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Biosynthesis of the biphenyl phytoalexin aucuparin in *Sorbus aucuparia* cell cultures treated with *Venturia inaequalis*

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ABSTRACT

Aucuparin is the most widely distributed biphenyl phytoalexin in the rosaceous subtribe Pyrinae, which includes the economically important fruit trees apple and pear. The biphenyl scaffold is formed by biphenyl synthase, which catalyzes biosynthesis of 3,5-dihydroxybiphenyl. Conversion of this precursor to aucuparin (3,5-dimethoxy-4-hydroxybiphenyl) was studied in cell cultures of *Sorbus aucuparia* after treatment with an elicitor preparation from the scab-causing fungus *Venturia inaequalis*. The sequence of the biosynthetic steps detected was *O*-methylation – 4-hydroxylation – *O*-methylation. The two alkylation reactions were catalyzed by distinct methyltransferases, which differed in pH and temperature optima as well as stability. Biphenyl 4-hydroxylase was a microsomal cytochrome P450 monooxygenase, whose activity was appreciably decreased by the addition of established P450 inhibitors. When fed to *V. inaequalis*-treated *S. aucuparia* cell cultures, radioactively labeled 3,5-dihydroxybiphenyl was not only incorporated into aucuparin but also into the dibenzofuran eriobofuran, the accumulation of which paralleled that of aucuparin. However, biphenyl 2′-hydroxylase activity proposed to be involved in dibenzofuran formation was detected in neither microsomes nor cell-free extracts in the presence of NADPH and 2-oxoglutarate, respectively. Nevertheless, a basis for studying biphenyl biosynthesis at the gene level is provided.

1. Introduction

The rosaceous subtribe Pyrinae, formerly known as subfamily Maloideae, includes a number of economically valuable fruit trees, such as apple (*Malus* × *domestica*) and pear (*Pyrus communis*) (Campbell et al., 2007). In 2010, the world-wide production of apple and pear was valued 64 and 13 milliard US$, respectively (FAO, 2010). However, the numerous cultivars of the fruit crops are afflicted by many diseases, leading to dramatic losses of both trees and fruits. The most devastating diseases include fire blight, caused by the bacterium *Erwinia amylovora*, and scab, triggered by the fungus *Venturia inaequalis* (MacHardy, 1996; Vanneste, 2000). The annual losses due to the fire blight disease and the costs of control are valued at over US$ 100 million in the USA alone (Norelli et al., 2003). In terms of control expenditure, apple scab is the most costly apple disease (Carisse and Bernier, 2002). Due to the great economic impact of these diseases on fruit production, the interactions between the fruit trees and the devastating pathogens are intensely studied to enhance the disease resistance potential of the economically valuable cultivars (Norelli et al., 2003).

One plant defense strategy is formation of phytoalexins (Dixon, 2001). Within the Rosaceae, the Pyrinae are the only taxon that produces biphenyls and dibenzofurans as *de novo* formed defense compounds when subjected to biotic and abiotic stresses (Fig. 1; Kokubun and Harborne, 1995). So far, 10 biphenyls and 17 dibenzofurans were isolated from 14 of the 30 Pyrinae genera and demonstrated to be induced by infection or elicitation (Chizzali and Beerhues, 2012). Their antibacterial and antifungal activities against pathogens were established, however, the underlying mechanisms of antimicrobial action are not yet elucidated (Chizzali et al., 2012b; Kokubun et al., 1995). Biphenyls and dibenzofurans were also found in species outside the Pyrinae, however, their function here is to contribute to the constitutive barrier against microorganisms and herbivores rather than being infection-induced phytoalexins (Hüttner et al., 2010).

In infected plants of the Pyrinae, no single species was found to simultaneously accumulate both classes of phytoalexins in the same tissue, provoking the conclusion that the biosynthetic pathways of biphenyls and dibenzofurans are parallel rather than sequential (Kokubun and Harborne, 1995). Only recently,

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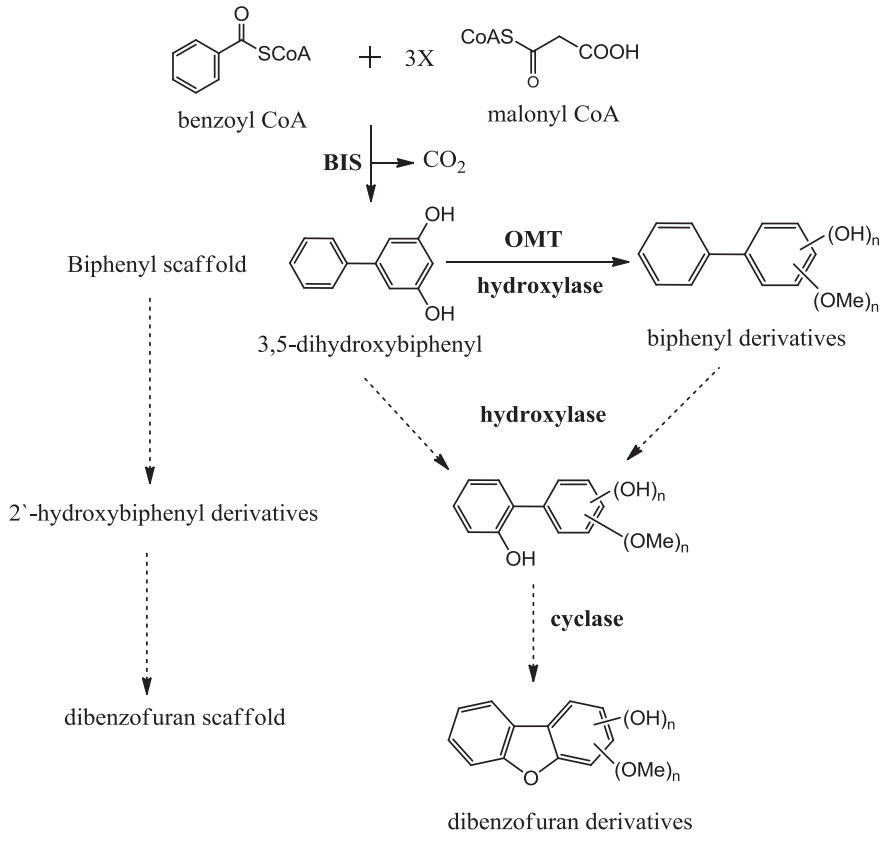

