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Elicited cell suspension cultures of apple (*Malus* × *domestica*) cv. Liberty produce biphenyl phytoalexins

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Abstract

Yeast extract treated cell suspension cultures from a scab (*Venturia inaequalis*) resistant apple cultivar, *Malus* × *domestica* cv. Liberty produce dibenzofuran and biphenyl compounds as part of their defense system against fungal invasion. We have isolated and identified three biphenyl derivatives, 4-hydroxy-3,5-dimethoxybiphenyl (aucuparin), 2′,4,-dihydroxy-3,5-dimethoxybiphenyl (2′-hydroxy-aucuparin) and 2′-*O*-β-D-glucopyranosyl-4-hydroxy-3,5-methoxybiphenyl (2′-*o*-β-D-glucopyranosylaucuparin) from the cells and the medium and show here their chemical properties. Although this is the first identification of 2′-glucopyranosyl-aucuparin, its aglycone, 2′-hydroxyaucuparin, and aucuparin have been reported previously [Kokubun, T., Harborne, J.B., Phytochemistry, 1995, 40, 1649–1654.] from fungus infected wood of *Malus* species. Production of an array of dibenzofuran and biphenyl derivatives in response to fungal attack may be an important part of the disease resistance mechanism of scab resistant apple cultivars.

Keywords: *Malus* × *domestica*; Rosaceae; Apple; Cell suspension; Phytoalexin; Biphenyl; Aucuparin; 2′-hydroxyaucuparin; 4-hydroxy-3,5-dimethoxybiphenyl; 2′,4-dihydroxy-3,5-dimethoxybiphenyl; 2′-*O*-β-D-Glucopyranosyl-4-hydroxy-3,5-dimethoxybiphenyl; 2′-*O*-β-D-Glucopyranosylacuparin; Elicitation

1. Introduction

A detailed investigation on phytoalexin induction in the leaves of Rosaceae indicated the presence of antifungal compounds in 47 species (Kokubun & Harborne, 1994). The majority of these compounds appeared to be constitutive metabolites. Although some members of this family have shown the accumulation of biphenyl (Erdtman, Eriksson, & Norin, 1963) or dibenzofuran (Burden, Kemp, Wiltshire, & Owen, 1984) derivatives in their sapwood, only one *Sorbus* species gave an induced phytoalexin response (Kokubun & Harborne, 1994).

In the sapwood of *Malus silvestris* three biphenyl derivatives were detected upon fungal infection (Kokubun & Harborne, 1995). These were reported to be aucuparin, and its 2′- and 4′-methoxy derivatives (Erdtman et al., 1963). *Malus domestica*, *M. baccata*, *M. fusca* and *M. sieboldii* failed to produce any of these compounds. These and subsequent investigations (Kokubun, Harborne, Eagles, & Waterman, 1995a,b) on other members of the Rosaceae lead the authors to conclude that either biphenyl or dibenzofuran derivatives are formed in the different species, but not both simultaneously. It was reported that *Malus* species are always biphenyl producers, while *Pyrus* sp. produce exclusively dibenzofurans (Kokubun & Harborne, 1995).

We have investigated phytoalexin production in scab (*Venturia inaequalis*)-resistant and -susceptible apple cell suspension cultures. In a previous paper (Hrazdina, Borejsza-Wysocki, & Lester, 1997) we have reported the identification of malusfuran (4-*O*-β-D-glucopyranosylerio bofuran), a dibenzofuran glucoside as the major phytoalexin in scab-resistant apple cultivars. Here we report the identification of biphenyl compounds such as aucuparin, its 2′-hydroxy and 2′-glucopyranosyl derivatives that are most likely components of the phytoalexin array of scab resistant apple cell cultures.

