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Differential Effect of Elicitors on Biphenyl and Dibenzofuran Formation in *Sorbus aucuparia* Cell Cultures

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The Rosaceous subtribe Pyrinae (formerly subfamily Maloideae) is well-known for its economically important fruit trees, such as apple and pear, and also includes *Sorbus aucuparia*. Elicitor-treated *S. aucuparia* cell cultures are used to study the biosynthesis of the Pyrinae-specific phytoalexins, biphenyls and dibenzofurans. Three biphenyls (aucuparin, noraucuparin, 2′-hydroxyaucuparin) and a dibenzofuran (eriobofuran) were isolated and structure elucidated using GC-MS and NMR. A second dibenzofuran of low abundance was tentatively assigned as noreriobofuran. Treatment of *S. aucuparia* cell cultures with yeast extract induced the formation of aucuparin as the major phytoalexin. In contrast, addition of preparations from the fire blight bacterium, *Erwinia amylovora*, and the scab-causing fungus, *Venturia inaequalis*, resulted in accumulation of eriobofuran as the major inducible constituent. Methyl jasmonate was a poor elicitor. The observations are suggestive of a biogenic relationship between biphenyls and dibenzofurans. Elicitor-treated *S. aucuparia* cell cultures provide an interesting in vitro system for studying biphenyl and dibenzofuran metabolism in the economically valuable Pyrinae.

KEYWORDS: *Sorbus aucuparia*; Pyrinae; biphenyl; dibenzofuran; phytoalexin; elicitor; cell culture

INTRODUCTION

Sorbus aucuparia (rowan, mountain ash) is a species of the Rosaceous subtribe Pyrinae (formerly subfamily Maloideae) (*1*), which includes a number of economically important fruit trees, such as apple (*Malus domestica*) and pear (*Pyrus communis*). *S. aucuparia* is grown as an ornamental tree, fruit crop, and medicinal plant (*2*,*3*). In response to pathogen attack, the Pyrinae form biphenyls and dibenzofurans as phytoalexins (**Figure 1**) (*4*). Notably, species of other Rosaceous taxa failed to produce biphenyls and dibenzofurans after infection (*4*). The ability to produce these two classes of inducible defense compounds is thus confined to the Pyrinae. Interestingly, biphenyls and dibenzofurans commonly accumulate in the sapwood (*4*). So far, 9 biphenyls and 17 dibenzofurans have been isolated from the Pyrinae (*4*−*11*).

In a few species of the Pyrinae, biphenyl and dibenzofuran phytoalexins were also found in tissues other than the sapwood. Diseased *S. aucuparia*, *Photinia glabra*, and *Rhaphiolepsis umbellata* formed the defense compounds in leaves (*4*, *12*−*15*). In *Eriobotrya japonica*, the biphenyl aucuparin was detected in the cortex and a dibenzofuran structure was found in the leaves; no phytoalexins were present in the sapwood (*4*, *11*, *12*). In rare cases, biphenyls and dibenzofurans were not detected as inducible phytoalexins but as constitutive compounds. The heartwood of healthy *S. aucuparia* contained aucuparin and 2′-methoxyaucuparin, and the bark of *Crataegus monogyna* formed two dibenzofurans (*8*, *16*, *17*). Outside the Pyrinae, biphenyls and dibenzofurans also occur as constitutive metabolites, that is, phytoanticipins. They were isolated from members of numerous families, such as Fabaceae, Alliaceae, Polygalaceae, Clusiaceae, and Berberidaceae (*18*−*22*).

Pathogen-infected Pyrinae form either biphenyls or dibenzofurans in the sapwood (*4*). Therefore, these authors concluded that the two classes of phytoalexins, although structurally closely related, are formed by parallel, rather than by sequential, pathways. *Malus* was always a biphenyl producer, and *Pyrus* exclusively formed dibenzofurans (*4*). Diseased *M. domestica*, *Malus sieversii,* and *Malus sylvestris* contained up to three biphenyls (*4*). Three dibenzofurans were isolated from the sapwood of *P. communis* (perry pear) after infection with the fungus *Chondrostereum purpureum*, the causative agent of silver leaf disease (*23*, *24*).

Co-occurrence of the two classes of phytoalexins in the Pyrinae has so far been observed only with in vitro cultures of *M. domestica* (*10*, *25*). Cell cultures derived from a scab-resistant apple cultivar (Liberty) simultaneously accumulated biphenyls and dibenzofurans in response to elicitation with either yeast extract or a preparation from the scab-causing fungus. The major phytoalexins isolated were the biphenyl aucuparin and the dibenzofuran malusfuran. Interestingly, cell cultures derived from a scab-susceptible cultivar (McIntosh) did not respond to elicitation with phytoalexin formation. Very recently, biphenyls

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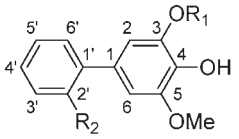
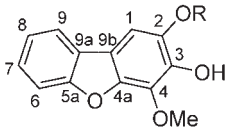


	Biphenyl	R_1	R_2
1	aucuparin	Me	H
2	noraucuparin	H	H
3	2'-hydroxyaucuparin	Me	OH



	Dibenzofuran	R
4	eriobofuran	Me
5	noreriobofuran	H

Figure 1. Chemical structures of biphenyls and dibenzofurans isolated from elicitor-treated *S. aucuparia* cell cultures.

and dibenzofurans have been isolated from the trunk of *Berberis koreana* (Berberidaceae); however, the tissue-specific localization of the compounds remains open (*22*).