Fig. 1. Survey of biphenyl and dibenzofuran biosynthesis. BIS, biphenyl synthase; OMT, *O*-methyltransferase. Solid lines, established reactions; dashed lines, hypothetic reactions.

fire-blight-infected shoots of the apple cultivar 'Holsteiner Cox' and the pear cultivar 'Conference' have been reported to form both biphenyls and dibenzofurans in the transition zone, which is located between the healthy and necrotic parts of the infected stem (Chizzali et al., 2012a,b, 2013). Before, *in vitro* systems had already been shown to produce the two classes of compounds simultaneously. Cell cultures of the scab-resistant apple cultivar 'Liberty' accumulated three biphenyls and one dibenzofuran upon elicitation with yeast extract (Borejsza-Wysocki et al., 1999; Hrazdina et al., 1997). Cell cultures of *Sorbus aucuparia* formed various patterns of phytoalexins in response to the type of elicitor added (Hüttner et al., 2010). The biphenyl aucuparin was the major phytoalexin after the addition of yeast extract, whereas the dibenzofuran eriobofuran was the major inducible compound when *E. amylovora* and *V. inaequalis* preparations were added. In addition, the two latter elicitors induced maximum phytoalexin levels. These recent observations provided the basis for postulating sequential rather than parallel biosynthetic pathways of biphenyl and dibenzofuran formation (Chizzali and Beerhues, 2012; Hrazdina and Borejsza-Wysocki, 2003; Hüttner et al., 2010).

The carbon skeleton of the compounds is formed by biphenyl synthase (BIS), a type III polyketide synthase, which catalyzes iterative condensation of benzoyl-CoA with three molecules of malonyl-CoA to give a tetraketide intermediate which then undergoes aldol condensation and decarboxylation to yield 3,5-dihydroxybiphenyl (Fig. 1; Liu et al., 2004, 2007, 2010). The conversion of this precursor to aucuparin may proceed via three alternative routes, which differ in the sequence of the methylation and hydroxylation steps (Fig. 2). Either two *O*-methylation steps are followed by hydroxylation or vice versa. As a third alternative, the methylation steps frame the hydroxylation reaction. Furthermore, all metabolites involved are potential candidates to be converted to the corresponding dibenzofurans by 2′-hydroxylation and subsequent intramolecular cyclization (Fig. 1).

Here we report biochemical elucidation of the biosynthetic pathway leading from 3,5-dihydroxybiphenyl to aucuparin in *S. aucuparia* cell cultures treated with *V. inaequalis* extract. Furthermore, tracer experiments resulted in incorporation of radioactively labeled 3,5-dihydroxybiphenyl not only in aucuparin but also in the dibenzofuran eriobofuran.

2. Results

2.1. Accumulation of phytoalexins after treatment with V. inaequalis

The biphenyl aucuparin and the dibenzofuran eriobofuran are the major phytoalexins present in *S. aucuparia* cell cultures after treatment with *V. inaequalis* extract, as previously observed (Hüttner et al., 2010). Here, we have studied the time course of their accumulation. Formation of aucuparin and eriobofuran started 9 and 12 h, respectively, after the onset of the treatment and the phytoalexin levels increased up to 96 and 120 h post-elicitation, respectively (Fig. 3). Control cell cultures which received only an equal volume of water failed to form the defense compounds.

2.2. Synthesis and feeding of radioactive 3,5-dihydroxybiphenyl

To study if aucuparin and eriobofuran are derived from 3,5-dihydroxybiphenyl, as proposed by Hüttner et al. (2010), the latter compound was enzymatically synthesized as radioactive tracer. Recombinant BIS1 of *S. aucuparia* (Liu et al., 2007) was incubated with benzoyl-CoA and [2-^{14}C]malonyl-CoA, resulting in the formation of ^{14}C-labeled 3,5-dihydroxybiphenyl (Fig. 1). This tracer was

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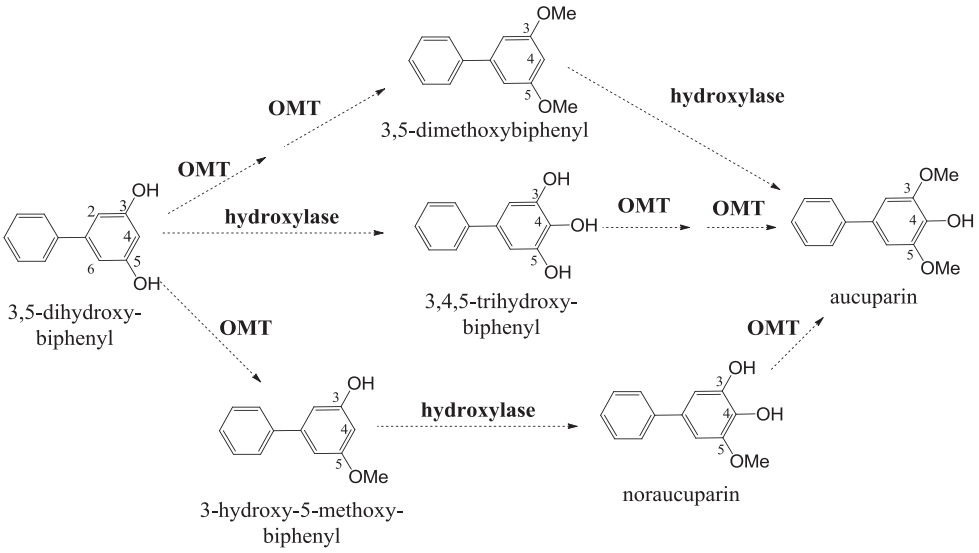

Fig. 2. Proposed alternative routes of aucuparin formation starting from 3,5-dihydroxybiphenyl.

