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TWO PHYTOALEXIN GLYCOSIDES FROM POTATO TUBERS INFECTED WITH *PHOMA*

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; *Phoma exigua* var. *foveata*; coumarin glucoside; sesquiterpene glucoside; phytoalexins.

Abstract—Four coumarins and seven isoprenoid compounds have been identified in potato tubers infected with *Phoma exigua* var. *foveata*. Among these were the 7-*O*-β-D-glucopyranoside of 7-hydroxy-6,8-dimethoxycoumarin (isofraxidin) and the sesquiterpene 2-(11,12-dihydroxy-11-methylethyl)-6,10-dimethyl-spiro[4,5]dec-6-en-8-one and its 12-*O*-β-D-glucopyranoside, which apparently have not been previously identified in potato tubers. At least two diastereoisomers of the latter glucoside were present. Analysis of eight fluorescent compounds in different parts of infected potatoes was performed by an improved HPLC technique.

INTRODUCTION

The literature contains many reports about the accumulation in potato of phytoalexins of the coumarin type in response to various infections. These compounds are found in the blue-fluorescing zones of potato tissues [1–4]. The most extensive studies relate to the pathogen *Phytophthora infestans*, in which umbelliferone (**1**), esculetin (**2**), scopoletin (**3**) and scopolin (**4**) [2, 3] have been found. These compounds and skimmin were also reported in potatoes inoculated with *Ceratosystis fimbriata* [5, 6], while **3** and esculin (**5**) are reported from infection with *Phoma exigua* and *Fusarium coeruleum* [7]. Some of these compounds have been separated by HPLC by Court [8].

Sesquiterpenoids constitute another group of phytoalexins which accumulate in the potato after infection [9]. Thus, rishitin (**6**) [10], rishitinol (**7**) [11], phytuberin (**8**) and deacetylphytuberin (**9**) [12], solavetivone (**10**) and anhydro-β-rotunol (**11**) [13], lubimin [14], epilubimin [15], hydroxylubimin [16] and cyclodehydroisolubimin [17] have been identified previously.

In connection with studies in progress on various types of extractives in *Solanum tuberosum* L. cv Bintje, and their fungitoxic effect (against *Phoma exigua* var. *foveata*), four coumarins and seven isoprenoid phytoalexins have been isolated from the blue-fluorescing zone. These compounds as well as a HPLC technique for the analysis of coumarins will be discussed in this paper.

RESULTS AND DISCUSSION

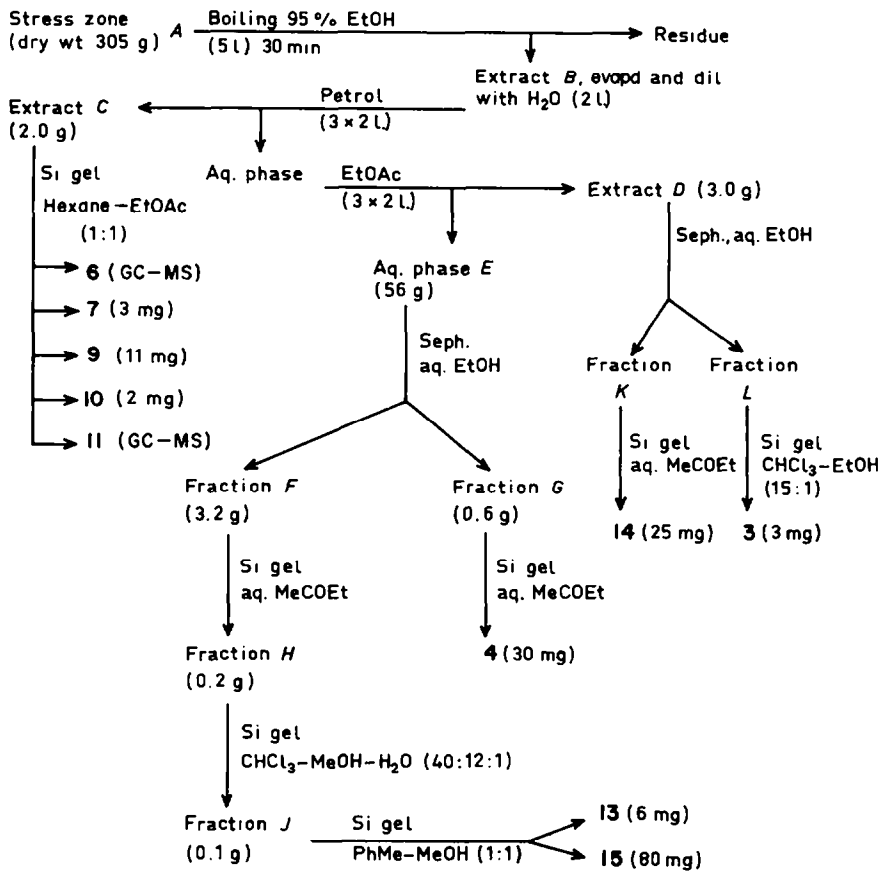
The extraction and fractionation procedure is outlined in Scheme 1. Three light-blue fluorescent compounds—scopoletin (**3**), scopolin (**4**), and a compound proved to be isofraxidin-7-glucoside (**13**)—were isolated by column chromatography from the blue-fluorescing zone of potato tubers (*Solanum tuberosum* L. cv Bintje) infected with *Phoma exigua* var. *foveata*. Umbelliferone (**1**) and an unknown fluorescent compound (**x**) were also observed but were not isolated in a pure state.