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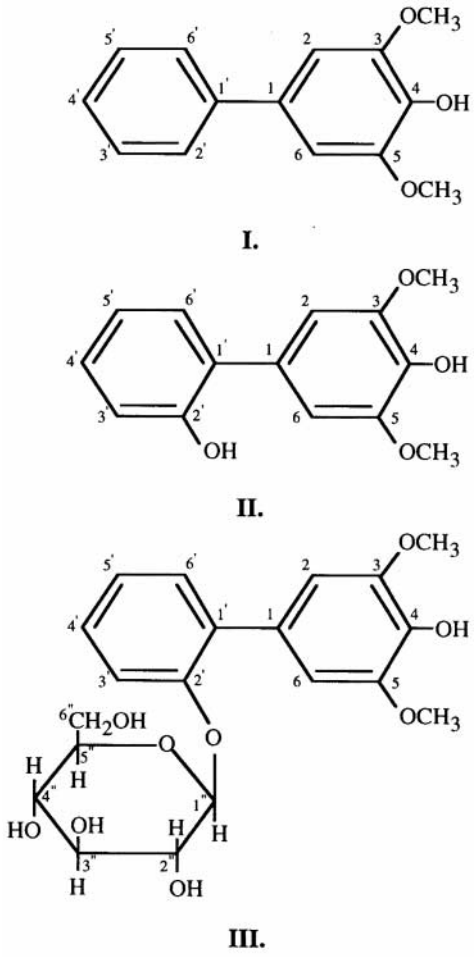

2. Results and discussion

Although members of the Maloidae were reported to accumulate biphenyl and dibenzofuran derivatives (Kokubun & Harborne, 1995), *Malus* species seemed to accumulate exclusively biphenyl phytoalexins such as aucuparin, 2′-methoxyaucuparin and 4′-methoxyaucuparin. Substituted dibenzofuran derivatives, although accumulating in such closely related species as *Pyrus* and *Sorbus*, were not found in the investigated *Malus* species. We have recently established cell suspension cultures of a scab (*Venturia inaequalis*) resistant apple (*Malus* × *domestica*) cultivar 'Liberty' and investigated their response to elicitor treatment (Hrazdina et al., 1997).

Table 1
Chromatographic and spectral properties of aucuparin(I), 2′-hydroxyaucuparin(II) and 2′-*O*-β-D-glucopyranosylaucuparin (III)

R_f values in solvent	I	II	III
A	0.73	0.38	0
B	0.39	0.34	0
C	0.94	0.88	0.31
D	0.91	0.86	0.74
E	–	0.57	0.76
λ_{max} in MeOH	272 (3.07)	260 (3.42)	263 (3.92)
(log ϵ) nm		290 (3.34)	281 sh[a] (3.80)

[a]sh — shoulder; for solvent composition see Section 3.

Scab-resistant 'Liberty' cells responded to elicitor treatment with the synthesis of six new compounds not present in unelicited cells or those of the scab susceptible cultivar McIntosh. The compound produced in the largest amount was identified as 'malusfuran', a dibenzofuran glucoside. We have isolated another three compounds from both the cells and the culture medium and identified their structure as aucuparin (I), 2′-hydroxyaucuparin (II) and 2′-*O*-β-D-glucopyranosyl-aucuparin (III).

The spectral properties of the compounds were similar to those reported for aucuparin and 2′-hydroxyaucuparin, indicating a biphenyl structure. While the TLC R_f values of two compounds indicated that they were most probably aglycones, the migration properties of the third compound indicated that this may contain a sugar substituent (Table 1). Acidic hydrolysis did not change the R_f values of the first two compounds, however, hydrolysis of the third compound produced an aglycone that was similar in its spectral and chromatographic properties to 2′-hydroxyaucuparin. Positive-ion electrospray mass spectrum of the first compound showed an $(M + H)^+$ ion at m/z, 231 indicating that this compound may be aucuparin. The quasi-molecular ion of the second compound was observed at m/z 247, in agreement with a hydroxyaucuparin structure. The electrospray mass spectrum of the aglycone obtained from the third compound showed a molecular ion at m/z 247 indicating a similar structure as compound two. The electrospray mass spectrum of the third compound showed an intense sodium adduct ion at m/z 431, indicating a molecular mass of 408, suggesting the structure of a glucosyl derivative of hydroxyaucuparin.

Nuclear magnetic resonance spectroscopic analysis confirmed the structure of the compounds as aucuparin (Table 2), 2′-hydroxyaucuparin (Table 3) and 2′-*O*-β-D-glucopyranosylaucuparin (Table 4). The ^1H NMR spectrum of aucuparin showed a total of 7 aromatic protons. Resonances at 7.63, 7.40, and 7.27 ppm were assigned to an AA′BB′C spin system. The remaining singlet at 6.87 ppm assigned to H-2 and H-6 indicating chemically equivalent sites containing *ortho* methoxyl groups at positions H-3 and H-5. A single hydroxyl proton observed at a chemical shift of 8.46 ppm was therefore assigned to the C-4 position. This assignment for the hydroxyl group was confirmed with the gHMBC experiment which showed a three-bond coupling of $^3J_{CH} = 5.0$ Hz between H-2/H-6 and the C-4 ^{13}C resonance at 135.5 ppm (Ernst, Wray, Chertkov, & Sergeyev, 1977).