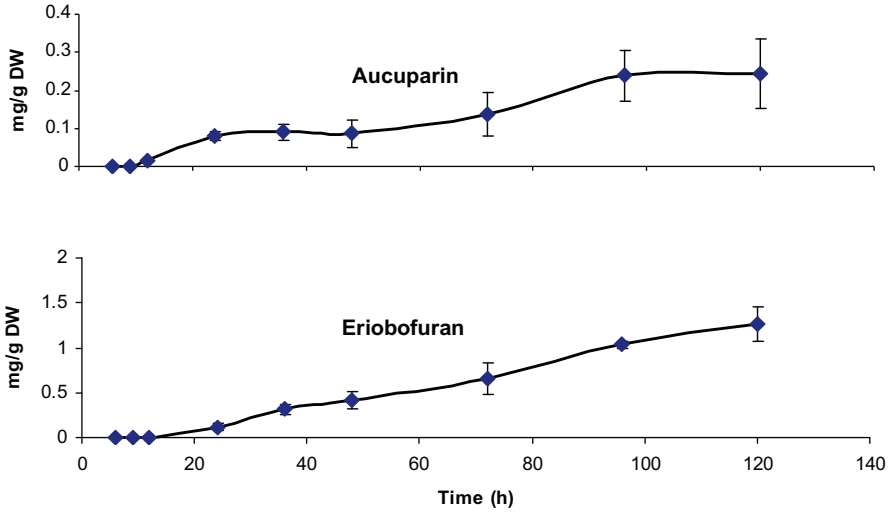

Fig. 3. Accumulation of aucuparin and eriobofuran in *S. aucuparia* cell cultures after the addition of *V. inaequalis* extract. Quantification was relative to 4-phenylphenol as internal standard. SDs are indicated ($n = 3$).

fed to *S. aucuparia* cell cultures after treatment with *V. inaequalis* extract. Using radiodetector-coupled HPLC, accumulation of both labeled aucuparin and labeled eriobofuran was detected. After 3 d of tracer feeding, the incorporation rates of 3,5-dihydroxybiphenyl into aucuparin and eriobofuran were 0.6% and 3.3%, respectively, reflecting the ratio of the aucuparin and eriobofuran levels (Fig. 3). Feeding of [U–^{14}C]benzoic acid in a parallel experiment achieved incorporation rates of 1.1% and 4.6%. No glycosylated compounds are detectable after elicitor treatment, as demonstrated previously by enzymatic and acidic hydrolyses of extracts (Hüttner et al., 2010).

2.3. Detection of O-methyltransferase (OMT1) activity

To study if either *O*-methylation or hydroxylation is the first biosynthetic step, non-radioactive 3,5-dihydroxybiphenyl was chemically synthesized according to Nilsson and Norin (1963) and incubated with both desalted cell-free extract in the presence of *S*-adenosyl-L-methionine (SAM) and microsomes in the presence of NADPH. The protein fractions were prepared from *S. aucuparia* cell cultures treated with *V. inaequalis* for 15 h. While the microsomal fraction failed to convert 3,5-dihydroxybiphenyl, cell-free extract was capable of forming a new compound, as demonstrated by HPLC-DAD analysis. This product was absent from assays that either lacked SAM or contained heat-denatured protein. The enzymatic product was identified as 3-hydroxy-5-methoxybiphenyl by co-chromatography with chemically synthesized reference compound. When 3-hydroxy-5-methoxybiphenyl itself was incubated with desalted cell-free extract and SAM, no further *O*-methylation to give 3,5-dimethoxybiphenyl was observed (Table 1). Among the dibenzofurans tested, 2,4-dihydroxydibenzofuran was *O*-methylated to give 2-hydroxy-4-methoxydibenzofuran, which in turn did not undergo a second methylation to yield 2,4-dimethoxydibenzofuran. Similarly, 4-hydroxy-2-methoxydibenzofuran did not experience *O*-methylation. All dibenzofurans used were chemically synthesized and their structures were confirmed by mass spectrometry and NMR spectroscopy (^{1}H, ^{13}C including DEPT-135, HSQC, HMBC, NOESY or NOEDIFF). While the K_m values for 3,5-dihydroxybiphenyl and 2,4-dihydroxydibenzofuran were similar, the V_{max} values differed by factor 5 (Table 2). The pH and

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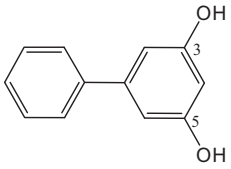
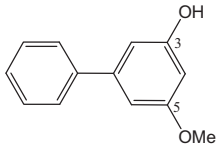
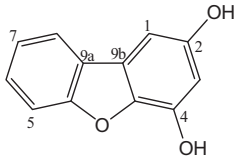
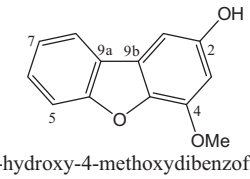
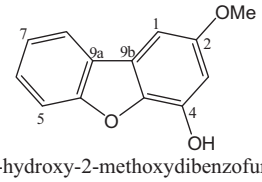
Table 1
Substrate specificity of OMT1 activity catalyzing the first methylation reaction.

Substrate	Relative activity (%)
 3,5-dihydroxybiphenyl	100[a]
 3-hydroxy-5-methoxybiphenyl	n.d.
 2,4-dihydroxydibenzofuran	20.4
 2-hydroxy-4-methoxydibenzofuran	n.d.
 4-hydroxy-2-methoxydibenzofuran	n.d.

[a] 0.49 nkat; n.d., not detectable.

Table 2
Kinetic parameters of OMT activity in cell-free extracts.

Substrate	K_m (μM)	V_{max} (nkat)
3,5-Dihydroxybiphenyl	0.85	0.49
2,4-Dihydroxydibenzofuran	1.17	0.10
Noraucuparin	4.46	0.12

temperature optima found for the OMT activity were 8.5 and 40 °C, respectively.

2.4. Detection of biphenyl 4-hydroxylase (B4H) activity

3-Hydroxy-5-methoxybiphenyl was incubated with the microsomal fraction from *V. inaequalis*-treated *S. aucuparia* cell cultures

Table 3
Cofactor dependence of B4H and effect of inhibitors.

Conditions	Relative activity (%)
Standard assay	100[a]
−NADPH	1.1
+NADH	119
+0.5 μM miconazole	43.9
+10 μM miconazole	1.4
+10 μM cytochrome *c*	6.9
+50 μM cytochrome *c*	1.3

[a] 0.44 nkat.

Table 4
Effect of freeze–thaw on OMT activity in cell-free extracts. SDs are indicated ($n = 3$).

