health, Sapporo, Japan.

TLC bioassay. Antifungal activity of chromatographic frs were monitored by TLC bioassay. Developed silica gel TLC sheets were sprayed with a dense conidial suspension of *Bipolaris leersiae* in a potato–glucose medium and incubated in a moist box at 25° for 2 days. Fungitoxic spots appeared white against a dark grey background.

Induction and isolation of phytoalexins. Roots of northern type (4.1 kg) were cut into slices (4 mm thick), kept in moist boxes at 25° for 12 hr, and then inoculated with *P. cichorii* (*ca* 10^8 cells ml^{-1}). After incubation for 3 days at 25°, they were air-dried (800 g) and extracted $\times 3$ with Me_2CO to yield an extract (72.4 g). This was sepd into 8 frs (F-1–F-8) by CC on silica gel using CH_2Cl_2, Et_2O, EtOAc and MeOH. Frs F-2 (5.23 g) and F-3 (0.62 g), eluted with CH_2Cl_2 and Et_2O, respectively, were combined and sepd into 6 frs (F-2-1–F-2-6) by CC on silica gel using hexane with increasing amounts of EtOAc. A part (120 mg) of F-2-3 (420 mg), eluted with 30% EtOAc, gave isoimperatorin (**6**, 13 mg) [9] after HPLC on μ-Porasil (EtOAc–hexane, 1:24) and then on NOVA-Pak C_{18} (MeOH–H_2O, 13:7). F-2-5 (1.99 g), eluted with 50–70% EtOAc in hexane, was recrystallized from EtOAc–CH_2Cl_2 to give bergapten (**3**, 87 mg) [9]. The mother liquor was sepd into 4 frs (F-3-1–F-3-4) by CC on silica gel using Me_2CO–CH_2Cl_2 (1:99). F-3-2 (1.66 g) was chromatographed on silica gel with EtOAc–hexane (3:7) to give a fr. (546 mg), which gave psoralen (**1**, 172 mg) [9] after recrystallization from MeOH. A part (40 mg) of the mother liquor (370 mg) gave imperatorin (**5**, 12 mg) [9] after HPLC on NOVA-Pak C_{18} (MeOH–H_2O, 1:1). F-3-3 (32 mg) gave xanthotoxin (**2**, 23 mg) [9] after HPLC on NOVA-Pak C_{18} (MeCN–H_2O, 1:3). F-4 (13.96 g), eluted with Et_2O, gave demethylsuberosin (**4**, 25 mg) [10] after sequential chromatography: silica gel CC (MeOH–CH_2Cl_2, 1:49), silica gel CC (EtOAc–hexane, 3:7 to 1:1), Sephadex LH-20 (MeOH), NOVA-Pak C_{18} (MeOH–H_2O, 7:3). F-4 also gave falcarindiol (**7**, 2.07 g) [8] after silica gel CC (EtOAc–hexane, 3:7). Antifungal scores on TLC bioassay: **1**, ++; **2**, +++; **3**, +; **4**, +++; **5**, ±; **6**, ±; **7**, +++.

Psoralen (**1**). $C_{11}H_6O_3$, mp 165–166°. $[M]^+$ *m/z* 186.0300. ^1H NMR ($CDCl_3$): δ 6.38 (1H, *d*, $J = 9.8$ Hz, H-3), 6.83 (1H, *dd*, $J = 2.0$, 1.0 Hz, H-3′), 7.46 (1H, *br s*, H-8), 7.68 (1H, *s*, H-5), 7.70 (1H, *d*, $J = 2.0$ Hz, H-2′), 7.80 (1H, *d*, $J = 9.8$ Hz, H-4). ^{13}C-NMR ($CDCl_3$): δ 99.8 (*d*), 106.4 (*d*), 114.6 (*d*), 115.4 (*s*), 119.9 (*d*), 124.9 (*s*), 144.1 (*d*), 146.9 (*d*), 152.0 (*s*), 156.4 (*s*), 161.0 (*s*). UV λ_{max}^{MeOH} nm (ε): 246 (22 600), 291 (10 400), 327 (6400). IR ν_{max}^{KBr} cm^{-1}: 1725, 1635, 1577, 1136, 824.