Table 2
^1H and ^{13}C chemical shift assignments for aucuparin(I) in DMSO-d_6. Chemical shift values are referenced to TMS by assigning the solvent resonances to 2.50 ppm for ^1H and 39.50 ppm for ^{13}C

Position	^1H chemical shift (ppm)	^{13}C chemical shift (ppm)
1′		131.7
2′	7.63	126.1
3′	7.40	128.1
4′	7.28	125.8
5′	7.40	128.1
6′	7.63	126.1
1		131.7
2	6.87	103.8
3		148.4
4	8.46	135.5
5		148.4
6	6.87	103.8
OCH_3	3.82	54.9

The ^1H spectrum of 2′-hydroxyaucuparin was similar to that of aucuparin but included an additional hydroxyl proton at 9.34 ppm and a total of 6 aromatic protons consisting of an ABCD spin system on one ring. Placement of the second hydroxyl group on the same ring as the four aromatic protons was confirmed with the gradient heteronuclear multiple-bond correlation (gHMBC) experiment which displayed connectivities between the hydroxyl ^1H at 9.34 ppm and the ^{13}C resonances at 153.9, 127.3 and 115.9 ppm.

The ^1H spectrum of glucopyranosylaucuparin revealed the presence of six aromatic protons. The resonances at 7.35 ppm and 7.19 ppm displayed doublet of doublets coupling patterns and were assigned to H-6′ and H-3′, respectively. An *ortho*-coupling ($^2J_{HH} = 7.60$ Hz) with H-5′ and a *meta*-coupling ($^3J_{HH} = 1.64$ Hz) with H-4′ was observed for H-6′, and an *ortho*-coupling ($^2J_{HH} = 8.26$ Hz) with H-4′ and a *meta*-coupling of ($^3J_{HH} = 0.90$ Hz) with H-5′ was observed for H-3′. A singlet at 6.91 ppm corresponded to equivalent protons H-2 and H-6.

Table 3
^1H and ^{13}C chemical shift assignments for 2′-hydroxyaucuparin (II) in DMSO-d_6

Position	^1H chemical shift (ppm)	^{13}C chemical shift (ppm)
1′		128.6
2′	9.34	153.9
3′	6.89	115.9
4′	7.10	127.3
5′	6.83	119.0
6′	7.25	130.1
1		128.1
2	6.78	106.7
3		147.5
4	8.32	134.6
5		147.5
6	6.78	106.7
OCH_3	3.76	56.0

Table 4
^1H and ^{13}C chemical shift assignments for 2′-*O*-β-D-glucopyranosylaucuparin(III) in DMSO-d_6. Chemical shift values are referenced to TMS by assigning the solvent resonances to 2.50 ppm for ^1H and 39.50 ppm for ^{13}C

Position	^1H chemical shift (ppm)	^{13}C chemical shift (ppm)
1		127.7
2	6.91	107.1
3		147.5
4		134.7
5		147.5
6	6.91	107.1
1′		130.4
2′		153.9
3′	7.19	114.9
4′	7.24	127.8
5′	7.04	121.8
6′	7.35	130.2
1″	5.05	100.1
2″	3.18	73.6
3″	3.33	77.2
4″	3.14	69.6
5″	3.29	76.9
6″	3.65	60.6
3,5-OCH_3	3.77	56.0

Gradient-enhanced heteronuclear multiple-quantum correlation (HMQC) and gHMBC experiments (Hurd & John, 1991; Ruiz-Cabello et al., 1992) were used to establish the ^{13}C assignments and the positions of the substituents of the aromatic rings. Three-bond gHMBC connectivities of the H-4′ and H-6′ resonances with the quaternary ^{13}C resonance at $\delta = 153.9$ ppm placed the glucose moiety at position C-2′. A ^1H–^1H coupling constant of 7.79 Hz for the anomeric signal suggests that a β-type glycosidic linkage exists.

