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Biosynthesis of tetraoxygenated phenylphenalenones in *Wachendorfia thyrsiflora*

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ABSTRACT

The biosynthetic origin of 1,2,5,6-tetraoxygenated phenylphenalenones and the sequence according to which their oxygen functionalities are introduced during the biosynthesis in *Wachendorfia thyrsiflora* were studied using two approaches. (1) Oxygenated phenylpropanoids were probed as substrates of recombinant *W. thyrsiflora* polyketide synthase 1 (WtPKS1), which is involved in the diarylheptanoid and phenylphenalenone biosynthetic pathways, (2) Root cultures of *W. thyrsiflora* were incubated with ^{13}C-labelled precursors in an $^{18}O_2$ atmosphere to observe incorporation of the two isotopes at defined biosynthetic steps. NMR- and HRESIMS-based analyses were used to unravel the isotopologue composition of the biosynthetic products, lachnanthoside aglycone and its allophanyl glucoside. Current results suggest that the oxygen atoms decorating the phenalenone tricycle are introduced at different biosynthetic stages in the sequence *O*-1 → *O*-2 → *O*-5. In addition, the incubation of *W. thyrsiflora* root cultures with ^{13}C-labelled lachnanthocarpone established a direct biosynthetic precursor–product relationship with 1,2,5,6-tetraoxygenated phenylphenalenones.

1. Introduction

Phenylphenalenones are a group of polycyclic natural products occurring in the Musaceae (banana) (Luis et al., 1994; Kamo et al., 1998; Hölscher and Schneider, 1998; Otálvaro et al., 2007) and related plant families Strelitziaceae (*Strelitzia reginae*, the bird of paradise plant) (Hölscher and Schneider, 2000), Haemodoraceae (e.g. *Anigozanthos* species, the kangaroo paw) (Cooke and Thomas, 1975; Hölscher and Schneider, 1997; Hölscher and Schneider, 1999; Opitz et al., 2002), and Pontederiaceae (e.g. *Eichhornia crassipes*, the water hyacynth) (DellaGreca et al., 2008; Hölscher and Schneider, 2005). Bioassay studies have demonstrated the antifungal properties of (phenyl)phenalenones (Quiñones et al., 2000; Otálvaro et al., 2007). The remarkable structural diversity of phenylphenalenones is mainly due to their variable oxygenation pattern. Anigorufone (**3a**) (Fig. 1), which occurs in most phenylphenalenone-producing plants, is one of the simplest 2-hydroxy-9-phenylphenalen-1-ones. Recently, the biosynthetic origin of the 1-keto-2-hydroxy motif of phenylphenalenones was elaborated using ^{13}C/^{18}O labelling experiments and isotopologue analysis based on nuclear magnetic resonance (NMR) spectroscopy and high resolution electro-spray mass spectrometry (HRESIMS) (Munde et al., 2011). It was shown that the hydroxyl group at C-2 of 9-phenylphenalenones had been introduced at the stage of a linear diarylheptanoid, and the carbonyl oxygen atom originated from the hydroxyl group of the 4-coumaroyl moiety.

Not only 2-hydroxy-9-phenylphenalen-1-ones such as anigorufone (**3a**) but also compounds such as lachnanthoside aglycone (**2a**) and its allophanyl glucoside (**1a**) (Fig. 1) with two more hydroxyl groups in the phenalenone nucleus occur in *Wachendorfia thyrsiflora* (Fang et al., 2011), *Xiphidium caeruleum* (Opitz et al., 2002) and some other Haemodoraceae (Cooke and Edwards, 1980). One of the additional oxygen atoms is located at C-6, and, in most phenylphenalenones, the other atom is attached to C-5 but can also be found at C-4. The oxygen functionality at C-6 very likely comes from the carbonyl thioester of 4-coumaroyl–CoA, which in the course of phenylphenalenone biosynthesis condenses with a diketide to form a diarylheptanoid (Brand et al., 2006). Unlike the origin of the oxygen atom at C-6, nothing is known about the introduction of the oxygen at C-5.

In order to probe for the origin of the oxygen functionalities and the sequence according to which they are introduced to the phenylphenalenone scaffold in *W. thyrsiflora*, two independent approaches were used. First, the substrate specificity of WtPKS1 was investigated. The recombinant enzyme was incubated with α-oxygenated phenylpropanoyl *N*-acetyl cysteamine (NAC) derivatives [phenylpyruvoyl–NAC, 3-(4-hydroxyphenyl)pyruvoyl–NAC, (*R*)- and (*S*)-phenyllactoyl–NAC), (*R*)- and (*S*)-3-(4-hydroxyphenyl) lactoyl–NAC] as mimics of the corresponding CoA esters.

In a second approach, root cultures of *W. thyrsiflora* were supplemented with [2-^{13}C]L-phenylalanine ([2-^{13}C]Phe), [2-^{13}C]4-coumaric

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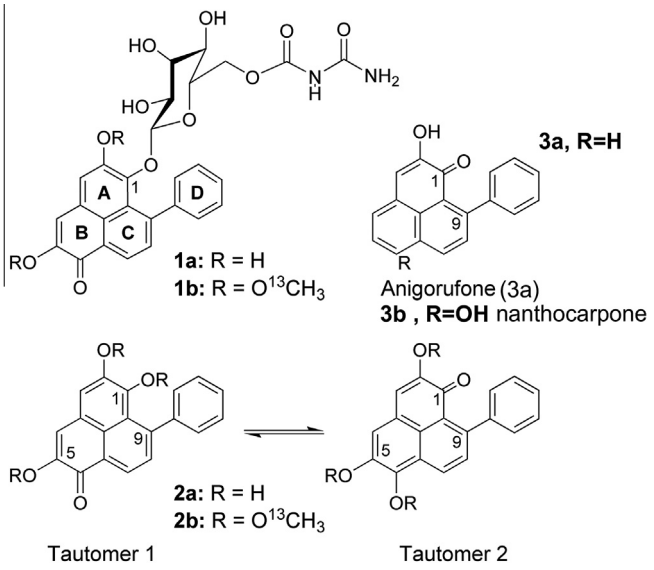

Fig. 1. Structures of 1,2,5,6-tetraoxygenated phenylphenalenones used as target compounds to study biosynthetic oxygenation in root cultures of *W. thyrsiflora* and anigorufone; the last compound exemplifies a 2-hydroxy-9-phenylphenalen-1-one. Because of tautomerism of **2a**, the carbonyl group of **2a**/**2b** appears at different positions in the phenalenone tricycle. For simplicity, the position *peri* to the phenyl ring is designated C-1 in the text and in structure drawings of all tautomeric 1,2,5,6-tetraoxygenated phenylphenalenones, regardless if this position is bearing a keto or hydroxyl functionality. Thus, the numbering used in this paper is different from that used in chemical names, which are as follows: **1a**, 6-*O*-[(6''-*O*-allophanyl-ß-D-glucopyranosyl]-2,5-dihydroxy-7-phenylphenalen-1-one; **1b**, 6-*O*-[(6''-*O*-allophanyl-ß-D-glucopyranosyl]-2,5-dimethoxy-7-phenylphenalen-1-one; **2a**, two tautomers of lachnanthoside aglycone, 2,5,6-trihydroxy-9-phenylphenalen-1-one and 2,5,6-trihydroxy-7-phenylphenalen-1-one; **2b**, two tri-*O*-methyl derivatives of lachnanthoside aglycone, [2,5,6-($O^{13}CH_3$)$_3$]2,5,6-trimethoxy-9-phenylphenalen-1-one and [2,5,6-($O^{13}CH_3$)$_3$]2,5,6-trimethoxy-7-phenylphenalen-1-one.

acid ([2-^{13}C]CA), and two ^{13}C-labelled diarylheptanoids, [6-^{13}C](4*E*,6*E*)-1-(4-hydroxyphenyl)-7-phenylhepta-4,6-dien-3-one (DAH-I), and [6-^{13}C](4*E*,6*E*)-1-(3,4-dihydroxyphenyl)-7-phenylhepta-4,6-dien-3-one (DAH-II), and simultaneously incubated in an $^{18}O_2$ atmosphere. Phenylphenalenones isolated from these precursor administration experiments were subjected to NMR- and HRESIMS-based isotopologue analysis. The results of the enzymatic studies and labelling experiments are discussed with respect to the biosynthetic sequence according to which oxygen functionalities are introduced to the phenylphenalenone scaffold.