The defensive role of biphenyls and dibenzofurans in woody tissue was demonstrated in inhibition assays. Phytoalexin concentrations supposed to be present at localized infection sites inhibited both spore germination and mycelial growth (*7*, *10*, *12*). For example, the dibenzofuran eriobofuran isolated from fungus-inoculated loquat leaves exhibited a strong inhibitory effect on spore germination and germ tube growth of *Pestalotia funereal*, a pathogenic fungus of *E. japonica* (*12*). Several biphenyls (aucuparin, 4′-methoxyaucuparin, rhaphiolepsin) inhibited spore germination and hyphal growth of the pathogenic fungi *Colletotrichum lindemuthianum* and *P. funereal* (*14*, *26*, *27*). In terms of fungitoxicity, biphenyls and dibenzofurans have activities comparable with those of the well-known isoflavonoid phytoalexins of the Fabaceae (*28*).

S. aucuparia cell cultures were established to initiate studies of biphenyl biosynthesis (*29*). The enzyme that forms 3,5-dihydroxybiphenyl was detected in yeast-extract-treated cells and named biphenyl synthase (BIS). Subsequently, a BIS cDNA was cloned and the recombinant enzyme was functionally expressed in *Escherichia coli* (*30*). BIS is a type III polyketide synthase and is rapidly and transiently induced at the transcriptional level by elicitation. The enzyme catalyzes the iterative condensation of benzoyl-CoA with three malonyl-CoAs to give a linear tetraketide intermediate, which then undergoes intramolecular aldol condensation and loss of the terminal carboxyl group to give 3,5-dihydroxybiphenyl (**Figure 2**). No downstream enzymes metabolizing this BIS product have so far been detected.

Here we report differential accumulation of biphenyl and dibenzofuran phytoalexins under the regimen of various elicitors in cell cultures of *S. aucuparia*.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical reagent purity and purchased from Fluka (Buchs, Switzerland) and Sigma-Aldrich (Steinheim, Germany). Solvents were of HPLC grade purity and used without further purification.

Cell Culture Conditions and Elicitation. Cell cultures of *S. aucuparia* were grown in the dark as described previously (*29*). Elicitor treatments were

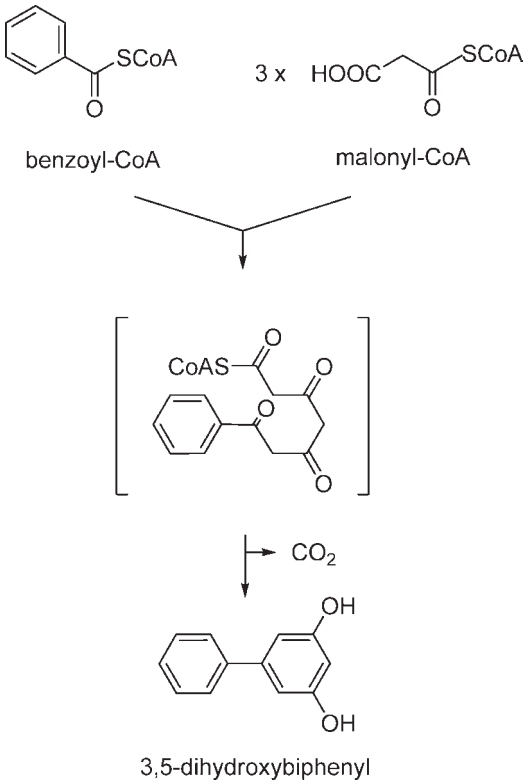


Figure 2. Biosynthesis of 3,5-dihydroxybiphenyl catalyzed by biphenyl synthase.

carried out at day 5 after transfer of cells into new medium. A chitosan stock solution (*31*) was used at a final concentration of 25 mg/L. Methyl jasmonate was diluted in ethanol (10 mM) and applied at a final concentration of 100 μM. Yeast extract (150 mg) was dissolved in water (1 mL) and added to cell cultures after sterile filtration (3 g/L). *Erwinia amylovora* was kindly supplied by the Julius Kühn-Institut (Institute for Breeding Research on Horticultural and Fruit Crops, Dresden, Germany). An aliquot (2 mL) of an autoclaved suspension (10^9 cfu/mL) was added to the cell cultures (50 mL). *Venturia inaequalis* was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The fungal elicitor was prepared as described previously (*32*) and applied at a final concentration of 50 mg/L. All elicitor concentrations used were found to achieve maximum effects in preliminary studies. Treated cells were harvested 48 h postelicitation when maximum phytoalexin levels were observed, and their constituents were extracted. All treatments were performed in triplicate, and average values of phytoalexin concentrations were calculated.

Extraction Procedure. Freshly harvested cells (8 g) were homogenized in 15 mL of methanol. After filtration, the residue was extracted twice with 10 mL methanol. For quantification, 4-phenylphenol (0.1 mg) was added as an internal standard to the methanolic extract, which then was concentrated to dryness under reduced pressure. The residue was dissolved in 1 mL of ethyl acetate, filtered through a 0.2 μm syringe filter, and analyzed by GC-MS. The medium of the cell cultures was extracted twice with 50 mL of ethyl acetate. The combined organic phases were dried over anhydrous sodium sulfate, filtered, and concentrated to dryness under reduced pressure. The residue was dissolved in 1 mL of ethyl acetate, filtered through 0.2 μm syringe filters, and analyzed by GC-MS.