Storage of cell-free extract at −80 °C	Relative OMT activity (% of max. each)	
	3,5-Dihydroxybiphenyl	Noraucuparin
With glycerol (20%, v/v)	100	97.5 ± 2.5
Without glycerol	32.3 ± 4.4	100

and NADPH, leading to formation of noraucuparin (Fig. 4). No product formation was found in assays that either lacked NADPH or contained heat-denatured microsomes. Identification of the enzymatic product was carried out by co-chromatography with a chemically synthesized and structurally confirmed reference compound (Hüttner et al., 2010). 3,5-Dihydroxybiphenyl and 3,5-dimethoxybiphenyl failed to be 4-hydroxylated when tested under identical conditions. Nor did 2,4-dihydroxydibenzofuran, 2-hydroxy-4-methoxydibenzofuran, and 4-hydroxy-2-methoxydibenzofuran serve as substrates. Formation of noraucuparin was hardly detectable in the absence of NADPH but was stimulated by the addition of NADH (Table 3). Well known inhibitors of cytochrome P450 enzymes, such as miconazole and cytochrome *c* (Ortiz de Montellano, 2005), led to inhibition of the hydroxylase activity in a concentration-dependent manner. The pH and temperature optima of B4H were 8.5 and 20 °C, respectively. Enzyme activity was linear for up to 90 min with protein concentrations up to 300 μg/assay. The K_m value for 3-hydroxy-5-methoxybiphenyl was 0.6 ± 0.1 μM and the V_{max} value was 0.38 nkat.

Unlike 4-hydroxylase activity, 2′-hydroxylase activity was not detectable. Microsomes failed to convert 3,5-dihydroxybiphenyl, 3-hydroxy-5-methoxybiphenyl, noraucuparin, and aucuparin (Fig. 2) into the corresponding 2′-hydroxylated products. Nor were the potential substrates hydroxylated by cell free-extracts in the presence of 2-oxoglutarate, ferrous, and ascorbate, which soluble dioxygenases rely on.

2.5. Detection of OMT2 activity

Noraucuparin was incubated with desalted cell-free extract and SAM, resulting in formation of a single product, which was identified as aucuparin by co-chromatography with an authentic reference compound (Hüttner et al., 2010). Incubation of aucuparin did not lead to further methylation at the 4-hydroxyl group to give

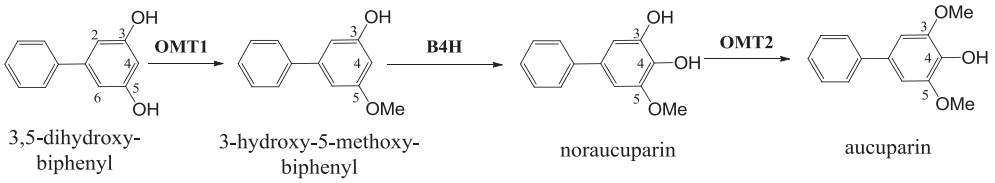


Fig. 4. Established sequence of biosynthetic reactions leading from 3,5-dihydroxybiphenyl to aucuparin in *V. inaequalis*-treated *S. aucuparia* cell cultures. OMT, *O*-methyltransferase; B4H, biphenyl 4-hydroxylase.

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3,4,5-trimethoxybiphenyl. The pH and temperature optima of the noraucuparin methylation reaction were 7.5 and 45 °C, respectively, and thus differed from the values observed with OMT1 (8.5 and 40 °C). OMT2 activity was linear for up to 35 min with protein concentrations up to 80 μg/assay. When either heat-denatured protein was incubated or SAM was omitted from the assay, no product formation was observed. The K_m value for noraucuparin was 4.5 ± 0.1 μM and the V_{max} value was 0.12 nkat (Table 2).

Upon freeze–thaw of desalted cell-free extracts in the presence of glycerol, both the 3,5-dihydroxybiphenyl- and the noraucuparin-converting OMT activities were stable (Table 4). However, when glycerol was omitted, the 3,5-dihydroxybiphenyl-metabolizing OMT1 activity was strongly decreased, whereas the noraucuparin-converting OMT2 activity remained unchanged.

3. Discussion

Our observation that 3,5-dihydoxybiphenyl is the precursor of both aucuparin and eriobofuran in *V. inaequalis*-treated *S. aucuparia* cell cultures indicates that biphenyls and dibenzofurans are formed via sequential rather than parallel pathways. Thus, BIS catalyzes the biosynthesis of the carbon skeletons of both classes of phytoalexins present in the Pyrinae. Three BIS cDNAs were previously cloned from elicitor-treated *S. aucuparia* cell cultures (Liu et al., 2007, 2010). Unlike BIS2 mRNA, the BIS1 and BIS3 transcript levels rapidly and strongly increased after elicitation, indicating that these two isoenzymes are involved in phytoalexin formation. So far, no 3,5-dihydoxybiphenyl has been isolated from any infected plant species although it exhibits pronounced antibacterial activity against *E. amylovora* (Chizzali et al., 2012b). Variable arrays of derivatives of this precursor may exhibit synergistic activities against a broad range of pathogens. While biphenyls have somewhat stronger antibacterial activity than structurally related dibenzofurans, the opposite tendency was observed for antifungal activity (Chizzali and Beerhues, 2012). Transgenic approaches aiming to introduce a single new phytoalexin into a susceptible plant resulted in neutralizing the severe symptoms associated with the infection but did not endow plant resistance (He and Dixon, 2000; Hipskind and Paiva, 2000). More than one phytoalexin compound or class appears to be essential to provide plants with resistance (Dixon, 2001).

The absence of detectable quantities of 3,5-dihydroxybiphenyl from infection sites and elicitor-treated cells indicates rapid conversion of this precursor to downstream products. The major products of this metabolism in *V. inaequalis*-treated *S. aucuparia* cell cultures are the biphenyl aucuparin and the dibenzofuran eriobofuran. Both compounds accumulate in parallel over 4–5 d post-elicitation. Therefore, *S. aucuparia* cell cultures provide a valuable system for studying biphenyl and dibenzofuran biosynthesis. Accumulation over several days was previously observed with malusfuran, the *O*-glucoside of 9-hydroxyeriobofuran, in yeast-extract treated cell cultures of the scab-resistant apple cultivar 'Liberty' (Hrazdina et al., 1997). Unlike cell culture systems, intact shoots contain only a limited number of phytoalexin-producing cells, as indicated by the exclusive phytoalexin accumulation in the transition zones of fire blight-infected stems of the apple cultivar 'Holsteiner Cox' and the pear cultivar 'Conference' (Chizzali et al., 2012b, 2013).