In addition, a 1,2,6-trioxygenated phenylphenalenone, lachnanthocarpone (**3b**), which is a potential biosynthetic precursor of tetraoxygenated phenylphenalenones, was administered in ^{13}C-labelled form to probe for direct precursor–product relationships.

2. Results

2.1. α-Oxygenated phenylpropanoids are substrates of WtPKS1 in vitro

In *W. thyrsiflora*, WtPKS1 (Genbank™ accession number AY727928) catalyzes the condensation of a phenylpropanoyl–CoA with one malonyl–CoA to form a diketide. The diketide is then condensed with another phenylpropanoyl–CoA to form a diarylheptanoid, which is further processed to form phenylphenalenones (Brand et al., 2006). As shown by competitive precursor administration experiments (Schmitt and Schneider, 1999) (which indicated reversible conversion of phenylpropionic acid and cinnamic acid) and as discussed by Brand et al. (2006), 4-coumaroyl–CoA seems to be the starter substrate of WtPKS1 *in vivo*. *In vitro*, however, WtPKS1 accepts various phenylpropanoyl–CoAs, including phenylpyruvoyl–CoA, as starter units. Hypothetically, an α-oxygenated 3-(4-hydroxyphenyl)propanoyl–CoA could function as a substrate of WtPKS1. If the hypothetical biosynthetic pathway shown in Fig. 2 operated in *W. thyrsiflora*, the α-oxygen of the phenylpropanoid side chain would end up at C-5 of the phenylphenalenone skeleton.

N-Acetyl cysteamine (NAC) esters have been reported instead of CoA esters to be the starter substrates of polyketide synthases (Cane et al., 1991; Jacobs et al., 1991). Therefore, activated NAC esters of (*R*)- and (*S*)-phenyllactate, (*R*)- and (*S*)-3-(4-hydroxyphenyl)lactate, phenylpyruvate and 3-(4-hydroxyphenyl)pyruvate were synthesized according to reported procedures (Gilbert et al., 1995; Pohl et al., 1998) and, instead of CoA esters, assayed with WtPKS1. Activated phenylpropionyl- and cinnamoyl derivatives were used as reference substrates to compare the relative activities obtained for the incubation of WtPKS1 with NAC esters and with CoA esters (Table 1). In a previous study, the phenylpropionyl–CoA ester was among the best substrates *in vitro* and the cinnamoyl–CoA ester was among the poorest (Brand et al., 2006). Assay conditions optimized for phenylpropionyl–CoA as a starter substrate were used as previously reported (see Experimental).

Although the relative reactivity of WtPKS1 with NAC esters was drastically reduced compared to CoA esters, products of enzymatic conversion were still readily detectable, demonstrating that the NAC esters are useful starter substrates. In all experiments, [2-^{14}C]malonyl–CoA was used as the extender substrate. The products were detected in the assay by their UV absorption at 280 nm and the radiolabel originating from [2-^{14}C]malonyl–CoA. With (*S*)-phenyllactoyl–NAC as a starter substrate, the condensation product with two malonyl–CoA units was identified as (*S*)-6-[1-hydroxy-2-(4-hydroxyphenyl)ethyl]-4-hydroxy-2*H*-pyran-2-one (**4**, R_t 28.2 min) by LCESIMS (*m/z* 249 $[M + H]^+$) (see Fig. 3b). A minor, according to its retention time, slightly more polar product was tentatively assigned to benzalacetone. According to LCESIMS data and the long retention times, the structures of enzymatic condensations with starter substrates possessing an unsubstituted phenyl ring were identified as (*R*)-6-(1-hydroxy-2-phenyl)ethyl-4-hydroxy-2*H*-pyran-2-one (**5**) (37.7 min, *m/z* 231 $[M-H]^-$), (*S*)-6-(1-hydroxy-2-phenyl)ethyl-4-hydroxy-2*H*-pyran-2-one (**6**) (37.7 min, *m/z* 231 $[M-H]^-$), and (*E*)-6-(1-hydroxy-2-phenyl)ethenyl-4-hydroxy-2*H*-pyran-2-one (**7**) (38.8 min, *m/z* 231 $[M + H]^+$) (Fig. 3).

WtPKS1 converts (*S*)-phenyllactoyl–NAC to the pyrone (**6**; rel. activity 101%) (Fig. 4) as efficient as phenylpropionyl–NAC (100%). In contrast, WtPKS1 was much less active (51%) with the *R*-enantiomer, (*R*)-phenyllactoyl–NAC, to produce **5**. With (*S*)-3-(4-hydroxyphenyl)lactoyl–NAC, a substrate having a hydroxyl group in 4-position of the phenyl ring, the activity of WtPKS1 was reduced to 34% compared to the non-hydroxylated substrate, (*S*)-phenyllactoyl–NAC, although it was still better accepted than cinnamoyl–NAC (24%). Phenylpyruvoyl–NAC (30%) is nearly as efficiently accepted as (*S*)-3-(4-hydroxyphenyl)lactoyl–NAC (34%). No product formation was observed with 3-(4-hydroxyphenyl)pyruvoyl–NAC and (*R*)-3-(4-hydroxyphenyl)lactoyl–NAC.

Pyrones have been formed by WtPKS1 *in vitro* (in addition to benzalacetones) from phenylpropanoids, but the products *in vivo* are diketides, which are converted to diarylheptanoids and, further downstream in the biosynthetic pathway, to phenylphenalenones. Hence, the formation of pyrones as a result of *in vitro* incubation experiments of WtPKS1 with α-oxygenated phenylpropanoid–NAC thioesters shows that some of them have the potential to function as precursors of the diarylheptanoid/phenylphenalenone biosynthesis. However, since the results with PKS type III enzymes *in vitro* generally do not provide convincing clues for the activity of the protein *in vivo*, alternative approaches were used to confirm or disprove the outcome of the enzymatic experiments.