^{1}H NMR of **13** indicated a coumarin glycoside structure substituted at positions 5–7 or 6–8. Hydrolysis with β-glucosidase gave free aglycone and glucose confirming the β-glucosidic linkage. The aglycone was identified as 7-hydroxy-6,8-dimethoxycoumarin (isofraxidin) (**12**), by comparison with an authentic sample (^{1}H NMR, MS, IR, UV). Compound **3** was identified by comparison with an authentic sample (^{1}H NMR, MS, IR, UV), and **4** was prepared [18] from **3** in order to confirm the structure.

Compound **12** has been reported in *Fraxinus excelsior* [19] and *Artemisia* species [20–22], *Calycanthus japonicus* [23] and *Erica cinerea* [24]. Its β-glucoside (**18**) has been reported in *C. floridus* under the name calycanthoside [25], and the α-isomer in *Eleutherococcus senticosus* [26]. To our knowledge, neither isofraxidin nor its glucoside has, however, been reported previously in *Solanum* species.

By using the method of Court [9] with some modifications, including use of a fluorescence detector, we have developed an analytical method and applied it to 8 coumarin phytoalexins. The results, including the amounts of different compounds estimated in our potato samples, are listed in Table 1.

By this sensitive method it was found that fresh peel contains small amounts of **3** and **4**, but no coumarins were detected in fresh pulp. The coumarins in the peel may come from sites of latent diseases, where the potato has begun to generate a blue-fluorescing zone. We analysed the *Phoma*-infected zone and found that



Scheme 1. Isolation of compounds **3**, **4**, **6**, **7**, **9–11** and **13–15** from the stress zone of potato tubers infected with *Phoma*. Seph. = CC on Sephadex LH-20, Si gel = CC on silica gel, aq. EtOH = elution with H_2O with increasing EtOH content (0–95%), aq. MeCOEt = 2-butanone saturated with H_2O.

the amounts of the various coumarins were generally lower than in the blue-fluorescing zone. We also analysed the blue-fluorescing zone from *Fusarium coeruleum* infection (Table 1). All components found in the *Phoma*-fluorescing zone, except the glucoside **13**, were present in the *Fusarium*-fluorescing zone but the unknown compound (**x**) occurred in much smaller amounts. When the same mixtures were analysed by HPLC using a UV detector, some of the fluorescent components were not detectable because of the complex product pattern. Finally, it is notable that umbelliferone (**1**), which was not isolated preparatively, could be detected in traces by the HPLC method.

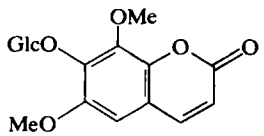
Table 1. HPLC data for fluorescent compounds relative to fluorescein and their occurrence in infected potato

Compound	R_f	Response factor	Occurrence (% of fr. wt) in potato infected by *Phoma* stress	*Phoma* rot	*Fusarium* stress
5	0.30	6.1	—	—	—
4	0.41	0.10	2.2	1.6	4.0
2	0.48	0.11	—	—	—
13	0.50	†	trace	—	—
x	0.53	1.0‡	6.4	1.2	0.6
1	0.63	8.1	0.03	0.04	0.03
12	0.64	0.21	—	—	—
3	0.65	11.4	0.15	0.10	0.13

*Retention relative to fluorescein.

†Isolated sample of high purity, not available in large enough quantities to obtain a response factor.

‡Arbitrarily chosen to get rel. abundance.