In our study, the pathway leading from 3,5-dihydroxybiphenyl to aucuparin in *V. inaequalis*-treated *S. aucuparia* cell cultures was elucidated at the enzyme level. Interestingly, the elucidated sequence of reactions was *O*-methylation – 4-hydroxylation – *O*-methylation (Fig. 4). The first methylation step yields 3-hydroxy-5-methoxybiphenyl, which failed to undergo further methylation at the 3-hydroxyl group. Instead, 3-hydroxy-5-methoxybiphenyl was found to be the only substrate for B4H which formed noraucuparin. Microsomal localization, NADPH dependency, and repression by CYP inhibitors indicated that B4H is a cytochrome P450 monooxygenase. Subsequently, a second OMT activity (OMT2) catalyzed methylation of the 3-hydroxyl group of noraucuparin to yield aucuparin. The presence of two distinct OMTs in *S. aucuparia* cell cultures was indicated by different pH and temperature optima and differential sensitivity to freeze–thaw in the absence of glycerol. In contrast to the strict regiospecificities of the *S. aucuparia* OMTs, some plant OMTs perform two sequential methylation steps on a single substrate, e.g. flavonoid OMTs (Cacace et al., 2003; Zhou et al., 2006) and resveratrol OMT (Schmidlin et al., 2008). In roses, two consecutively acting OMTs were detected and catalyze formation of 3,5-dimethoxytoluene from orcinol (Lavid et al., 2002; Scalliet et al., 2002, 2008). Both enzymes were able to methylate both intermediates but the catalytic efficiencies differed. Thorough studies of the *S. aucuparia* OMTs at the molecular level are needed to gain detailed insight into their properties.

In contrast to aucuparin biosynthesis, the conversion of biphenyls to dibenzofurans is not yet understood. Unlike 4-hydroxylase activity involved in aucuparin formation, 2′-hydroxylase activity proposed to participate in dibenzofuran biosynthesis (Fig. 1) could not be detected. Notably, 2′-hydroxyaucuparin has previously been isolated from elicitor-treated cell cultures of *S. aucuparia* and *M. domestica*, the latter cell culture also producing 2′-*O*-β-D-glucopyranosylaucuparin (Borejsza-Wysocki et al., 1999; Hüttner et al., 2010). In addition, 2′-hydroxyaucuparin was detected in the transition zones of fire-blight-infected shoots of the apple cultivar 'Holsteiner Cox' and the pear cultivar 'Conference' (Chizzali et al., 2012b, 2013). There are two classes of enzymes known to catalyze aromatic hydroxylation, namely microsomal cytochrome P450 monooxygenases and soluble 2-oxoglutarate-dependent dioxygenases. These enzymes, both of which use molecular oxygen, can replace each other in different species (Frey et al., 2003; Lukacin et al., 2001). However, neither enzyme activity could be detected in *V. inaequalis*-treated *S. aucuparia* cell cultures using cell-free extract together with 2-oxoglutarate, ferrous, and ascorbate and microsomes together with NADPH.

As an alternative to 2′-hydroxylation at the biphenyl level, the phenolic 2′-hydroxyl group might be introduced at an earlier biosynthetic stage. Utilization by BIS of *ortho*-hydroxybenzoyl-CoA (salicoyl-CoA) rather than benzoyl-CoA was previously believed to directly yield 2′,3,5-trihydroxybiphenyl. However, all recombinant BISs so far incubated with salicoyl-CoA formed 4-hydroxycoumarin after a single decarboxylative condensation with malonyl-CoA rather than 2′,3,5-trihydroxybiphenyl. This even holds true for BIS3 from *S. aucuparia* which preferred salicoyl-CoA over benzoyl-CoA as a starter molecule (Chizzali et al., 2012a; Liu et al., 2010). Furthermore, benzoic acid and cinnamic acid rather than salicylic acid were reported to be incorporated into aucuparin and malusfuran in elicitor-treated apple cell cultures (Hrazdina and Borejsza-Wysocki, 2003). These findings are in accord with the high incorporation of radioactively labeled 3,5-dihydroxybiphenyl into the dibenzofuran eriobofuran, indicating 2′-hydroxylation at the biphenyl level which, however, needs to be detected. Once formed, the 2′-hydroxylated biphenyl may be converted to the corresponding dibenzofuran via oxidative phenol coupling in analogy to the intramolecular cyclization of benzophenones to xanthones, catalyzed by cytochrome P450 enzymes (Peters et al., 1997).

4. Concluding remarks

Both classes of phytoalexins present in the Pyrinae are derived from 3,5-dihydroxybiphenyl, which in turn is formed from

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benzoyl-CoA and malonyl-CoA by BIS, the only enzyme of the biosynthetic pathway that has so far been studied at the biochemical and molecular levels. An interesting sequence of biosynthetic steps resulting in formation of the best known biphenyl phytoalexin, aucuparin, was elucidated. However, the branch point at which a biphenyl intermediate is channelled into dibenzofuran metabolism is still obscure. Regarding aucuparin biosynthesis, the way for further investigations at the molecular genetic level is now paved. Exploitation of biphenyl metabolism may provide new control strategies to improve the disease resistance of valuable apple and pear cultivars.

5. Experimental

5.1. Chemicals

MSTFA and benzoyl-CoA were purchased from ABCR (Karlsruhe, Germany) and Sigma–Aldrich (Steinheim, Germany). [2-^{14}C]malonyl CoA (55.2 mCi/mmol; 0.1 mCi/ml) was supplied by Hartmann Analytic (Braunschweig, Germany). Solvents were of HPLC grade and used without further purification. 3,5-Dihydroxybiphenyl, 3,4,5-trihydroxybiphenyl, 3,4,5-trimethoxybiphenyl, and 2′-hydroxyaucuparin were prepared, as described by Hüttner et al. (2010) and Chizzali et al. (2012b). The identities of the products were confirmed by spectroscopic analyses and were in accordance with published data (Song et al., 2006).

5.2. Syntheses

5.2.1. Synthesis of 2,4-dimethoxy-1-phenoxybenzene

Ullmann condensation of 1-bromo-2,4-dimethoxybenzene and phenol was based on a method published by Oliveira et al. (2003), except that additional amounts of phenol (0.14 mmol equivalents) were added 1, 2.5, 4, and 5.5 h after the onset of the reaction. The total reaction time was 7 h. The product was purified on a silica gel column using CH_2Cl_2/petroleum ether (40:60, v/v). The yield was 31% (5.36 g, 23.3 mmol).