Isolation of Biphenyls and Dibenzofurans. Phytoalexins were isolated from elicitor-treated cell cultures. Cells were harvested 48 h postelicitation and extracted as described under Extraction Procedure. Organic extracts of multiple inductions were pooled and preseparated on a silica gel column (10 × 2.5 cm; 20 g silica gel) to remove interfering lipids using pentane/ethyl acetate, 1:2 (v/v), as a solvent system. Fractions containing biphenyls and dibenzofurans were identified by GC-MS, combined, and subjected to HPLC for final purification. The system consisted of a 1525 binary HPLC pump, which was coupled with a 2487 dual absorbance detector and equipped with Breeze software 3.20 (Waters, Eschborn,

Table 1. ^{13}C NMR Spectroscopic Data (151 MHz) for Aucuparin (**1**) and Noraucuparin (**2**) and ^{1}H NMR Spectroscopic Data (600 MHz) for 2′-Hydroxyaucuparin (**3**)

	aucuparin (**1**)		noraucuparin (**2**)		2′-hydroxaucuparin (**3**)
position	δ_C, mult	δ_H (J in Hz)	δ_C, mult	δ_H (J in Hz)	δ_H (J in Hz)
1	132.9, C		133.6, C		
2	104.1, CH	6.80, s	107.7, CH	6.86, d (1.9)	6.66, s
3	147.3, C		144.0, C		
4	134.4, C		132.0, C		
5	147.3, C		147.1, C		
6	104.1, CH	6.80, s	102.2, CH	6.70, d (1.9)	6.66, s
1′	141.5, C		141.1, C		
2′	126.93, CH	7.54, m	126.86, CH	7.53, m	
3′	128.7, CH	7.42, m	128.7, CH	7.41, m	6.99, ddd (8.1, 1.2, 0.4)
4′	126.95, CH	7.32, m	126.94, CH	7.31, m	7.26, ddd (8.1, 7.4, 1.7)
5′	128.7, CH	7.42, m	128.7, CH	7.41, m	6.98, ddd (1.2, 7.4, 7.5)
6′	126.93, CH	7.54, m	126.86, CH	7.53, m	7.23, ddd (0.4, 1.7, 7.5)
OMe	56.4, CH_3	3.96, s	56.2, CH_3	3.95, s	3.92, s
OH		5.55, s		5.40, s	5.60, s
				5.34, s	5.32, s

Germany). Water containing 0.1% (v/v) formic acid (A) and methanol (B) served as the solvents on a Symmetry C_{18} (5 μm) column (150 mm long, 4.6 mm i.d.; Waters). The gradient was 50% B for 30 min, 50−90% B in 1 min, 90% for 5 min, 90−50% in 1 min, and 50% for 8 min at a flow rate of 0.8 mL/min. The detection wavelength was 280 nm. Corresponding peaks of multiple runs were collected, combined, and concentrated at room temperature under a stream of nitrogen gas. The individual fractions were analyzed by NMR and GC-MS.

Derivatization and Quantitative GC-MS Analysis. Aliquots of the organic extracts were transferred to micro GC vials and concentrated under a stream of nitrogen gas at room temperature. The residue was dissolved in 40 μL of MSTFA (ABCR, Karlsruhe, Germany) and derivatized at 60 °C for 30 min. The resulting mixtures were directly analyzed by GC-MS, as described previously (*33*), except that the split ratio ranged from 1:5 to 1:10. The quantitative determination of the individual compounds (**1**−**5**) was based on the internal standard 4-phenylphenol. A response factor of 1 was assumed for all compounds. Hence, the quantification reflects the relative amount of each compound. The standardized sample preparation allowed a fast and direct quantitative comparison of all individual induction experiments.

NMR. NMR spectra (600 MHz ^{1}H and 151 MHz ^{13}C) of $CDCl_3$ solutions of the natural products were obtained on a Bruker Avance II 600 spectrometer with a 5 mm TCI CryoProbe at a sample temperature of 20 °C. Internal chemical shift references were tetramethylsilane ($\delta_H = 0.00$ ppm) and the solvent (δ_C = 77.01 ppm). Sample concentrations were of the order of 1−5 mg/0.5 mL for **1**, **2**, and **4** and ≪1 mg/0.5 mL for **3**. The iterative analysis of the ^{1}H NMR spectrum of **3** was carried out with the program TopSpin 2.1 (Bruker BioSpin, Rheinstetten, Germany, 2008). NMR spectra of synthetic samples were recorded on a Bruker Avance DRX-400 spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C) at 25 °C.

Aucuparin (*1*)*:* ^{1}H and ^{13}C NMR (see **Table 1**); MS (70 eV), *m*/*z* (% rel abundance) 230 (100, $[M]^+$), 215 (44, $[M]^+ - 15$), 187 (50), 172 (17), 169 (8), 155 (5), 144 (8), 141 (8), 127 (8), 115 (18); MS of mono-TMS derivative (70 eV), *m*/*z* (% rel abundance) 302 (46, $[M]^+$), 287 (16, $[M]^+ - 15$), 273 (25), 272 (100), 230 (5), 229 (18), 199 (2), 155 (1), 136 (5), 115 (4), 99 (3), 89 (2), 73 (13), 45 (4); RI (ZB5-MS) 2086.

Noraucuparin (*2*)*:* ^{1}H and ^{13}C NMR (see **Table 1**); MS of di-TMS derivative (70 eV), *m*/*z* (% rel abundance) 360 (94, $[M]^+$), 345 (23, $[M]^+ - 15$), 331 (18), 330 (58), 315 (6), 287 (5), 272 (12), 229 (13), 197 (2), 147 (4), 133 (6), 99 (3), 73 (100), 59 (3), 45 (9); RI (ZB5-MS) 2119.

2′-Hydroxyaucuparin (*3*)*:* ^{1}H NMR (see **Table 1**); MS of di-TMS derivative (70 eV), *m*/*z* (% rel abundance) 390 (90, $[M]^+$), 375 (13, $[M]^+ - 15$), 361 (35), 360 (100), 345 (7), 330 (29), 287 (63), 256 (5), 204 (5), 147 (9), 105 (13), 77 (18), 75 (19), 73 (54), 45 (8); RI (ZB5-MS) 2190.

Eriobofuran (*4*)*:* ^{1}H and ^{13}C NMR (see **Table 2**); ^{13}C NMR (150 MHz, $CDCl_3$); MS of mono-TMS derivative (70 eV), *m*/*z* (% rel abundance) 316 (50, $[M]^+$), 301 (7, $[M]^+ - 15$), 287 (23), 286 (100), 271 (12), 243 (15), 213 (2), 187 (4), 161 (2), 143 (4), 129 (3), 101 (2), 73 (13), 45 (3); RI (ZB5-MS) 2223.