Xanthotoxin (**2**). $C_{12}H_8O_4$, mp 146–148°. $[M]^+$ *m/z* 216.0401. ^1H NMR ($CDCl_3$): δ 4.30 (3H, *s*, OMe), 6.37 (1H, *d*, $J = 9.3$ Hz, H-3), 6.83 (1H, *d*, $J = 2.4$ Hz, H-3′), 7.36 (1H, *s*, H-5), 7.70 (1H, *d*, $J = 2.4$ Hz, H-2′), 7.77 (1H, *d*, $J = 9.3$ Hz, H-4). ^{13}C NMR ($CDCl_3$): δ 61.4 (*q*), 106.8 (*d*), 112.9 (*d*), 114.7 (*d*), 116.5 (*s*), 126.2 (*s*), 132.8 (*s*), 143.0 (*s*), 144.4 (*d*), 146.7 (*d*), 147.7 (*s*), 160.5 (*s*). UV λ_{max}^{MeOH} nm (ε): 218 (25 300), 248 (24 600), 299 (12 400). IR ν_{max}^{KBr} cm^{-1}: 1710, 1620, 1586, 1155, 821.

Bergapten (**3**). $C_{12}H_8O_4$, mp 188–190°. $[M]^+$ *m/z* 216.0393. ^1H NMR ($CDCl_3$): δ 4.27 (3H, *s*, OMe), 6.26 (1H, *d*, $J = 9.8$ Hz, H-3), 7.02 (1H, *d*, $J = 2.5$ Hz, H-3′), 7.12 (1H, *s*, H-8), 7.59 (1H, *d*, $J = 2.5$ Hz, H-2′), 8.15 (1H, *d*, $J = 9.8$ Hz, H-4). ^{13}C NMR ($CDCl_3$): δ 60.1 (*q*), 93.8 (*d*), 105.1 (*d*), 106.4 (*s*), 112.5 (*d*), 122.7 (*s*), 139.3 (*d*), 144.8 (*d*), 149.6 (*s*), 152.7 (*s*), 158.4 (*s*), 161.2 (*s*). UV λ_{max}^{MeOH} nm (ε): 222 (22 600), 250 (16 500), 310 (13 600). IR ν_{max}^{KBr} cm^{-1}: 1731, 1625, 1580, 1124, 834.

Demethylsuberosin (**4**). $C_{14}H_{14}O_3$, mp 128–131°. $[M]^+$ *m/z* 230.0943. ^1H NMR ($CDCl_3$): δ 1.74 (3H, *s*), 1.78 (3H, *s*), 3.37 (2H, *d*, $J = 6.8$ Hz), 5.32 (1H, *t*-like, $J = 6.8$ Hz), 6.23 (1H, *d*, $J = 9.3$ Hz), 7.05 (1H, *s*), 7.19 (1H, *s*), 7.65 (1H, *d*, $J = 9.3$ Hz). ^{13}C NMR ($CDCl_3$): δ 17.9 (*q*), 25.8 (*q*), 28.3 (*t*), 103.1 (*d*), 112.0 (*d*), 112.1 (*s*), 121.1 (*d*), 126.1 (*s*), 128.2 (*d*), 134.8 (*s*), 144.5 (*d*), 154.1 (*s*), 158.8 (*s*), 162.7 (*s*). UV λ_{max}^{MeOH} nm: (ε) 334 (12 700), 256 (3100), 248 (3600), 222 (13 500). IR ν_{max}^{KBr} cm^{-1}: 3227, 1695, 1617, 1131, 821.