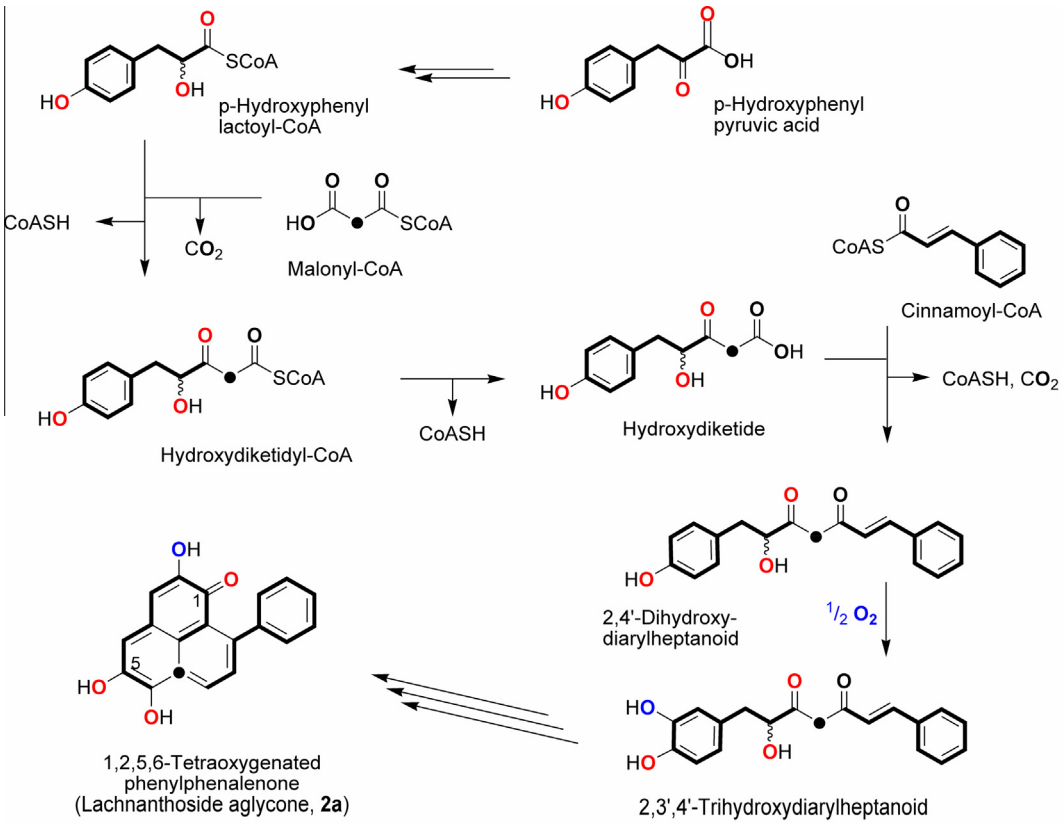

Fig. 2. Hypothetical "early hydroxylation" biosynthesis of 1,2,5,6-tetraoxygenated phenylphenalenones as probed by the incubation of WtPKS1 with α-oxygenated phenylpropanoyl thioesters and incubation of *W. thyrsiflora* root cultures with the corresponding ^{13}C-labelled precursors. The hypothetical fate of oxygen atoms is coded by color. Oxygen atoms which are suggested to be kept during phenylphenalenone biosynthesis are highlighted in red; lost oxygen atoms are displayed in bold; and oxygen atoms incorporated from O_2 are highlighted in blue. Phenylpropanoid units are marked with bold C–C bonds. ● indicates the origin of the marked atom from C-2 of malonate (Hölscher and Schneider, 1995b).

Table 1
Relative activities of WtPKS1 for cinnamoyl– and phenylpropionyl–CoA and –NAC esters as starter substrates.

Starter substrate	Relative activity [%]
Cinnamoyl–CoA	8
Cinnamoyl–NAC	2
Phenylpropionyl–CoA	100
Phenylpropionyl–NAC	7

See experimental section for conditions.

2.2. Early precursor administration experiments

Three ^{13}C-labelled α-oxygenated 3-(4-hydroxyphenyl)propanoic acids, [2-^{13}C](*S*)-3-(4-hydroxyphenyl)lactic acid, [2-^{13}C](*R*)-3-(4-hydroxyphenyl)lactic acid, and [2-^{13}C]3-(4-hydroxyphenyl) pyruvic acid, were synthesized and administered to root cultures of *W. thyrsiflora* in order to probe for their incorporation into phenylphenalenones. However, enhancement of ^{13}C signals was observed neither in the NMR spectra of the root extracts nor in isolated lachnanthoside aglycone (**2a**) and its allophanyl glucoside (**1a**). As shown by HPLC of aliquots taken regularly from the medium, significant absorption of the α-oxygenated precursor into the roots did not take place. Therefore, the results of these precursor administration experiments were inconclusive, and the biosynthesis of 2-oxygenated diarylheptanoids and 5-oxygenated phenylphenalenones remained unclear.

2.3. HRESIMS- and NMR-based ^{13}C/^{18}O isotopologue analysis

Unlike the administration of α-oxygenated 3-(4-hydroxyphenyl)propanoic acids, precursor administration experiments with radio-labelled phenylalanine and 4-coumaric acid showed that the label in the medium decreased within 1 day to 84% for [2,3,4,5,6-^{3}H]Phe (1.5 mg, 0.37 kBq) and 43% for [U–^{14}C]CA (1.5 mg, 0.34 kBq), suggesting absorption into the root tissue. The absorption of L-Phe and hydroxycinnamic acids from the medium into the cultured roots is consistent with previous studies, in which the corresponding ^{13}C-labelled precursors were smoothly incorporated into phenylphenalenones in *W. thyrsiflora* (Opitz and Schneider, 2003; Brand et al., 2006) and root cultures of the related species, *Anigozanthos preissii* (Hölscher and Schneider, 1995b; Schmitt et al., 2000).

Recent ^{13}C/^{18}O labelling experiments have demonstrated the usefulness of isotopologue analysis to infer the biosynthetic origin of the oxygen atoms in positions 1 and 2 of simple phenylphenalenones (Munde et al., 2011). Thus, ^{13}C/^{18}O-labelled isotopologues of compounds **1a** and **2a** from *W. thyrsiflora* were also considered to encode useful biosynthetic information about the sequence according to which oxygen functionalities are introduced into phenylphenalenones.

The administration of ^{13}C-labelled precursors to root cultures, which were simultaneously incubated in an $^{18}O_2$ atmosphere, followed by the isolation of phenylphenalenones **1a** and **2a**, and *O*-$^{13}CH_3$-methylation of the hydroxyl groups, was used to confirm the previously reported origin of the 1-keto-2-hydroxy motif