Noreriobofuran (*5*, *tentatively identified*)*:* MS of di-TMS derivative (70 eV), *m*/*z* (% rel. abundance) 374 (87, $[M]^+$), 359 (12 $[M]^+ - 15$), 345 (33), 344 (100), 329 (9), 301 (7), 286 (10), 271 (7), 243 (9), 187 (3), 147 (3), 133 (4), 75 (10), 73 (66), 45 (10); RI (ZB5-MS) 2251.

Preparation of 3,4,5-Trimethoxybiphenyl (6). 5-Bromo-1,2,3-trimethoxybenzene (2.47 g; 10 mmol) and tetrakis(triphenylphosphine) palladium (720 mg, 0.6 mmol) were dissolved in toluene (75 mL) and stirred under nitrogen atmosphere at room temperature. Thirty milliliters of Na_2CO_3 (2 M) followed by a solution of phenylboronic acid (1.34 g, 11 mmol) in 40 mL of ethanol was added dropwise and the mixture refluxed for 2 h. After cooling to room temperature, the excess of boronic acid was quenched by the addition of 3 mL of hydrogen peroxide solution (30%). The mixture was made basic by the addition of 30 mL of NaOH (2 M) and extracted with CH_2Cl_2. The organic phase was concentrated under reduced pressure and purified by flash chromatography on silica gel (ethyl acetate/*n*-pentane, 1:1). The synthesis was based on a published procedure (*34*).

^{1}H NMR (400 MHz, $CDCl_3$), δ 7.55 (m, 2H), 7.44 (m, 2H), 7.34 (m, 1H), 6.78 (s, 2H), 3.92 (s, 6H), 3.90 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$), δ 153.4, 141.4, 137.6, 137.2, 128.7, 127.4, 127.1, 104.5, 60.9, 56.2; MS (70 eV), *m*/*z* (% rel abundance) 244 (100, $[M]^+$), 129 (70), 214 (2), 201 (21), 186 (9), 171 (9), 152 (3), 141 (8), 127 (4), 115 (20); RI (ZB5-MS) 2010.

Preparation of 3,4,5-Trihydroxybiphenyl (7). Methyl groups were removed by adding 3 equiv of BBr_3 in CH_2Cl_2 to **6** cooled to −80 °C. The reaction was terminated by the addition of 75 mL of H_2O, and the organic phase was washed with 30 mL of 1 M $Na_2S_2O_3$ and then with 25 mL of water. After removal of the organic solvent, **7** was purified on silica gel (ethyl acetate/*n*-pentane, 1:1). The synthesis was based on a published procedure (*35*).

^{1}H NMR (400 MHz, $CDCl_3$), δ 7.49 (m, 2H), 7.39 (m, 2H), 7.31 (m, 1H), 6.74 (s, 2H), 5.33 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$), δ 144.1, 140.5, 133.8, 131.2, 128.7, 127.0, 126.7, 108.1; MS of tri-TMS derivative (70 eV), *m*/*z* (% rel abundance) 418 (100, $[M]^+$), 403 (7, $[M]^+ - 15$), 330 (6), 315 (53), 278 (20), 269 (3), 195 (3), 147 (5), 133 (6), 73 (42). RI (ZB5-MS) 2133.

Preparation of Aucuparin (1). Two hundred and fifty milligrams of **7** was dissolved in 25 mL of 10 mM NaOH and stirred on ice while 15 equiv of $(CH_3O)_2SO_2$ was added dropwise. After 30 min, the reaction was stopped by the addition of 1 mL of HCl (2 M). Compounds were extracted twice by the same volume of ethyl acetate (*36*). Aucuparin (**1**) was separated from the side products by column chromatography on silica gel (ethyl acetate.*n*-pentane, 1:9).

^{1}H NMR (400 MHz, $CDCl_3$), δ 7.54 (m, 2H), 7.43 (m, 2H), 7.32 (m, 1H), 6.80 (s, 2H), 5.55 (s, 1H), 3.96 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$), δ 147.3, 141.5, 134.4, 132.9, 128.7, 126.945, 126.937, 104.1, 56.4; MS (70 eV), *m*/*z* (% rel abundance) 230 (100, $[M]^+$), 215 (44, $[M]^+ - 15$), 187 (50), 172 (17), 169 (8), 155 (5), 144 (8), 141 (8), 127 (8), 115 (18); RI (ZB5-MS) 2094; MS of mono-TMS derivative (70 eV), *m*/*z* (% rel abundance) 302 (46, $[M]^+$), 287 (15, $[M]^+ - 15$), 273 (26), 272 (100), 230 (4), 229 (18), 199 (2), 155 (1), 136 (5), 115 (4), 99 (2), 89 (2), 73 (12), 45 (3); RI (ZB5-MS) 2087.

RESULTS AND DISCUSSION

Elicitation of Phytoalexin Formation in *S. aucuparia* Cell Cultures. In addition to the previously used yeast extract (3 g/L) (*29*), four elicitors were tested for their potential to elicit phytoalexin biosynthesis in cultured *S. aucuparia* cells. Although widely used, methyl jasmonate (100 μM) and chitosan (25 mg/L) were poor elicitors, whereas an autoclaved suspension of *E. amylovora* (4×10^{10} cfu/L) and a preparation from *V. inaequalis* (50 mg/L) induced high levels of phytoalexins. The bacterium *E. amylovora* is the causative agent of fire blight, the most devastating disease of fruit trees (*37*). Fire blight leads to serious losses around the world and is still spreading due to the lack of a chemical that can appropriately control the disease. The fungus *V. inaequalis* causes scab and has invaded all apple-growing regions (*38*). Apple scab leads to significant yield losses and crop failures. In *S. aucuparia* cell cultures, application of these