Imperatorin (**5**). $C_{16}H_{14}O_4$, mp 97.5–99°. $[M]^+$ *m/z* 270.0897. ^1H NMR ($CDCl_3$): δ 1.72 (3H, *s*), 1.74 (3H, *s*), 5.01 (2H, *d*, $J = 7.3$ Hz), 5.61 (1H, *t*-like, $J = 7.3$ Hz), 6.37 (1H, *d*, $J = 9.3$ Hz, H-3), 6.81 (1H, *d*, $J = 2.4$ Hz, H-3′), 7.36 (1H, *s*, H-5), 7.69 (1H, *d*, $J = 2.4$ Hz, H-2′), 7.76 (1H, *d*, $J = 9.3$ Hz, H-4). ^{13}C NMR ($CDCl_3$): δ 18.1 (*q*), 25.8 (*q*), 70.2 (*t*), 106.7 (*d*), 113.1 (*d*), 114.7 (*d*), 116.1 (*s*), 119.8 (*d*), 125.9 (*s*), 131.7 (*s*), 139.8 (*s*), 143.9 (*s*), 144.3 (*d*), 146.6 (*d*), 148.6 (*s*), 160.6 (*s*). UV λ_{max}^{MeOH} nm: (ε) 218 (22 800), 249 (20 600), 301 (10 600). IR ν_{max}^{KBr} cm^{-1}: 1722, 1707 1587, 1150, 838.

Isoimperatorin (**6**). $C_{16}H_{14}O_4$, mp 104–108°. $[M]^+$ *m/z* 270.0905. ^1H NMR ($CDCl_3$): δ 1.70 (3H, *s*), 1.80 (3H, *s*), 4.92 (2H, *d*, $J = 6.8$ Hz), 5.54 (1H, *t*-like, $J = 6.8$ Hz), 6.27 (1H, *d*, $J = 9.8$ Hz, H-3), 6.96 (1H, *d*, $J = 2.4$ Hz, H-3′), 7.15 (1H, *s*, H-8), 7.60 (1H, *d*, $J = 2.4$ Hz, H-2′), 8.16 (1H, *d*, $J = 9.8$ Hz, H-4). ^{13}C NMR ($CDCl_3$): δ 18.3 (*q*), 25.8 (*q*), 69.8 (*t*), 94.2 (*d*), 105.1 (*d*), 107.5 (*s*), 112.6 (*d*), 114.2 (*s*), 119.1 (*d*), 139.6 (*d*), 139.8 (*s*), 144.9 (*d*), 149.0 (*s*), 152.7 (*s*), 158.1 (*s*), 161.3 (*s*). UV λ_{max}^{MeOH} nm: (ε) 222 (21 700), 250 (16 000),

259 (14 300), 267 (13 900), 310 (12 400). IR ν_{max}^{KBr} cm^{-1}: 1729, 1628, 1604, 1582, 1129, 821.

Time-course study. Roots of northern type were cut transversely into slices (4 mm thick) and incubated at 25° for 12 hr under humid conditions. Aged slices were inoculated with *P. cichorii* or irradiated on both sides with a 20 W germicidal lamp (0.56 m W cm^{-2}) for 10 min and then incubated for an additional 12, 24, 48 and 72 hr under the same conditions. Slices were then dried, powdered and treated as follows. To respective powdered samples (500 mg) in screw-capped test tubes was added 10 ml of MeOH–H_2O (4:1). The mixt. was sonicated for 20 min at room temp. and then centrifuged (3000 rpm, 10 min). The upper layer was subjected to HPLC analysis. Column: LiChrospher 100 RP-18 endcapped column (4 × 250 mm) at 40°. Mobile phase: MeCN–H_2O (stepwise gradient, 7:13 for 12 min, 12:13 for 18 min, 17:8 for 20 min and 4:1 for 10 min). Flow rate: 1 ml min^{-1}. UV detector (248 nm) R_ts (min): **1**, 10.1; **2**, 10.9; **3**, 14.7; **5**, 26.9; **6**, 34.6.

Phytoalexin induction in southern type G. littoralis. Roots were sliced, aged for 12 hr, inoculated with *P. cichorii*, incubated for 72 hr and processed as described above. HPLC analyses were carried out under the same conditions.

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