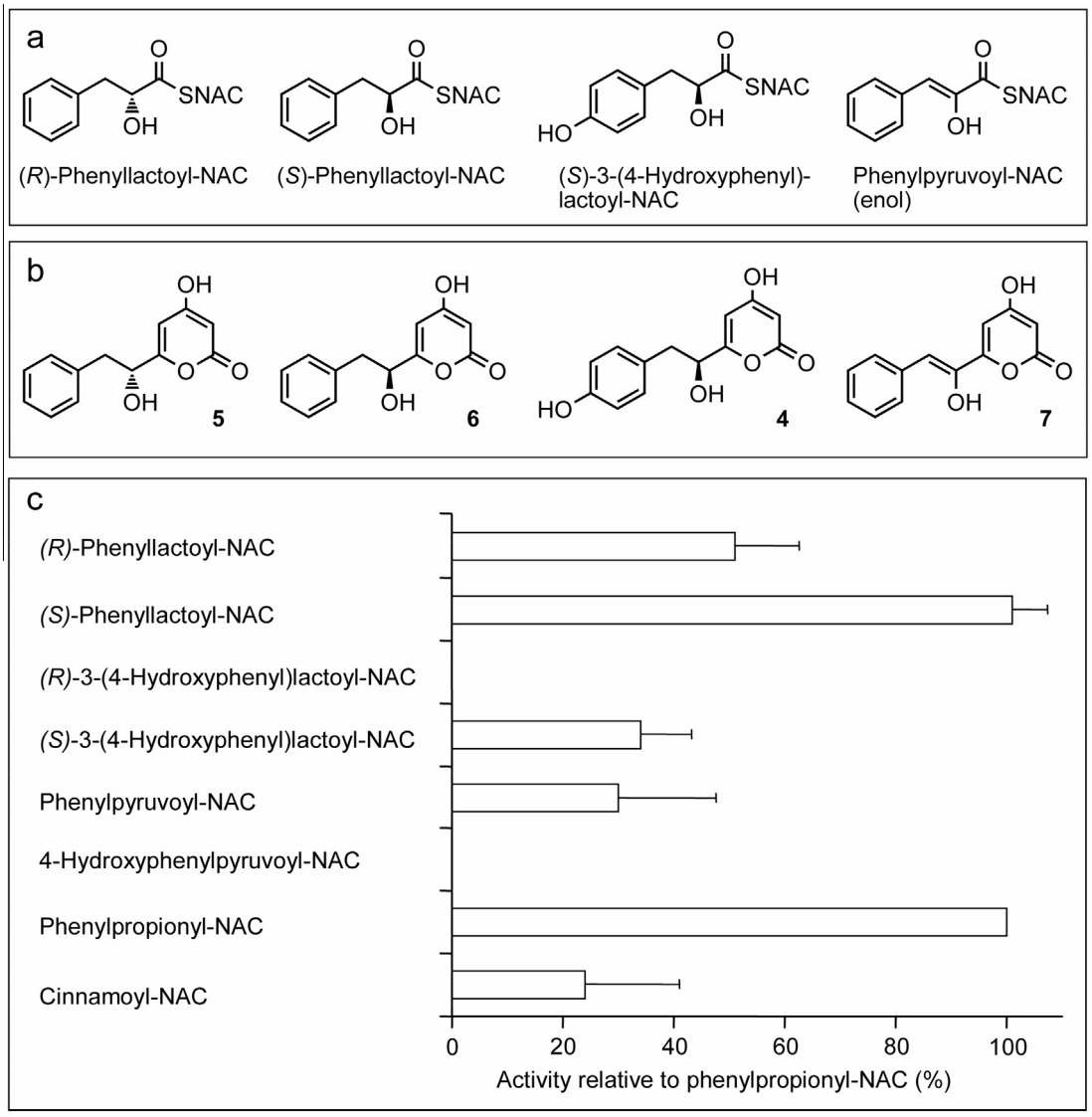

Fig. 3. (**a**) Starter substrates and (**b**) their corresponding products formed by incubation with [2-^{14}C]malonyl–CoA and WtPKS1. Products of two condensations are (*S*)-6-[1-hydroxy-2-(4-hydroxyphenyl)ethyl]-4-hydroxy-2*H*-pyran-2-one (**4**), (*R*)-6-(1-hydroxy-2-phenyl)ethyl-4-hydroxy-2*H*-pyran-2-one (**5**), (*S*)-6-(1-hydroxy-2-phenyl)ethyl-4-hydroxy-2*H*-pyran-2-one (**6**), and (*E*)-6-(1-hydroxy-2-phenyl)ethenyl-4-hydroxy-2*H*-pyran-2-one (**7**). (c) Enzymatic activity of WtPKS1 with different NAC esters, relative to the activity with phenylpropionyl–NAC (100%). Mean values of three experiments.

(Munde et al., 2011) by NMR and MS. Whether the additional oxygen atoms in positions 5 and 6 of 1,2,5,6-tetraoxygenated phenylphenalenones **1a** and **2a** are incorporated as a unit with α-oxygenated 4-hydroxyphenylpropanoids or come from an alternative late hydroxylation was the subject of the double labelling experiments.

[2-^{13}C]Phe, [2-^{13}C]CA, [6-^{13}C]DAH-I, and [6-^{13}C]DAH-II were used as ^{13}C-labelled precursors. Labels derived from [2-^{13}C]Phe into C-5 and C-8 (for a comment on numbering see caption of Fig. 1) of the allophanyl glycoside **1a** were reportedly incorporated (Brand et al., 2006) and confirmed in the present investigation for [2-^{13}C]Phe and [2-^{13}C]CA as precursors of phenylphenalenones in *W. thyrsiflora* (data not shown). ^{18}O-Induced ^{13}C NMR signals of C-2 and C-5 of **1a** were not detected, indicating the low level of ^{18}O that was incorporated. In order to enhance the sensitivity with which ^{18}O is detected by isotope-induced ^{13}C NMR signals, samples of the target compound obtained from precursor administration experiments were methylated with $^{13}CH_2N_2$, thus attaching a ^{13}C atom to each hydroxyl group, including those groups containing ^{18}O atoms incorporated from atmospheric $^{18}O_2$. The ^{13}C NMR spectra of the 2,5-di-O$^{13}CH_3$ derivative **1b** obtained from [2-^{13}C]Phe and [2-^{13}C]CA, respectively, exhibited weak but distinct 2-^{18}O$^{13}CH_3$ and 5-^{18}O$^{13}CH_3$ signals; these were shifted to a slightly higher field compared to the large 2-^{16}O$^{13}CH_3$ ($^1\Delta\delta^{18}O(^{13}C) = -29$ ppb) and 5-^{16}O$^{13}CH_3$ signals ($^1\Delta\delta^{18}O(^{13}C) = -25$ ppb) (Fig. 4, a and b). The Δδ values are in the order of magnitude of α-induced isotope shifts (Risley and van Etten, 1990). ^{18}O-Induced ^{13}C NMR shifts of the same magnitude were also observed for C-2 and C-5 of compound **1b** after administering [6-^{13}C]DAH-I and [6-^{13}C]DAH-II (Fig. 4, c and d). These results demonstrated that the two hydroxyl groups at C-2 and C-5 of **1a/b** are derived from atmospheric oxygen. The sequence of oxygen incorporation, however, remained unknown because the ^{13}C NMR spectra suggested that the occurrence of ^{18}O in positions 2 and 5 seems to be independent of the administered precursor (Fig. 4). The reason that ^{18}O appeared to be attached to C-2 and C-5, regardless of the administered precursor, is that NMR of isotopologue mixtures does not necessarily distinguish between individual isotopologues, i.e.,

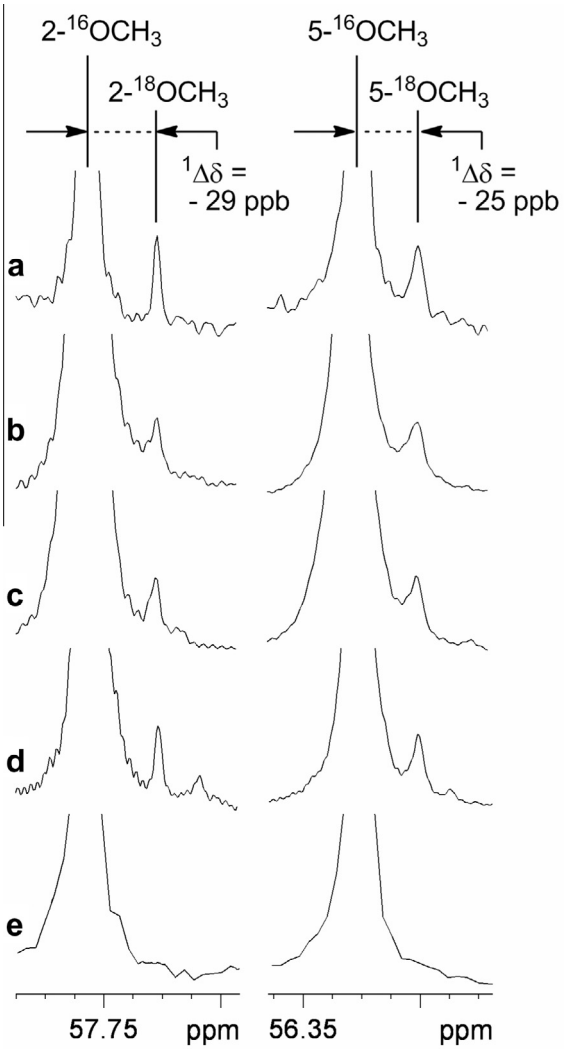

Fig. 4. Partial ^{13}C NMR spectra (125 MHz, acetone-d_6, regions of *O*-methyl signals) of compound **1b** prepared from **1a** by *O*-methylation using $^{13}CH_2N_2$ (99% ^{13}C). Samples of compound **1a** were obtained from *W. thyrsiflora* root cultures incubated in an atmosphere of $^{18}O_2$ and supplemented with ^{13}C-labelled precursors: (**a**) [2-^{13}C]Phe, (**b**) [2-^{13}C]CA, (**c**) [6-^{13}C]DAH-I, (**d**) [6-^{13}C]DAH-II. Spectrum (**e**) was recorded from unlabelled **1b** (reference).