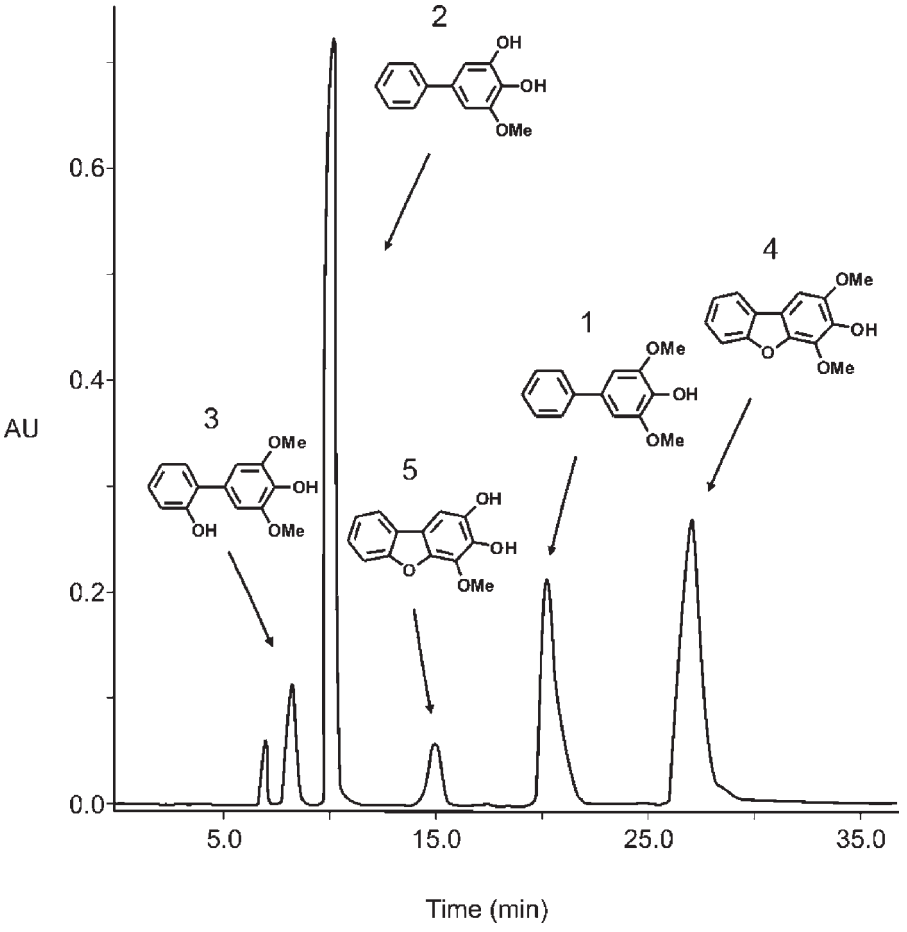


Figure 3. RP-HPLC separation of biphenyls and dibenzofurans present in methanolic extracts from elicitor-treated *S. aucuparia* cell cultures.

elicitors resulted in the accumulation of three biphenyls and two dibenzofurans.

Structure Elucidation of Biphenyl and Dibenzofuran Phytoalexins. Due to the high phytoalexin levels, extracts from elicitor-treated cells were used to isolate three biphenyls and a dibenzofuran by a combination of column chromatography on silica gel and semipreparative high-performance liquid chromatography (HPLC) on C_{18} reversed-phase material (RP-C18; **Figure 3**). The isolated compounds were analyzed and characterized by NMR and GC-MS after trimethylsilyl (TMS) derivatization. ^{13}C NMR and ^{1}H NMR spectra including two-dimensional HSQC and HMBC spectra were obtained for aucuparin (**1**), noraucuparin (**2**), and eriobofuran (**4**) (**Tables 1** and **2**). The isolated amount of 2′-hydroxyaucuparin (**3**) was only sufficient to obtain ^{1}H NMR data (**Table 1**).

An authentic reference of aucuparin (**1**) was prepared via chemical synthesis. A tetrakis(triphenylphosphine)palladium-catalyzed Suzuki coupling generated 3,4,5-trimethoxybiphenyl. All methoxy groups were removed by a BBr_3 treatment, and the resulting 3,4,5-trihydroxybiphenyl was partially methylated with dimethyl sulfate. The resulting mixture of methylated biphenyls was separated on silica gel to yield pure **1**. The identity of **1** with aucuparin was concluded from the comparison of the ^{1}H NMR spectrum measured in $CDCl_3$ solution (**Table 1**) with literature data (*6*, *25*). In addition, assignments were achieved using 2D NMR techniques (HSQC and HMBC spectra). A number of discrepancies between our ^{13}C NMR data and reported data (*25*) were found, namely, four smaller chemical shift deviations (1.1−1.5 ppm) and two larger ones (2.7 and 6.0 ppm), the latter being too large for a solvent effect. Also, the previously published order of assignment of the C-1′ and C-4 NMR signals (*25*) is reversed compared to ours, which we have secured by HMBC correlations between C-1′ and H-2,6 and H-3′,5′, on the one hand, and between C-4 and H-2,6 and 4-OH, on the other hand.