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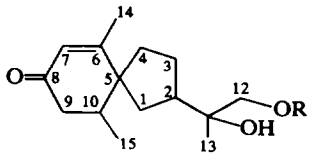
A number of isoprenoids were also isolated or identified (Scheme 1). Compound **15** ($[\alpha]_D^{23}$ −76°) was isolated and hydrolysed with β-glucosidase, yielding equal amounts of an aglycone and glucose. This result, and the ^1H NMR spectrum of the acetylated glucoside **15a** with the anomeric proton at δ 3.56 (J = 7.5 Hz), indicated a β-D-glucopyranoside [27]. The isolated aglycone **14**, ($[\alpha]_D^{23}$ −55.0°) was identified (^{13}C NMR ^1H NMR, MS and IR) as the recently reported sesquiterpene, 2-(11,12-dihydroxy-11-methylethyl)-6,10-dimethylspiro[4,5]dec-6-en-8-one [28], isolated from tobacco. However, small differences were noticed in the ^{13}C NMR shifts, especially for carbons 1, 3, 4, 6



14 R = H
14a R = Ac
15 R = Glc
15a R = Glc(OAc)$_4$

and 9 (0.5–0.9 ppm), and in the ^{1}H NMR shift for the C-13 methyl group (0.07 ppm). Addition of the shift reagent Eu(fod)$_3$ to the acetylated aglycone **14a** split the ^{1}H NMR signals into two identical signals for each signal in the spectrum before adding the shift reagent. Since the shifts were very close to those reported by Anderson *et al.* [28], the differences are probably of sterochemical origin. The ^{1}H NMR spectrum in the presence of Eu(fod)$_3$ indicating a 4:1 mixture of two diasteroisomers. Four acetyl signals appeared in the ^{1}H NMR spectrum of **15a** and one in that of **14a**, indicating that glucose is linked to the primary hydroxyl group (the tertiary one will not be acetylated during the conditions used). Compound **15** therefore consists of a mixture of two diastereoisomers of 2-(12-*O*-β-D-glucopyranosyl-11-hydroxy-11-methylethyl)-6,10-dimethylspiro[4,5]dec-6-en-8-one. The free aglycone **14** was also isolated from the blue-fluorescing zone.

Compound **6** was identified by comparison with an authentic sample (^{1}H NMR, MS). Compounds **7**, **9** and **10** were isolated and identified by means of literature data (^{1}H NMR, MS, IR, UV) [11–13] and anhydro-β-rotunol (**11**) was tentatively identified by GC–MS.

EXPERIMENTAL

General. Sephadex LH-20 and Merck Si gel 60 (230–400 mesh) were used for the column chromatography, being monitored by TLC on Si gel plates with the respective eluent (see Scheme 1), except for Sephadex LH-20 columns, where 2-butanone, saturated with H_2O, was used as TLC eluent. The TLC plates were inspected in UV light and sprayed with diazotized sulphanilic acid in 10% Na_2CO_3 or 3% vanillin in EtOH, followed by H_2SO_4 and heat. Solvents were freshly distilled before use and mixed on a vol. basis (v/v). ^{1}H and ^{13}C NMR spectra were measured at 100 and 22.53 MHz, respectively, and TMS was used as int. standard. MS was performed at 70 eV, and for GC–MS an OV-225 column was used at 150–250°, with the temp. increasing at 4°/min. The IR and UV spectra were measured in CCl_4 and MeOH solns, respectively. HPLC separations were made on a Waters liquid chromatograph, with a Model 660 solvent flow-programmer. The detector was a LBC-Fluoro-Monitor with excitation at 360 nm and emission at 400–700 nm. The column used was a 4 mm i.d. × 30 cm Bondapak C_{18} and gradient elution programme 9 with 20–50% MeOH in 0.05 M KH_2PO_4 for 10 min and a flow rate of 2 ml/min were used. About 5 mg unfractionated EtOH-extract of various potato zones (compare Table 1) and a soln containing 10 ng fluorescein were mixed and filtered through a 0.5 Millipore filter. Authentic samples of **6** and **12** were obtained as gifts, compound **4** was prepared from **3** [18] and compounds **1–3** and **5** were commercial samples.

Extraction and purification. The blue-fluorescing zone (after inspection in UV light) from potato tubers infected with *Phoma exigua* var. *foveata* was cut out, homogenized and processed according to Scheme 1, yielding compounds **3**, **4**, **6**, **7**, **9–11** and **13–15**.

Identification. Compounds **3**, **4**, and **6** were identical (^{1}H NMR, MS, IR, UV, $[\alpha]_D$) to the respective reference samples.