it was not always evident from the NMR spectra whether the label belongs to the same or different isotopologues. This was the case because heavy isotopes used for labelling did not show isotope-induced shifts (e.g. 2-^{18}O and 5-^{13}C) or interact through spin–spin coupling (^{13}C-5 and ^{13}C-8) if they were located in remote positions.

Furthermore, differences in the uptake and transportation of precursors, pool size perturbation (Munde et al., 2011), and the low accuracy with which small $^{18}O^{13}CH_3$ signals versus large $^{16}O^{13}CH_3$ signals in the ^{13}C NMR spectra were integrated also made it difficult to quantify isotopologues and understand the sequence of ^{18}O incorporation. Moreover, ^{18}O-induced shifts were not observed for C-1 (in **1a/b** substituted with *O*-allophanyl-glc) and C-6 (C = O) because *O*-methylation with $^{13}CH_2N_2$ was impossible in these positions. Thus, NMR-based quantitative isotopologue analysis was supplemented by mass spectrometry, especially to determine $^{13}C/^{18}O$-labelled isotopologues.

According to the NMR spectroscopic results, the incorporation of ^{13}C from labelled precursors and ^{18}O from the gas phase (Fig. 4) into the allophanyl glucoside **1a** was low, suggesting large proportions of unlabelled and singly labelled (^{18}O or ^{13}C) isotopologues. This was confirmed by analysis of HRESIMS data (Table 2) of the di-$O^{13}CH_3$ derivative **1b** of the biosynthetically obtained allophanyl glucoside **1a**. Approximately 76% to 78% of the target molecules accounted for the isotopologues **1bA** and another approximately 20% were natural abundance isotopologues, mainly due to 1.1% ^{13}C in non-enriched positions of the molecule (not shown in Table 2). In addition to isotopologue **1bA** containing ^{13}C only in the diazomethane-derived *O*-methyl groups, isotopologue groups **B** – **H** were identified in the case of compound **1b**.

The isotopologues of group **1bB** contained one additional ^{13}C either at C-5 or C-8 but did not incorporate ^{18}O and therefore were not useful for studying oxygenation. Unlike **1bB**, the isotopologues **1bC**, **1bE**, and **1bG** did not contain ^{13}C, except in the diazomethane-derived $O^{13}CH_3$ group, but incorporated one (**1bC**), two (**1bE**), or three ^{18}O atoms (**1bG**), respectively, from the gas phase, demonstrating that the experimental system works. The value of isotopologues containing only ^{18}O for determining the sequence of oxygenation was limited because they were not formed from the ^{13}C-labelled precursors. The isotopologue group **1bH** was also unable to provide new information about the sequence of oxygenation because, except for the carbonyl oxygen at C-6 (which was derived from the carbonyl oxygen of phenylpropanoic acid), ^{16}O atoms of the oxygen functionalities were uniformly substituted by ^{18}O.

Therefore, to understand how the ^{18}O is incorporated, only the isotopologue groups **1bD** and **1bF** had to be considered; these groups possess one ^{13}C atom in the carbon skeleton and either one (**1bD**) or two ^{18}O atoms (**1bF**) in the functional groups. As previously reported (Brand et al., 2006) and confirmed here by means

Table 2
$^{13}C/^{18}O$ Isotopologue composition of labelled **1b** as determined by HRESIMS (positive ion mode) and ^{13}C NMR (125 MHz). Compound **1a** was isolated from root cultures of *W. thyrsiflora* after these were incubated with ^{13}C-labelled precursors ([2-^{13}C]Phe, [2-^{13}C]CA, [6-^{13}C]DAH-I, [6-^{13}C]DAH-II) in an atmosphere of $^{18}O_2$ followed by *O*-methylation with $^{13}CH_2N_2$ to afford **1b**.

Isotopologue							Isotopologue of **1b** obtained from the experiment with							
							[2-^{13}C]Phe		[2-^{13}C]CA		[6-^{13}C]DAH-I		[6-^{13}C]DAH-II	
No.	Molecular composition of aglycone cation					*m/z* $[M+H]^+$ (calcd.)	*m/z* $[M+H]^+$ (found)	Relative abundance (%)[a]	*m/z* $[M+H]^+$ (found)	Relative abundance (%)[a]	*m/z* $[M+H]^+$ (found)	Relative abundance (%)[a]	*m/z* $[M+H]^+$ (found)	Relative abundance (%)[a]
	^{12}C	^{13}C	^{1}H	^{16}O	^{18}O									
1bA	19	2	17	4	0	335.1188	335.1201	78.22	335.1202	78.46	335.1201	76.20	335.1197	76.27
1bB	18	3	17	4	0	336.1222	336.1232	0.55	-	0.00	336.1233	1.22	336.1227	2.06
1bC	19	2	17	3	1	337.1231	337.1254	0.42	337.1256	0.07	337.1256	0.83	337.1252	0.26
1bD	18	3	17	3	1	338.1264	338.1273	0.41	338.1274	0.29	**338.1275**	**0.98**	**338.1269**	**0.42**
1bE	19	2	17	2	2	339.1273	339.1285	0.19	339.1283	0.51	339.1283	0.46	339.1276	0.66
1bF	18	3	17	2	2	340.1307	340.1315	0.33	340.1316	0.28	**340.1314**	**0.21**	-	0.00
1bG	19	2	17	1	3	341.1316	341.1345	0.10	341.1326	0.81	341.1325	0.44	341.1318	0.82
1bH	18	3	17	1	3	342.1349	342.1358	0.11	–	0.00	–	0.00	–	0.00

[a] Isotopologues containing the level of naturally abundant ^{13}C were eliminated.

of analysis of ^{13}C NMR and HSQC spectra (data not shown), ^{13}C from [2-^{13}C]Phe appears at C-5 or C-8. The experiment with [2-^{13}C]CA had similar results, although the formation of [8-^{13}C]-isotopologues was not possible in this case and the ^{13}C-label had to have been located exclusively at C-5. The NMR-spectroscopic detection of ^{18}O in 2-OH and/or 5-OH (Fig. 4) and HRESIMS data (Table 2) in the experiments with [2-^{13}C]Phe and [2-^{13}C]CA did not clarify whether 2-OH or 5-OH comes first, i.e., the introduction of the oxygen functionality at C-2 may occur before or after oxygenation at C-5.