MS (70 eV), *m*/*z* (% rel abundance) 230 (100, $[M]^+$), 215 (15, $[M]^+$-15), 200 (2, $[M]^+$-30), 153 (11), 125 (10), 77 (14, $[Ph]^+$); RI (VF5-MS) 1866. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 7.26 (m, 2 H, H-3′, 5′), 6.99 (m, 1 H, H-4′), 6.95 (d, J = 8.7 Hz, 1 H, H-6), 6.89 (m, 2 H, H-2′, 6′), 6.58 (d, J = 2.8 Hz, 1 H, H-3), 6.45 (dd, J = 8.7, 2.8 Hz, 1 H, H-5), 3.81 (s, 3 H, 4-OMe), 3.78 (s, 3 H, 2-OMe); ^{13}C NMR (101 MHz, $CDCl_3$, δ = 77.01 ppm) 158.83 (C-1′), 157.28 (C-4), 152.54 (C-2), 138.18 (C-1), 129.38 (C-3′, 5′), 122.32 (C-6), 121.81 (C-4′), 116.09 (C-2′, 6′), 104.19 (C-5), 100.66 (C-3), 55.94 (2-OMe), 55.62 (4-OMe).

5.2.2. Synthesis of 2,4-dimethoxydibenzofuran

2,4-Dimethoxy-1-phenoxybenzene was subjected to oxidative coupling using $Pd(OAc)_2$ and acetic acid (Oliveira et al., 2003). The crude product was purified on a silica gel column using CH_2Cl_2/petroleum ether (25:75, v/v). The yield was 12% (810 mg, 3.55 mmol).

MS (70 eV), *m*/*z* (% rel abundance) 228 (100, $[M]^+$), 213 (26, $[M]^+$-15), 199 (7, $[M]^+$-29), 185 (40), 170 (26), 142 (11), 126 (10), 114 (16); (RI (VF5-MS) 2079. ^{1}H NMR (600 MHz, $CDCl_3$, TMS) 7.89 (ddd, J = 7.7, 1.3, 0.7 Hz, 1 H, H-9), 7.59 (dt, J = 8.3, 1.6 Hz, 1 H, H-6), 7.44 (ddd, J = 8.3, 7.3, 1.3 Hz, 1 H, H-7), 7.32 (ddd, J = 7.7, 7.3, 1.0 Hz, 1 H, H-8), 6.99 (d, J = 2.3 Hz, 1 H, H-1), 6.62 (d, J = 2.3 Hz, 1 H, H-3), 4.03 (s, 3 H, 4-OMe), 3.91 (s, 3 H, 2-OMe); ^{13}C NMR (151 MHz, $CDCl_3$, δ = 77.01 ppm) 156.71 (C-2), 156.54 (C-5a), 145.83 (C-4), 140.22 (C-4a), 127.04 (C-7), 125.17 (C-9b), 124.59 (C-9a), 122.51 (C-8), 120.58 (C-9), 112.02 (C-6), 99.36 (C-3), 94.31 (C-1), 56.17 (4-OMe), 55.99 (2-OMe).

5.2.3. Synthesis of 2,4-dihydroxydibenzofuran, 2-hydroxy-4-methoxydibenzofuran, and 4-hydroxy-2-methoxydibenzofuran

Demethylations were achieved using a modified method of Bao et al. (2009). A CH_2Cl_2 solution of 2,4-dimethoxydibenzofuran (5.2.2; 545 mg, 2.39 mmol) was mixed with freshly prepared magnesium iodide etherate solution (7.17 mmol). After evaporation under reduced pressure, the residue was heated at 80 °C for 10 h under a stream of argon gas. The reaction was stopped by the addition of a saturated solution of NH_4Cl (50 ml; Anioł et al., 2008; as alternative to $Na_2S_2O_3$ and washing with $NaHCO_3$). The resulting solution was acidified using aqueous HCl (5%). The aqueous phase was extracted with CH_2Cl_2 (3 × 50 ml) and dried over anhydrous Na_2SO_4. The reaction products were fractionated on a silica gel column using petroleum ether/EtOAc (65:35, v/v) to yield fractions A and B with R_f values 0.8 and 0.24, respectively.

Fraction A (323 mg) was further purified on a silica gel column using petroleum ether/EtOAc (8:2, v/v) to yield 4-hydroxy-2-methoxydibenzofuran (267 mg, 1.24 mmol, 52.2% yield, R_f = 0.64) and 2-hydroxy-4-methoxydibenzofuran (12 mg, 0.056 mmol, 2.3% yield, R_f = 0.4).

5.2.3.1. 4-Hydroxy-2-methoxydibenzofuran

MS of mono-TMS derivative (70 eV), *m*/*z* (% rel abundance) 286 (100, $[M]^+$), 271 (53, $[M]^+$-15), 256 (62, $[M]^+$-30), 240 (9), 228 (9), 185 (10), 126 (10), 73 (18); RI (ZB5-MS) 2086. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 7.89 (ddd, J = 7.7, 1.3, 0.6 Hz, 1 H, H-9), 7.54 (ddd, J = 8.7, 1.0, 0.7 Hz, 1 H, H-6), 7.44 (ddd, J = 8.4, 7.2, 1.3 Hz, 1 H, H-7), 7.32 (td, J = 7.5, 1.0 Hz, 1 H, H-8), 7.00 (d, J = 2.4 Hz, 1 H, H-1), 6.66 (d, J = 2.4 Hz, 1 H, H-3), 5.5 (br. s, OH), 3.88 (s, 3 H, 2-OMe); ^{13}C NMR (101 MHz, $CDCl_3$, δ = 77.01 ppm) 156.76 (C-2), 156.54 (C-5a), 141.31 (C-4), 139.14 (C-4a), 127.23 (C-7), 125.49 (C-9b), 124.79 (C-9a), 122.70 (C-8), 120.88 (C-9), 111.84 (C-6), 102.26 (C-3), 95.89 (C-1), 56.07 (2-OMe).