Table 2. ^{13}C NMR Spectroscopic Data (151 MHz) for Eriobofuran (**4**)

position	δ_C, mult	δ_H (*J* in Hz)
1	96.2, CH	7.11, s
2	144.7, C	
3	137.5, C	
4	132.6, C	
4a	142.6, C	
5a	156.1, C	
6	111.6, CH	7.55, ddd (8.2, 0.9, 0.7)
7	125.7, CH	7.37, ddd (8.2, 7.3, 1.3)
8	122.6, CH	7.29, ddd (0.9, 7.3, 7.6)
9	119.7, CH	7.82, ddd (0.7, 1.3, 7.6)
9a	124.7, C	
9b	116.0, C	
2-OMe	56.8, CH_3	3.95, s
4-OMe	61.0, CH_3	4.26, s
OH		5.83, s

The ^{1}H and ^{13}C NMR spectra of **2** were also fully assigned by 2D techniques and showed that the compound is noraucuparin (**Table 1**). This biphenyl has only recently been isolated from *Berberis koreana* (*22*). However, the ^{13}C NMR spectrum reported shows large deviations from our data, which were also measured in $CDCl_3$ solution (five chemical shift deviations of 2.5−5.2 ppm). The Supporting Information accompanying ref *22* contains the ^{13}C NMR spectrum of **2**, which is of low quality, in particular with regard to the signal-to-noise ratio. Hence, mistaking spurious

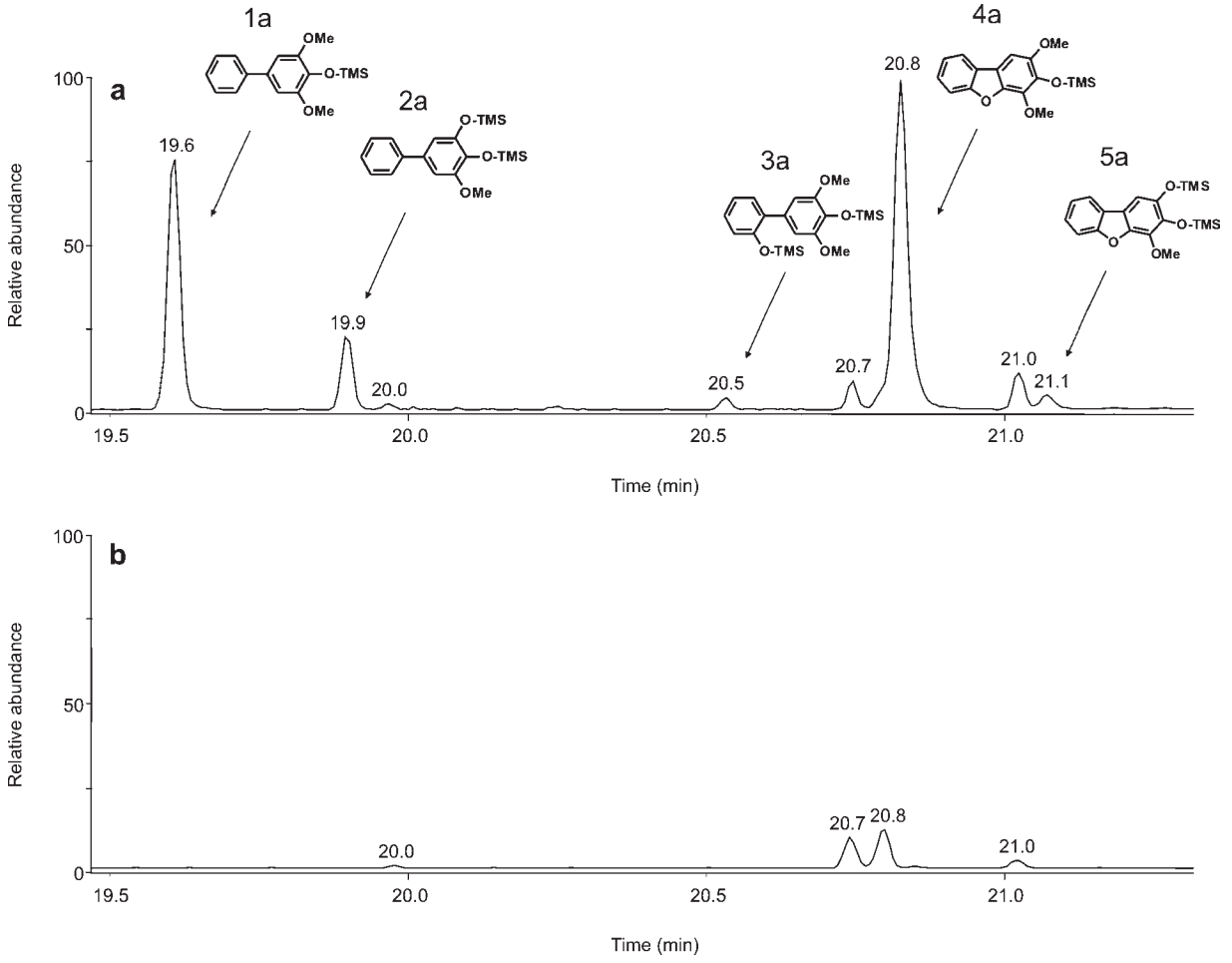


Figure 4. GC-MS analysis of methanolic extracts from *E. amylovora*-treated (**a**) and untreated (**b**) *S. aucuparia* cell cultures. Compounds were separated as trimethylsilyl (TMS) derivatives numbered **1a**−**5a**.

noise peaks for chemical shifts may have caused the deviations mentioned, although their cumulation is rather surprising.

The isolated amount of **3** was too small to obtain a ^{13}C NMR spectrum, despite using a cryo-probehead at 600 MHz. The ^{1}H NMR spectrum ($CDCl_3$ solution) was simulated and iteratively refined. It proved **3** to have the structure of 2′-hydroxyaucuparin (**Table 1**). The literature so far reports a 60 MHz spectrum of a solution in acetone-d_6 and a 500 MHz spectrum in DMSO-d_6 (*25*, *39*).

Compound **4** was shown to have the structure of eriobofuran by complete assignment of its ^{1}H and ^{13}C NMR spectra in $CDCl_3$ solution using 2D techniques (HSQC and HMBC; **Table 2**). There was good agreement with the chemical shifts and signal assignments reported for a $CDCl_3$ solution (*22*). However, major ^{13}C chemical shift differences (four deviations in the range of 1.9−3.0 ppm and three in the range of 1.1−1.6 ppm) were found in comparison to earlier data (*7*).