Compound **13**. Amorphous. ^{1}H NMR (CD_3OD): δ 3.3–4.0 (*m*), 3.90 (*s*, 3H), 4.04 (*s*, 3H), 5.18 (*d*, 1H, anomeric proton, J = 7 Hz), 6.41 (*d*, 1H, J = 10 Hz), 7.06 (*s*, 1H), 7.95 (*d*, 1H, J = 10 Hz). Hydrolysis of **13** with β-glucosidase overnight at room temp., extraction with EtOAc (4×), drying (Na_2SO_4) and evapn gave the aglycone **12**, identical (^{1}H NMR, MS, IR, UV) with the reference sample. The aq. phase was evapd and glucose was identified by PC and GLC as a TMSi derivative.

Compound **10**. Amorphous. MS *m/e* (rel. int.): 218 (M^+) (25), 203 (13), 190 (24), 175 (24), 161 (32), 147 (36), 137 (33), 108 (68), 105 (38), 91 (48).

Compound **11**. Amorphous. MS *m/e* (rel. int.): 216 (M^+) (17), 201 (20), 188 (7), 173 (35), 159 (21), 145 (32), 135 (68), 105 (20), 91 (38).

Compound **14**. Amorphous, $[\alpha]_D^{23}$ −55.0° (*c* 0.1, EtOH). ^{1}H NMR ($CDCl_3$): δ 0.97 (*d*, 3H, J = 7 Hz), 1.18 (*s*, 3H), 1.6–2.4 (*m*, 8*H*), 1.95 (*d*, 3H, J = 1 Hz), 2.67 (*m*, 2*H*), 3.43 (*d*, 1H, J = 12 Hz), 3.55 (*d*, 1H, J = 12 Hz), 5.76 (*br*, 1H). ^{13}C NMR ($CDCl_3$): δ 15.9 (—Me), 21.0 (—Me), 21.7 (—Me), 27.4 (—CH_2—), 34.0 (—CH_2—), 36.9 (—CH_2—), 38.5 (>CH—), 42.8 (—CH_2—), 46.1 (>CH—), 49.9 (—C(—)—), 69.3 (—OCH_2—), 73.6 (—OC(—)—), 125.4 (=CH—), 167.1 (=C<), 199.2 (O=C<). MS *m/e* (rel. int.): 252.173 (M^+) (3.6% of base peak, Calc. for $C_{15}H_{24}O_3$: 252.1725), 237 (3.7), 221 (32), 203 (26), 161 (28), 137 (69). IR: ν_{max} cm^{-1}: 1670, 1615^{-1}. (lit. 1670, 1610 cm^{-1} [28]). UV: λ_{max} nm: 242. (lit.: EtOH 240 nm, [28]).

Acetate **14a**. The aglycone **14** (10 mg) was acetylated (Ac_2O–Py) and purified on a short Si gel column (EtOAc), giving pure acetate (**14a**). ^{1}H NMR ($CDCl_3$): δ

	H_7	$H_{12,12'}$	H_{13}	H_{14}	H_{15}	H_{Ac}
14a	5.76	4.00, 4.02	1.21	1.95	0.97	2.11
14a + Eu(fod)$_3$	7.08 + 7.25	4.96, 5.04 (*br*) (*br*)	1.69 + 1.71	2.38 + 2.42	1.42 + 1.47	2.52 + 2.56

IR: ν_{max} cm^{-1}: 1745, 1670, 1610.

Compound **15**. Amorphous, $[\alpha]_D^{23}$ −75.7° (*c* 0.1, EtOH). ^{1}H NMR (CD_3OD): δ 0.96 (*d*, 3H, J = 7 Hz), 121 (*s*, 3H), 1.5–2.4 (*m*, 8H), 1.99 (*d*, 3H, J = 1 Hz), 2.70 (*m*, 2H), 3.3–4.0 (*m*), 4.30 (*d*, 1H, anomeric proton, J = 7 Hz), 5.74 (*br*, 1H). UV: λ_{max} nm: 242. Hydrolysis with β-glucosidase overnight at room temp., extraction with EtOAc (4×), drying and evapn gave the aglycone **14**, amorphous, identical (^{1}H NMR, MS, IR, UV) with the isolated sample (preceding paragraph).

Acetate **15a**. Compound **15** (40 mg) was acetylated and purified in a similar way to **14**, and the acetate **15a** was obtained. ^{1}H NMR ($CDCl_3$): δ 3.56 (*d*, 1H, anomeric proton, J = 7 Hz), 1.99 (*s*), 2.02 (*s*), 2.03 (*s*), 2.08 (*s*): 4Ac. IR: ν_{max} cm^{-1}: 1756, 1670, 1610.

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