5.2.3.2. 2-Hydroxy-4-methoxydibenzofuran

MS of mono-TMS derivative (70 eV), *m*/*z* (% rel abundance) 286 (100, $[M]^+$), 271 (64, $[M]^+$-15), 256 (4, $[M]^+$-30), 126 (12), 73 (20); RI (ZB5-MS) 2133. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 7.86 (ddd, J = 7.7, 1.3, 0.7 Hz, 1 H, H-9), 7.59 (dt, J = 8.3, 0.8 Hz, 1 H, H-6), 7.45 (ddd, J = 8.2, 7.3, 1.3 Hz, 1 H, H-7), 7.32 (ca. td, $J \approx 8$, 1 Hz, 1 H, H-8), 6.95 (d, J = 2.3 Hz, 1 H, H-1), 6.58 (d, J = 2.3 Hz, 1 H, H-3), 4.03 (s, 3 H, 4-OMe); ^{13}C NMR (101 MHz, $CDCl_3$, δ = 77.01 ppm) 156.66 (C-5a), 152.21 (C-2), 145.87 (C-4), 140.16 (C-4a), 127.26 (C-7), 125.6 (C-9b), 124.29 (C-9a), 122.55 (C-8), 120.74 (C-9), 112.03 (C-6), 99.16 (C-1), 97.63 (C-3), 56.27 (4-OMe).

Fraction B (120 mg) was purified on a silica gel column using CH_2Cl_2/MeOH/HCOOH (5.0:0.15:0.05, v/v/v) to yield 2,4-dihydroxydibenzofuran (70 mg, 0.35 mmol, 13.68% yield).

5.2.3.3. 2,4-Dihydroxydibenzofuran

MS of di-TMS derivative (70 eV), *m*/*z* (% rel abundance) 344 (100, $[M]^+$), 329 (40, $[M]^+$-15), 73 (75); RI (ZB5-MS) 2136. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 7.86 (ddd, J = 7.4, 1.3, 0.6 Hz, 1 H, H-9), 7.54 (dt, J = 8.3, 0.4 Hz, 1 H, H-6), 7.45 (ddd, J = 8.4, 7.2, 1.3 Hz, 1 H, H-7), 7.32 (ddd, J = 7.7, 7.3, 1.0 Hz, 1 H, H-8), 6.95 (d, J = 2.3 Hz, 1 H, H-1), 6.58 (d, J = 2.3 Hz, 1 H, H-3), 5.47 (br. s, OH), 4.81 (br. s, OH); ^{13}C NMR (101 MHz, $CDCl_3$, δ = 77.01 ppm) 156.6 (C-5a), 152.27 (C-2), 141.32 (C-4), 139.05 (C-4a), 127.41 (C-7), 125.8 (C-9b), 124.53 (C-9a), 122.76 (C-8), 121.02 (C-9), 111.85 (C-6), 102.57 (C-3), 98.26 (C-1).

5.2.4. Synthesis of eriobofuran

The procedure resembled that described for the dioxygenated dibenzofurans (5.2.1–5.2.3). The intermediates 1,2,3-trimethoxy-4-phenoxybenzene and 2,3,4-dimethoxydibenzofuran were prepared by Ullmann synthesis and oxidative coupling, respectively.

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The MS and NMR data of both reaction products were identical to those published by Kemp et al. (1983). Demethylation of 2,3,4-trimethoxydibenzofuran was carried out using magnesium iodide etherate. MS and NMR data of eriobofuran were identical to those reported by Hüttner et al. (2010).

5.2.5. Synthesis of aucuparin

Demethylation of 3,4,5-trimethoxybiphenyl (400 mg, 1.64 mmol) was achieved using magnesium iodide etherate solution (30 equivalents). The solutions of both reactants in diethyl ether were mixed and evaporated to dryness. The residue was heated on an oil bath at 80 °C for 11 h under a stream of argon gas and protected from light. The residue was dissolved in water, and $Na_2S_2O_3$ was added to remove I_2. The solution was acidified and extracted with CH_2Cl_2 (2 × 50 ml). The organic layer was washed with $NaHCO_3$ solution (100 ml) and brine solution (100 ml) and dried over anhydrous Na_2SO_4. The concentrated residue was purified on a silica gel column using CH_2Cl_2. The yield was 76.9% (290 mg, 1.26 mmol). The spectroscopic properties were in accordance with published data (Hüttner et al., 2010).

5.2.6. Synthesis of noraucuparin

Aucuparin (240 mg, 1.04 mmol) was dissolved in 10 ml CH_2Cl_2. Cold (4 °C) BBr_3 solution (1 ml, 1 M in CH_2Cl_2) was added (Bao et al., 2009). The reaction mixture was stirred at room temperature for 3 h under moisture-free atmosphere. Then additional BBr_3 solution (0.5 ml, 1 M in CH_2Cl_2) was added and the reaction was continued for another 3 h. The reaction was stopped by dropwise addition of water. The resulting mixture was acidified with aqueous HCl (5%). The organic layer was separated and the aqueous layer was further extracted with CH_2Cl_2 (2 × 10 ml). The organic layer was evaporated and noraucuparin was purified on a silica gel column using CH_2Cl_2 (100 mg; 0.46 mmol, 45% yield). The spectroscopic properties were identical to published data (Hüttner et al., 2010).

5.3. Cell cultures, elicitation, and analysis of phytoalexin accumulation

Cell cultures of *S. aucuparia* were grown in the dark at 25 °C, as described before (Liu et al., 2004). Mycelium of *V. inaequalis* was extracted with water (pH 2) for 1 h at 100 °C, as described by Zhang et al. (2000). The resulting elicitor solution, whose pH was readjusted to 5, was added to 5-day-old cell cultures (50 ml) at a final concentration of 50 mg/l. Aliquots (10 ml) were taken at various times under sterile conditions, and cells were collected by centrifugation and lyophilized. Phytoalexins were previously found to accumulate intracellularly (Hüttner et al., 2010). After determination of the dry weight, cells were extracted three times with methanol (5 ml each). The combined methanol extract was evaporated to dryness. Preparation of samples for GC–MS analysis and quantitation of compounds were carried out as described previously considering the real response factors of the compounds (Hüttner et al., 2010). Three biological samples and up to three technical replicates each were studied.

5.4. Enzymatic preparation of radiolabeled 3,5-dihydroxybiphenyl

BIS1 of *S. aucuparia* was heterologously produced in *Escherichia coli* and isolated by affinity chromatography, as described earlier (Liu et al., 2007). The enzyme assay (200 µl) consisted of 7 µl [2-^{14}C]malonyl CoA (1.27 nmol, 0.07 µCi), 1.15 nmol malonyl-CoA, 7.4 µM benzoyl-CoA, and 3 µg BIS1 in 0.1 M potassium phosphate buffer (pH 7). After incubation for 30 min at 35 °C, the reaction was stopped by adding 40 µl HCl (10%) and the product was extracted twice with 200 µl ethylacetate each. The extracts from 20 incubations were combined and evaporated to dryness. The product was redissolved in a volume of methanol (~150 µl), its identity was confirmed by co-chromatography (HPLC) with a sample of authentic reference compound, and its radioactivity was measured by scintillation counting (Beckman LS 6500). Due to the relatively low yield and the moderate stability of the product, further purification was omitted and the tracer was fed to the cell cultures right away.