The concentration of a second dibenzofuran detected by GC-MS was too low for isolation and NMR characterization. However, on the basis of the MS data and the context of structures that were unambiguously identified in cultured *S. aucuparia* cells, this compound was tentatively assigned as noreriobofuran (**5**).

GC-MS Quantification of Differentially Induced Phytoalexins. To improve the chromatographic peak shape and the sensitivity, aliquots of the methanolic extracts from elicitor-treated *S. aucuparia* cell cultures were derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), giving trimethylsilyl (TMS) derivatives of the compounds of interest. On the basis of the NMR results, the purified compounds were used as authentic reference substances to generate MS and retention index data for the TMS derivatives. The internal standard, 4-phenylphenol, was added to each workup before extraction. A response factor of 1 was assumed. This method was used to study qualitative and quantitative changes in the pattern of biphenyls and dibenzofurans that were induced in *S. aucuparia* cell cultures by the addition of various elicitors (**Figure 4**). The standardized conditions allowed for a direct quantitative comparison of the results obtained with different elicitors.

As previously observed (*29*), addition of yeast extract to *S. aucuparia* cell cultures led to the accumulation of one major constituent (8.0 μg/g DW; **Figure 5**). This compound was here identified as aucuparin (**1**). Prior to elicitor treatment, cultured cells were devoid of detectable amounts of biphenyls and dibenzofurans. Chitosan was a relatively poor elicitor; however, it mainly induced the formation of noraucuparin (**2**; 2.5 μg/g DW). A dramatic qualitative change in the pattern of the accumulated phytoalexins was observed with an autoclaved suspension of *E. amylovora* and a preparation from *V. inaequalis* (**Figure 5**). These elicitors strongly induced the biosynthesis of eriobofuran (**4**). This dibenzofuran was the major constituent after treatment with both the bacterial and the fungal pathogens. The concentrations of **1** and **2** were also relatively high. Thus, the two Pyrinae-specific pathogens turned out to be the most efficient elicitors of phytoalexin formation in *S. aucuparia* cell cultures. After treatment with *E. amylovora*, the concentration of **4** reached 8.3 μg/g DW, and the levels of **1** and **2** were 5.2 and 3.1 μg/g DW, respectively. Compounds **3** and **5** were minor components. Methyl jasmonate induced only traces of biphenyls. Previously,

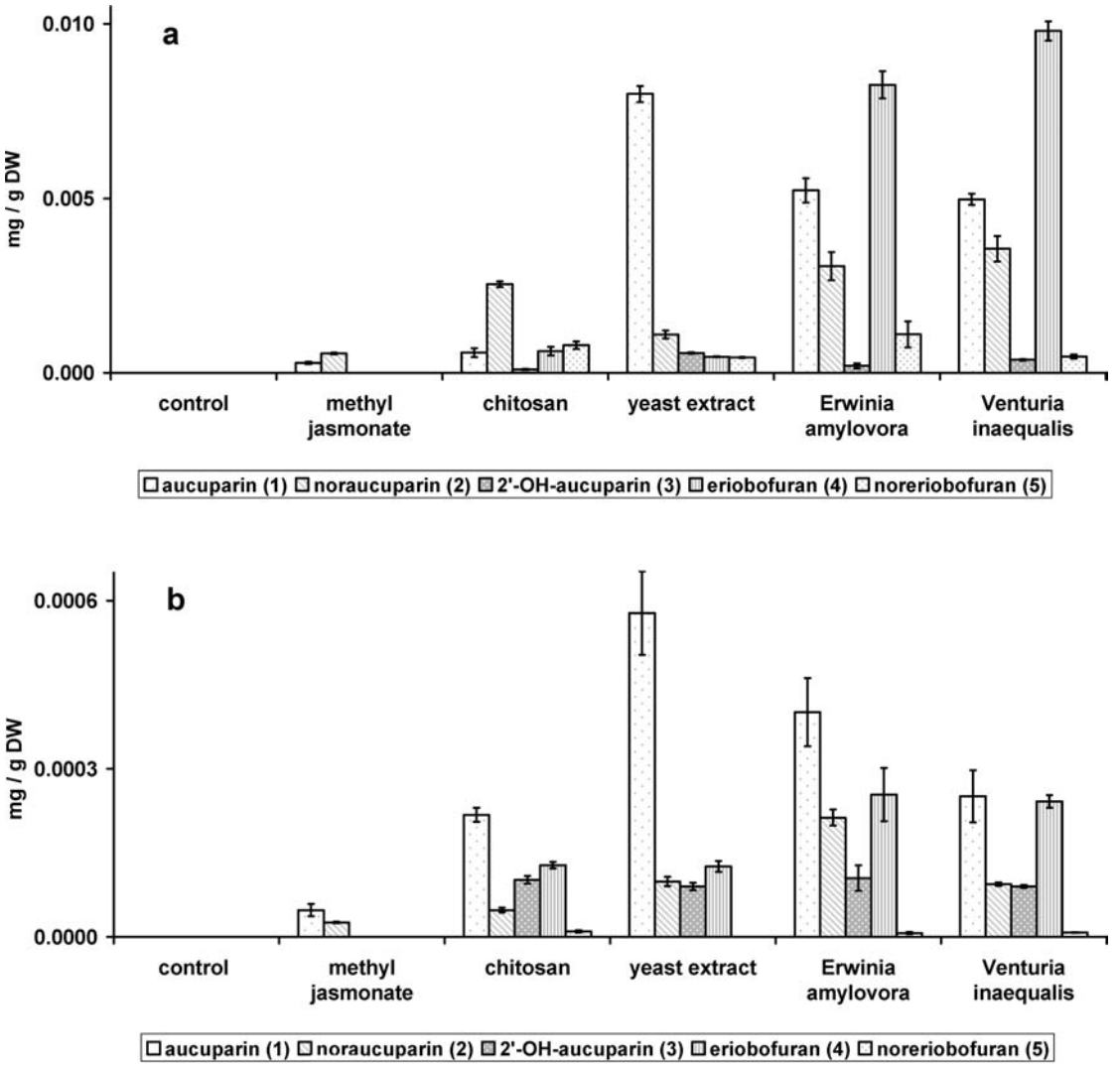


Figure 5. Effect of elicitors on biphenyl and dibenzofuran accumulation in cells (**a**) and medium (**b**) of *S. aucuparia* cell cultures (note the different scales). The results are based on a relative quantification using 4-phenylphenol as an internal standard. SDs are indicated (n = 3).