^{13}C chemical shift values for aromatic resonances in addition to HMBC connectivities between the proton resonance at 6.91 ppm and ^{13}C resonances at 147.5 ppm, 134.7 ppm, 130.4 ppm and 127.7 ppm confirm the existence of equivalent protons in the H-2 and H-6 positions. Two-bond HMBC connectivities of H-2 and H-6, and C-3 and C-5 confirm assignment of the six equivalent methoxyl protons ($\delta = 3.77$ ppm) to the C-3 and C-5 positions of the ring. A three-bond connectivity ($^3J_{HH} = 6.7$ Hz) between H-2/H-6 with a quaternary ^{13}C resonance at 134.7 ppm results in the assignment of this chemical shift to C-4 which contains a hydroxyl substituent (Ernst et al., 1977).

Previously we have shown the accumulation of malusfuran, 2,4 methoxy-3-hydroxy-9-*O*-β-D-glucopyranosyldibenzofuran as the major phytoalexin in elicitor treated Liberty cells (Hrazdina et al., 1997). In the same paper we also reported the detection of five other compounds that are made by the cells in response to

elicitor treatment. We have identified the structure of three of these compounds as the biphenyls aucuparin (I), 2′-hydroxyaucuparin (II) and 2′-*O*-β-D-glucopyranosylaucuparin (III). That aucuparin, 2′-hydroxyaucuparin and 2-*O*-β-D-glucopyranosylaucuparin are biosynthetic intermediates in the malusfuran pathway is suggested by their close structural homology to malusfuran, and their transient accumulation in the cells and medium during the response of the cells. Their presence in the cells and in the culture medium indicates that they may serve as components of the scab-resistant apple plants phytoalexin array during the plants response to an attempted fungal invasion. Such a multicomponent phytoalexin response would effectively counter attempted fungal invasion. In such a response both the number of phytoalexins and their concentration is continuously changing, erecting new barriers for the pathogens invading mechanism.

3. Experimental

3.1. Plant material

Cell suspension cultures of Liberty (scab resistant) apple were initiated from friable, soft calli in liquid Murashige–Skoog (MS) basal salt medium on an orbital shaker (120 rpm) as reported earlier (Hrazdina et al., 1997). Cultures were shaken in the dark at 25°C, and cells were harvested and transferred to fresh medium every 7–10 days. Seven days old cells that were in the linear growth phase were elicited with yeast extract (3 g/l) and after 96 h of incubation in the dark cells were separated from the medium by filtration. Cells and media were frozen in liquid nitrogen and stored at −90°C.

3.2. Chemicals

Chemicals used for apple cell suspension cultures (i.e. MS-basal salt mixture; growth regulators; vitamins and myo-inositol); Dowex 1X2-100; β-mercaptoethanol and silica gel TLC plates were from Sigma, St. Louis, MO. Yeast extract was from Difco Laboratories, Detroit, MI. Solvents used for extraction and separation of the compound i.e. ethyl acetate, acetonitrile, methanol, hexane, glacial acetic acid were from Fisher; formic acid was from Aldrich Chem., Milwaukee, WI and sec-butyl alcohol was from Mallinckrodt Baker, Paris, KY.

3.3. Isolation of glucopyranosylaucuparin

3.3.1. From elicited cells

Frozen cells (320 g) were homogenized in a chilled blender with 500 ml of 0.1 M potassium phosphate buffer, pH 8.0, containing 6 mM β-mercaptoethanol for 15 min. The homogenate was centrifuged at 20,000 g for 20 min, Dowex 1X2-100 (9 g/100 ml) was added to the supernatant, stirred for 60 min at 4°C and removed by filtration. The resin was washed with 300 ml distilled water and extracted with methanol (3×300 ml while stirring for 60 min each). The methanolic extract was concentrated in vacuum to dryness. The residue was dissolved in 4 ml methanol, the solution was filtered through a 0.45 mm filter (Gelman Sciences, Ann Arbor, MI) and dried. The dry residue was dissolved in 500 μl methanol, applied as streaks to silica gel TLC plates and separated in hexane:ethyl acetate:methanol (4:4:2). The plates were dried, glucopyranosylaucuparin with the R_f of 0.31 was scraped off the plate, and eluted from the silica with 3×1 ml methanol. The methanolic extract was filtered through a 0.45 μm filter and dried. Yield: 8.4 mg (26 μg/g fr.wt.).