Evidence for the sequence according to which oxygen functionalities are introduced into the phenylphenalenone skeleton came from the administration of diarylheptanoids ([6-^{13}C]DAH-I and -II) and $^{18}O_2$. The ^{13}C labelling of C-6 in the linear seven-carbon-chain of the diarylheptanoids determined that only isotopologues of **1a** with a single ^{13}C atom, namely those at C-8, could be formed, which after *O*-[^{13}C]methylation using diazomethane results in the di-O$^{13}CH_3$ derivative **1b**. Therefore, ^{18}O incorporation and *O*-[^{13}C]methylation could result in isotopologues of the subgroups **1bB**, **1bD**, **1bF**, and **1bH**; of these, as noted above, only **1bD** and **1bF** were useful here.

In the experiment with [6-^{13}C]DAH-I, isotopologues of the **1bD** group possess one ^{18}O, which may be attached to C-2 (isotopologue **1bD-2**) or C-5 (isotopologue **1bD-5**). As determined by HRESIMS, the isotopologue **1bD** group was formed more efficiently (relative abundance ~ 1%; Table 2) than were the other $^{13}C/^{18}O$-labelled isotopologues, **1bF** and **1bH**. Although not fully conclusive, the abundance of **1bD** may suggest that the incorporation of ^{18}O takes place late in the biosynthetic pathway, and it seems unlikely that an α-oxygenated phenylpropanoid (early oxidation) would be used. The occurrence of isotopologue **1bF**, though present in a small quantity (0.21%) in the experiment with DAH-I, confirmed this suggestion. Since 8-^{13}C in **1bF** comes from [6-^{13}C]DAH-I, which has two unlabelled oxygen atoms (^{16}O) that end up at C-1 and C-6 of **1bF**, the two "new" oxygen atoms at C-2 and C-5 must be ^{18}O. This finding rules out the possibility that an α-oxygenated phenylpropanoid is a precursor. Thus, one ^{18}O atom was incorporated from the atmosphere to DAH-I and another ^{18}O atom was incorporated downstream in the pathway (Fig. 5). The product of the hydroxylation of DAH-I is likely DAH-II, a compound that was previously found to be an excellent precursor of phenylphenalenones (Hölscher and Schneider, 1995a).

In addition to resulting in some singly labelled isotopologues, the administration of [6-^{13}C]DAH-II in an ^{18}O atmosphere produced **1bD,** the only $^{13}C/^{18}O$ labelled isotopologue (Table 2). [6-^{13}C]DAH-II was already oxygenated at the positions, which in **1aD** and **1bD** ended up in the carbonyl group (C-6) and in the oxygen functionalities at C-1 and C-2, respectively. Therefore, the simultaneous occurrence of ^{18}O in positions 1, 5, and 6 and ^{13}C in C-8 was impossible. Only the isotopologue [8-^{13}C,5-$^{18}OCH_3$]-**1a** (**1aD-5**), containing ^{18}O at C-5 as the exclusive labelled oxygen, could be formed in the experiment with [6-^{13}C]DAH-II.

The complete amount (0.42%) of **1bD** (Table 2) generated by derivatization with $^{13}CH_2N_2$ was therefore attributed to a single isotopologue [8-^{13}C,5-$^{18}O^{13}CH_3$,2-$^{16}O^{13}CH_3$]-1 (**1bD-5**). The formation

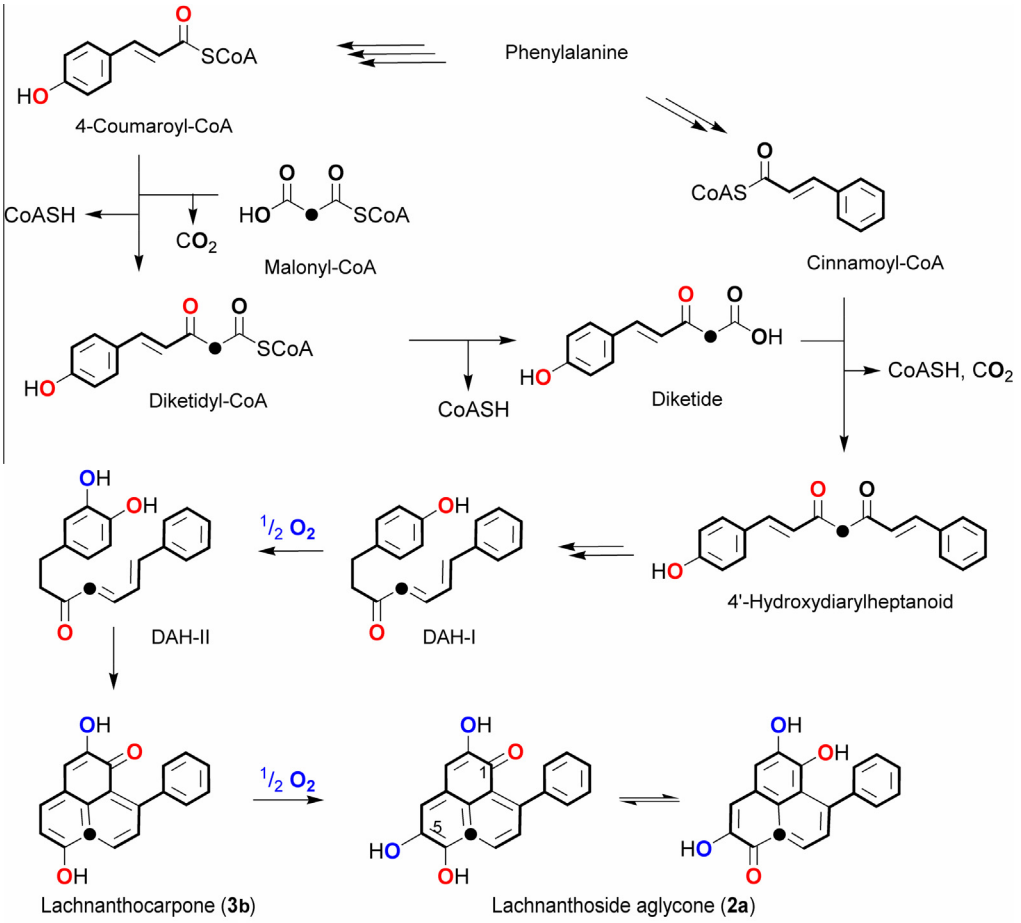

Fig. 5. "Late hydroxylation" biosynthetic pathway of 1,2,5,6-tetraoxygenated phenylphenalenones as evident from $^{18}O/^{13}C$-labelling experiments. The fate of oxygen atoms is coded by color. Oxygen atoms which are suggested to retain from the phenylpropanoid precursor through the entire phenylphenalenone biosynthesis are highlighted in red; lost oxygen atoms are displayed in bold, and oxygen atoms incorporated from O_2 are highlighted in blue. Phenylpropanoid units are marked with bold C–C bonds. ● indicates the origin of the marked atom from C-2 of malonate (Hölscher and Schneider, 1995b).

of isotopologues **1bF** and **1bH** was not possible from [6-^{13}C]DAH-II and indeed was not observed. This result provided evidence that the oxygen atom at C-5 of phenylphenalenones is introduced late in biosynthesis, probably by hydroxylation of lachnanthocarpone (Fig. 5).