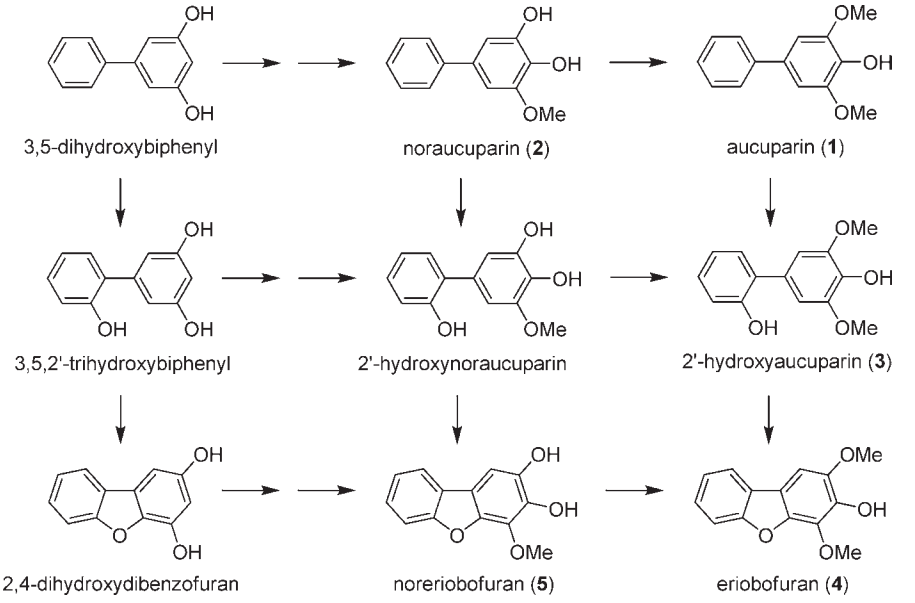


Figure 6. Proposed scheme of biosynthetic reactions leading to formation of biphenyls and dibenzofurans in elicitor-treated *S. aucuparia* cell cultures.

exogenously applied jasmonates were found to lead to appreciable formation of specific secondary metabolites in a number of cell cultures (*40*, *41*). In all of our elicitation experiments, the bulk of the induced biphenyls and dibenzofurans was found inside the

cells; the cell culture medium contained only minor amounts (**Figure 5**). The low levels of biphenyls and dibenzofurans in the medium (0.6−0.007 μg/g DW) may be due to cell lysis during the elicitation and cell harvesting processes. In contrast, cell cultures of *M. domestica* mainly excreted the phytoalexins into the medium (*10*, *25*).

Proposed Phytoalexin Biosynthetic Pathway in *S. aucuparia* Cell Cultures. The biphenyl scaffold is formed by BIS, which condenses benzoyl-CoA with three malonyl-CoAs to give 3,5-dihydroxybiphenyl. Subsequent hydroxylation and methylation steps are likely to yield noraucuparin (**2**) and aucuparin (**1**; **Figure 6**). The simultaneous accumulation of biphenyls and dibenzofurans in *S. aucuparia* cell cultures after treatment with *E. amylovora* and *V. inaequalis* is suggestive of a biogenic relationship between the two classes of phytoalexins. This conclusion is also based on the previously observed co-occurrence of biphenyls and dibenzofurans in elicitor-treated cell cultures of apple (*10*, *25*). A previous contradicting statement (*4*) resulted from the observation that intact plants accumulate either biphenyls or dibenzofurans. The dibenzofuran eriobofuran (**4**) is likely to arise via intramolecular cyclization from a 2′-hydroxylated biphenyl, such as 2′-hydroxyaucuparin (**3**), itself derived from aucuparin (**1**; **Figure 6**). An analogous pathway may yield noreriobofuran (**5**), although a corresponding 2′-hydroxylated intermediate has not yet been detected. Thus, BIS is likely to form the carbon skeleton of both biphenyls and dibenzofurans. Intra- and intermolecular C−O coupling reactions were previously detected in benzophenone and benzylisoquinoline metabolisms, respectively, and are catalyzed by cytochrome P450 enzymes (*42*−*44*).

In conclusion, the induced accumulation of antimicrobial secondary metabolites is generally one of the best-studied pathogen defense mechanisms of plants (*45*). However, the phytoalexin response of the Pyrinae is poorly understood, despite the economic value of a number of fruit crops. To protect these trees from diseases that commonly come along with long-time monocropping is a stimulating challenge. Application of pesticides for fruits is strictly regulated, and the development of pathogen insensitivities is an increasing problem (*46*). Therefore, it seems important for the future to increase the natural resistance of the economically used varieties. One strategy among the multiple pathogen defense mechanisms of plants is the formation of phytoalexins. Elicitor-treated *S. aucuparia* cell cultures not only form biphenyls and dibenzofurans simultaneously but also accumulate these phytoalexins differentially in response to the different types of elicitors added. This observation reflects the plasticity of plant secondary metabolism. The cell culture−elicitor system of reduced complexity is an interesting tool for studying the biochemical and molecular genetic aspects underlying the biosynthesis of the Pyrinae-specific phytoalexins. Data thus obtained lay the foundation for future studies of the interactions between differentiated plants and intact pathogens.

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