5.5. Feeding of radiolabeled 3,5-dihydroxybiphenyl

Cultured cells (0.3 g) were inoculated into 5 ml LS medium in 25 ml Erlenmeyer flasks. On day 5, the extract of *V. inaequalis* was added and, after 4.5 h, the methanolic solution of radiolabeled 3,5-dihydroxybiphenyl (117 µl; 0.52 µCi) was fed under sterile conditions. Cells were harvested by centrifugation 72 h after the onset of elicitation and homogenized in methanol (7 ml). After centrifugation, the residue was extracted twice with methanol (4 ml each) by vortexing for 1 min. The combined methanol extract was evaporated to dryness and the residue was redissolved in 100 µl methanol (HPLC grade). Aliquots were used for scintillation counting and HPLC analysis.

5.6. Radiodetector-coupled HPLC

The HPLC apparatus (Agilent 1200 series) was coupled with both a UV detector (Agilent 1200 Variable Wavelength Detector) and a radiodetector (RAMONA star 2; Raytest, Germany). A Lichrospher C_{18} column (250 × 4.6 mm i.d., 5 µm; Wicom, Germany) was used. The solvent for isocratic analysis consisted of 55% (v/v) water containing *o*-phosphoric acid (0,1%, v/v) and 45% (v/v) methanol. Data acquisition and analysis were carried out using the GINA star 4 software (Raytest, Germany).

5.7. Protein extraction

Cultured cells (30 g) were harvested 15 h after elicitation and homogenized in 6 ml 0.1 M Tris–HCl buffer (pH 7) containing 10 mM DTT and 10 µM PMSF in a mortar for 15 min. The homogenate was centrifuged at 9.000*g* for 25 min and the supernatant was passed through a PD_{10} column (GE Healthcare). The resulting cell-free protein extract was used for testing OMT and hydroxylase activities. To prepare the microsomal fraction, the crude extract was centrifuged at 100.000*g* for 90 min. The microsomal pellet was resuspended in 0.1 M Tris–HCl buffer (pH 7) containing 14% (w/v) sucrose, 3.5 mM β-mercaptoethanol and 10 µM PMSF. The microsomal pellet could be stored at −80 °C without appreciable decrease in enzyme activity. Protein contents were determined colorimetrically using Bradford's reagent and a bovine serum albumin calibration curve (Bradford, 1976).

5.8. Enzyme assays

To optimize the assay conditions, the incubation temperature was varied between 25 and 60 °C, except for B4H: 7–60 °C. The pH values tested ranged from 6 to 11 using potassium phosphate buffer (6–7), Tris–HCl buffer (7–8.5), and glycine–NaOH buffer (9–11). The optimum incubation time and protein concentration were also determined. To study kinetic parameters, first a wide range of substrate concentrations were used for rough determination. Subsequently, concentrations of 0.2–5 × K_m were used for accurate measurement (0.62–50 µM OMT substrates; 0.12–6 µM B4H substrate). The standard assays were as follows:

The B4H assay (200 µl) consisted of 6 µM 3-hydroxy-5-methoxybiphenyl and 1 mM NADPH in 0.1 M Tris–HCl buffer (pH 7). The reaction was initiated by the addition of 80 µg microsomal protein. After incubation for 30 min at 22 °C, the reaction was

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stopped by the addition of 40 µl HCl (10%). The reaction product was extracted twice with ethyl acetate (200 µl each) and the combined organic phase was evaporated to dryness. The residue was dissolved in 60 µl methanol and analyzed by HPLC, as described under 5.9.

The OMT assay (200 µl) consisted of 15 µM substrate (3,5-dihydroxybiphenyl, noraucuparin), 50 µM SAM, and 1.5 mM ascorbic acid in 0.1 M Tris–HCl buffer (pH 8.5). The reaction was initiated by the addition of 40 µg protein. After incubation for 20 min at 37 °C, the reaction was stopped and the product was extracted and analyzed by HPLC, as described under 5.9. To test the stability of OMT activities, aliquots of the cell-free protein extract, prepared in 0.1 M Tris–HCl buffer pH 7 (5.7), were stored with and without glycerol (20%, v/v) at −80 °C. After 6 months, the aliquots were thawed on ice and their OMT activities were determined under standard assay conditions.

The 2-oxoglutarate-dependent dioxygenase assay (200 µl) consisted of 5 mM 2-oxoglutarate, 0.5 mM ferrous sulphate and 5 mM ascorbic acid in 0.1 M Tris–HCl buffer (pH 7). In addition, it contained the potential subtrates 3,5-dihydroxybiphenyl, 3-hydroxy-5-methoxybiphenyl, noraucuparin, and aucuparin at concentrations from 6 µM to 1.25 mM. The reaction was initiated by the addition of 100 µg protein. After incubation for 30 min at 30 °C, the reaction was stopped and the products were extracted and analyzed by HPLC as described under 5.9.

5.9. HPLC analysis of enzymatic products

HPLC was carried out on an Elite Lachrome system (Darmstadt, Germany) coupled to a diode array detector (L-2455) and equipped with a HyperClone ODS column (C_{18}, 250 × 4.6 mm, 3 µ; Phenomenex). Data acquisition and analysis were carried out using the EzChrome Elite software (Agilent). The solvents consisted of water containing 0.1% *o*-phosphoric acid (A) and methanol (B). The gradient was 50–80% B in 14 min, 80% B for 10 min, 80–100% B in 2 min, and 100% B for 2 min at a flow rate of 0.3 ml/min. The detection wavelengths were 254 and 269 nm for biphenyls and 285 and 300 nm for dibenzofurans and 2′-hydroxylated biphenyls. For the analysis of 2,4-dihydroxydibenzofuran and its methylated product, 2-hydroxy-4-methoxydibenzofuran, the following gradient was used: 45% B for 2 min, 45–65% B in 8 min, 65–76% B in 10 min, 76–100% B in 2 min, and 100% B for 3 min at a flow rate of 0.3 ml/min. The detection wavelengths were 252 and 285 nm.

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