In addition to compound **1a**, lachnanthoside aglycone (**2a**) was isolated from root cultures, which were administered with [2-^{13}C]Phe, [2-^{13}C]CA, [6-^{13}C]DAH-I and [6-^{13}C]DAH-II, respectively, and simultaneously incubated under an ^{18}O atmosphere. Again, as observed by ^{13}C NMR and HRESIMS analysis of non-derivatized tautomeric **2a** and its O^{13}CH$_3$-derivatives **2b**, isotope enrichment was low in experiments with all precursors, and the data obtained with [2-^{13}C]Phe, [2-^{13}C]CA, and [6-^{13}C]DAH-I were not conclusive (data not shown). However, ^{13}C/^{18}O-labelled tautomers of lachnanthoside aglycone were detected after the administration of [6-^{13}C]DAH-II and $^{18}O_2$ and were quantified after *O*-methylation with $^{13}CH_2N_2$ as isotopologues **2bD** (Table 3). The occurrence of ^{18}O in **2bD** from the experiment with [6-^{13}C]DAH-II again indicated the incorporation of the oxygen into position 5 late in biosynthesis (Fig. 5). This confirmed the result, which was found for the allophanyl glucoside **1a** and, after *O*-methylation, for **1b**.

2.4. Administration of [phenyl–$^{13}C_6$]lachanthocarpone

After evidence for late oxygenation at C-5 had been obtained by ^{13}C/^{18}O stable isotope labelling experiments, lachnanthocarpone (**3b**) was considered a good candidate to function as the immediate precursor of the 1,2,5,6-tetraoxygenated phenylphenalenone **2a**. Compound **3b** has been previously reported as a constituent of *W. paniculata* (Edwards, 1974). In order to probe for a precursor–product relationship, [*phenyl*–$^{13}C_6$]lachanthocarpone (**3b**) (Otálvaro et al., 2004) was administered to root cultures of *W. thyrsiflora* as described in Experimental, after which compounds **1a** and **2a** were isolated. The NMR spectrum of **1a** (Fig. 6) showed low but significant ^{13}C enrichment in the lateral phenyl ring. The enhancement of the

Table 3
^{13}C/^{18}O Isotopologue composition of the tri-O^{13}CH$_3$ derivatives **2b** was determined by HRESIMS (positive ion mode) and ^{13}C NMR (125 MHz). Lachnanthoside aglycone (**2a**) was isolated from the root cultures of *W. thyrsiflora* after these were incubated with [6-^{13}C]DAH-II in an atmosphere of $^{18}O_2$ followed by *O*-methylation with $^{13}CH_2N_2$ to afford two derivatives of **2b**.

Isotopologue							Isotopologues of **2b** obtained from the experiment with [6-^{13}C]DAH-II			
							Tri-O^{13}CH$_3$ derivative 1 (1-OH, 6-C = O)		Tri-O^{13}CH$_3$ derivative 2 (1-C = O, 6-OH)	
No.	Molecular composition of molecular cation[a]					*m/z* [M + H]$^+$ (calcd.)	*m/z* [M + H]$^+$ (found)	Relative abundance (%)[b]	*m/z* [M + H]$^+$ (found)	Relative abundance (%)[b]
	^{12}C	^{13}C	^{1}H	^{16}O	^{18}O					
2bA	19	3	18	4	0	372.1204	372.1208	78.67	372.1205	78.49
2bB	18	4	18	4	0	373.1237	373.1238	0.76	373.1235	0.77
2bC	19	3	18	3	1	374.1247	374.1258	0.53	374.1255	0.77
2bD	18	4	18	3	1	375.1279	**375.1278**	**0.20**	**375.1276**	**0.10**
2bE	19	3	18	2	2	376.1288	376.1288	0.15	376.1282	0.18

Isotopologue groups **2bF** ($^{12}C_{18}{}^{13}C_4{}^{1}H_{18}{}^{16}O_2{}^{18}O_2Na^+$) and **2bG** ($^{12}C_{19}{}^{13}C_3{}^{1}H_{18}{}^{16}O_2{}^{18}O_2Na^+$) were not detected.
[a] Molecular ions were observed as sodium adducts.
[b] Isotopologues containing the level of naturally abundant ^{13}C were eliminated.

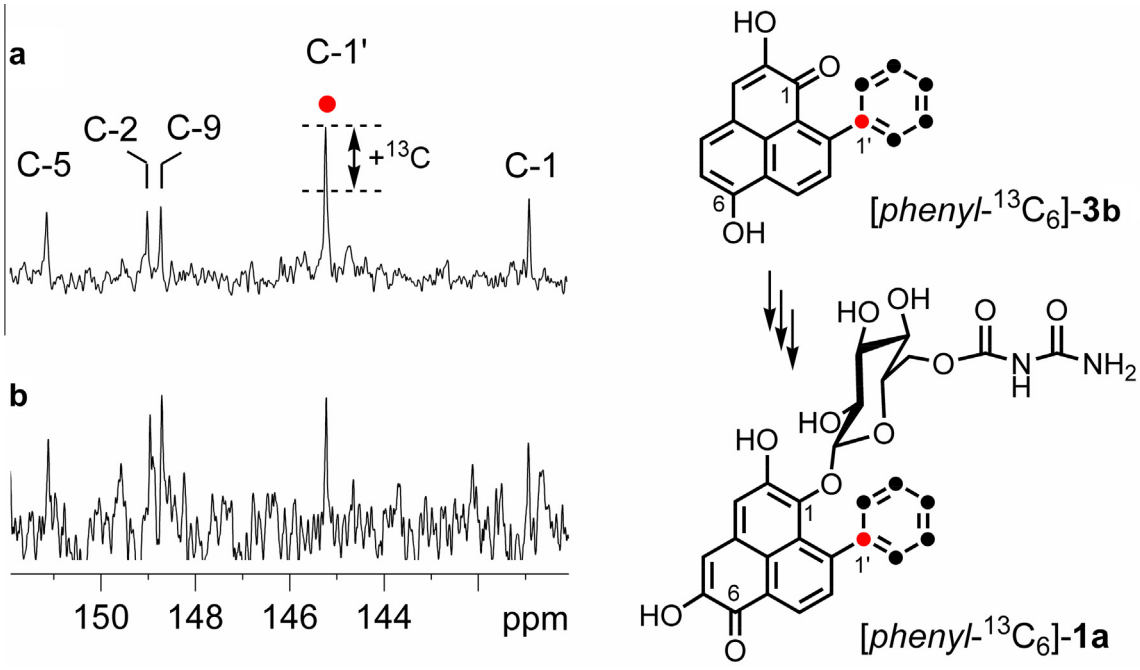

Fig. 6. Partial ^{13}C NMR spectra (125 MHz, acetone-d_6, region of quaternary carbon signals) of **1a** obtained from *W. thyrsiflora* fed with (a) [*phenyl*–$^{13}C_6$]lachnanthocarpone (**3b**). The spectrum shows an enhanced signal for C-1', indicating that ^{13}C was incorporated from the labelled precursor. Spectrum (b) was recorded from unlabelled **1a** (reference). For the numbering of carbon atoms, see also the legend for Fig 1. Red dot: positions enriched with ^{13}C, corresponding signal of [*phenyl*–$^{13}C_6$]**1a** shown in spectrum (a). Black dots: positions enriched with ^{13}C (signals not shown in ^{13}C NMR spectra).

^{13}C NMR signal of the quaternary C-1 in the spectrum (a) compared to the corresponding signal in spectrum (b) of the non-labelled reference indicates ^{13}C-enrichment of this particular carbon atom. The signals of other phenyl carbon atoms of **1a** (C-2'-C-6') (not shown) were enriched as well but are less descriptive due to overlap with other ^{13}C resonances, signal broadening, and magnetic inequivalence (Opitz et al., 2002). In addition to ^{13}C NMR, HRESIMS indicated $^{13}C_6$-labelled isotopologues of **1a** ($[C_{21}{}^{13}C_6H_{25}O_{11}N_2]^+$, found: 559.1654, calcd: 559.1537), confirming labelling of the lateral phenyl ring. The ^{13}C enrichment of 2.0% is in the same order of magnitude as determined by ^{13}C NMR (Fig. 6). HRESIMS also established biosynthetic $^{13}C_6$-labelling of lachnanthoside aglycone (**2a**) (2.0% ^{13}C enrichment, $[C_{13}{}^{13}C_6H_{13}O_4]^+$, found: 311.1006, calcd: 311.1015) from [*phenyl*–$^{13}C_6$]lachnanthocarpone (**3b**) as a precursor. Unfortunately the amount of **2a** was insufficient for recording a ^{13}C NMR spectrum.