3.3.2. From the medium

The medium after removal of the cells (5.6 l) was extracted with ethyl acetate (3 l), the ethyl acetate layer dried over solid sodium sulfate, filtered and evaporated to dryness. The residue was dissolved in 4 ml methanol, filtered, dried and redissolved in 500 μl methanol for further purification by TLC on silica gel plates as above. Yield of glucopyranosylaucuparin was 6.1 mg.

3.4. Isolation of aucuparin and 2′-hydroxyaucuparin

To obtain larger amounts of (I) and (II) for structure identification, a similar volume (5 l) of the medium was extracted with ethyl acetate as above, and the extract was evaporated to dryness. The residue was dissolved in 1 ml MeOH and subjected to chromatography on a silica gel column (1×15 cm, Silica Gel 60, Merck, Darmstadt, Germany) that was equilibrated in hexane. The column was eluted stepwise with hexane (40 ml) and a mixture of hexane:ethyl acetate (1:1) (40 ml). The hexane: ethyl acetate fraction was evaporated to dryness, the residue was dissolved in 7 ml ethyl acetate and 21 ml of hexane was added. After storing at 4°C for 72 h a precipitate was filtered off and the filtrate was concentrated to approximately 1 ml. The concentrate was applied to silica gel TLC plates and the plates were developed in a mixture of chloroform:acetone (19:1). UV-absorbing bands with the R_f of 0.73 and 0.43 respectively, were scraped off the plate, eluted with 3 ml of the above solvent and evaporated to dryness. Yield: 9.5 mg aucuparin (I) and 10.2 mg 2′-hydroxyaucuparin (II).

3.5. Acidic hydrolysis of 2′-O-β-D-glucopyranosylaucuparin

One mg of 2′-*O*-β-D-glucopyranosylaucuparin was hydrolyzed in 10 ml of 1 M HCl as described (Hrazdina et al., 1997).

3.6. Thin layer chromatography

TLC was carried out on 250 μm silica gel glass plates containing fluorescent indicator using (A) chloroform/acetone (19:1); (B) hexane/ethyl acetate/methanol (60:40:1); (C) hexane/ethyl acetate/methanol (4:4:2); (D) *sec*. butyl alcohol/glacial acetic acid/water (70:2:28) and (E) water/glacial acetic acid (98:2) as solvents. Chromatograms were dried and viewed under UV (254 and 360 nm).

3.7. High pressure liquid chromatography

HPLC was performed on a Tracor HPLC system consisting of a 980A solvent programmer, a 950 chromatographic pump and a 970A variable wavelength detector. Separations were monitored and peaks quantitated by a Spectra-Physics computing integrator. Twenty μl of sample was analyzed by reversed phase C_{18} μBondapack column (4.6×250 mm; Alltech, Deerfield, IL). Conditions and solvent systems were identical to those used earlier for malusfuran analysis (Hrazdina et al., 1997).

3.8. UV spectra

UV spectra were recorded in methanol using a Spectronic Genesys-2 Spectrophotometer (Spectronic Instruments, Rochester, NY).

3.9. Mass spectrometry

Electrospray mass spectra were recorded on a Micromass Quattro I instrument. Samples were introduced as solutions in 50:50 acetonitrile: water (Cone voltage 40 V; Source temperature 90°C; Capillary voltage 3.8 kV).

Compound (I) (positive ion): *m*/*z*, [%]. 483 [30, $(2M + Na)^+$], 269 [40, $(M + K)^+$], 253 [100, $(M + Na)^+$], 231 [10, $(M + H)^+$]. Compound (II) (positive ion): 285 [43, $(M + K)^+$], 269 [100, $(M + Na)^+$]. 247 [10, $(M + H)^+$]. Compound (II) (negative ion): 491 [5, $(2M–H)^-$], 245 [100, $(M–H)^-$]. Compound (III) (positive ion): 839 [8, $(2M + Na)^+$], 431 [100, $(M + Na)^+$].

3.10. Nuclear magnetic resonance analysis

One- and two-dimensional homonuclear ^{1}H-NMR and gradient-enhanced ^{1}H and ^{13}C spectra were recorded on a Varian Unity 500 spectrometer operating at 499.93 MHz for proton and 125.72 MHz for carbon. $^{13}C\{^1H\}$ one dimensional spectra were recorded on a Varian VXR-400S spectrometer operating at 100.53 MHz for carbon and 399.76 MHz for proton. ^{1}H and ^{13}C chemical shift assignments are listed in Tables 2–4.

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