3. Discussion

Recombinant *W. thyrsiflora* polyketide synthase 1 (WtPKS1) accepts a wide variety of aromatic and aliphatic starter CoA esters, including phenylpropionyl–CoA and side-chain unsaturated phenylpropanoyl–CoA. This substrate specificity is consistent with the occurrence of 2-hydroxy-9-phenylphenalen-1-one (anigorufone **3a**) and its 2-*O*-methyl derivative (methoxyanigorufone), which in addition to other Haemodoracae, also occur in *W. thyrsiflora* (Fang et al., 2011). However, the most abundant constitutive phenylphenalones of whole plants and cultured roots of *W. thyrsiflora* not only contain the 1-keto-2-hydroxy motif, which is typical of simple phenylphenalenones, but are also oxygenated in additional positions. According to the occurrence of such 1,2,5,6-tetraoxygenated phenylphenalenones and the availability of root cultures, *W. thyrsiflora* was used to investigate the biosynthetic origin of the oxygen functionalities in the tricyclic phenalenone part of phenylphenalenones.

The administration of [1-^{13}C]phenylalanine to *A. preissii* root cultures has demonstrated (Opitz and Schneider, 2003) that the carbon atoms C-6 and C-7 (using the numbering scheme shown in Fig. 1) of phenylphenalenones are derived from C-1 of the phenylpropanoid precursors, and, although this has not been examined experimentally, it is very likely that the carbonyl in position 6 of 1,2,5,6-tetraoxygenated phenylphenalenones comes from the carboxyl group of the precursor forming the A/B ring. Moreover, the carbonyl oxygen atom of 9-phenylphenalenones (e.g. anigorufone **3a**) is derived from the hydroxyl group of 4-coumaroyl–CoA (Munde et al., 2011) as is the oxygen at C-1 of 1,2,5,6-tetraoxygenated phenylphenalenones (e.g. **1a**). Unlike the oxygen atoms at C-1 and C-6, the hydroxyl groups at C-2 and C-5 of compounds **1a** (and **2a**) do not come from the usual phenylpropanoid precursor, 4-coumaroyl–CoA. The introduction of the 2-OH group of anigorufone **3a** from atmospheric oxygen at the stage of DAH-I was established from $^{13}C/^{18}O$-labelling experiments with root cultures of *A. preissii* (Munde et al., 2011). Our results confirm that the 2-OH of 1,2,5,6-tetraoxygenated phenylphenalenones was also inserted at the stage of DAH-I.

Two pathways, an early and a late oxygenation pathway, for understanding the introduction of the oxygen atom at C-5 seemed possible. According to one, this oxygen could be introduced early in the biosynthesis as a unit with an α-oxygenated phenylpropanoid (Fig. 2). 3-(4-Hydroxyphenyl)pyruvic acid or 3-(4-hydroxyphenyl)lactic acid are putative intermediates of this pathway, and the activated esters, e.g. 3-(4-hydroxyphenyl)lactoyl–CoA, could be substrates of the PKS type III enzyme catalyzing the condensation with malonyl–CoA. WtPKS1 was an excellent candidate enzyme for this reaction because it shows broad substrate specificity and, among other starter substrates, also accepted phenylpyruvoyl–CoA, although the hydroxylated pyrone was produced instead of a diarylheptanoid *in vitro* (Brand et al., 2006). Indeed, α-oxygenated 4-hydroxyphenylpropanoids were accepted by WtPKS1, which is the first report of utilization of such substrates by PKS type III enzymes (Fig. 3). However, labelling experiments did not confirm the origin of the oxygen at C-5 from such substrates.

An alternative biosynthetic possibility was that not only the oxygen at C-2 of phenylphenalenones but also the oxygen at C-5 could be introduced at the stage of diarylheptanoids, more specifically to C-2 of the linear carbon chain of DAH-II. However, the question whether the oxygen ending up at C-5 of **1a** is introduced at the stage of DAH-II or further downstream in biosynthesis, after cyclization had yielded the phenalenone tricycle, finally was determined by administering [*phenyl*–$^{13}C_6$]lachnanthocarpone (**3b**) to root cultures of *W. thyrsiflora*. The incorporation of **3b** into lachnanthoside aglycone (**2a**) and its allophanyl glucoside **1a** proved conclusively that the hydroxylation at C-5 of the phenylphenalenone skeleton was the final step of the biosynthesis of 1,2,5,6-tetraoxygenated phenylphenalenones. However, formation of tetraoxygenated phenylphenalenones through additional routes cannot be excluded from this experiment.

The relatively low isotopic enrichment of **1a** and **2a** from atmospheric ^{18}O and ^{13}C-labelled precursors suggests the slow biosynthetic flux into phenylphenalenones in *W. thyrsiflora*. This seems to be a consequence of the constitutive character of the target compounds. Unlike the formation of anigorufone and methoxyanigorufone, the biosynthesis of compounds **1a** and **2a** is not inducible in *W. thyrsiflora* (Schüler et al., 2004; Brand et al., 2006), and the major proportion of these compounds is already present in the root cultures before incubation with labelled compounds starts. In order to detect low isotope enrichment with ^{18}O, the recently reported approach (Munde et al., 2011) of attaching O-$^{13}CH_3$ groups to the hydroxyl functionalities of phenylphenalenones by means of $^{13}CH_2N_2$, followed by recording isotope-induced ^{13}C NMR shifts of the obtained O-$^{13}CH_3$ derivatives, was used. In addition, HRESIMS was employed for isotopologue analysis. The synthesis of the substrates and labeled precursors has been reported in the Experimental and of [*phenyl*–$^{13}C_6$]lachnanthocarpone by Otálvaro et al. (2004).

4. Conclusions

The present work extends previously reported results on the biosynthetic origin of the oxygen functionalities of phenylphenalenones (Fig. 7). $^{13}C/^{18}O$ labelling together with sensitivity-enhanced NMR detection of isotope-induced chemical shift changes of $^{13}CH_2N_2$-derived O$^{13}CH_3$ groups and HRESIMS-based isotopologue analysis have been used successfully to probe the origin of oxygen functionalities and the biosynthetic sequence according to which oxygen functionalities are introduced to the polyoxygenated phenylphenalenone scaffold in root cultures of *W. thyrsiflora*.

The oxygen atoms, except those at C-6, of 1,2,5,6-tetraoxygenated phenylphenalenones were incorporated from atmospheric oxygen at different biosynthetic stages. The oxygen atoms ending up in C-1 and C-6 are incorporated together with the 4-coumaroyl unit (Fig. 7, panel b). Thereafter, the hydroxyl group in position 2 is introduced at the stage of DAH-I (Fig. 5). Isotope labelling experiments showed that the hydroxyl group in position 5 is introduced late in phenylphenalenone biosynthesis, at the stage of lachnanthocarpone (**3b**). Thus, not only the 4-coumaroyl–CoA-derived oxygen at C-1, which is introduced by cinnamate-4-hydroxylase, but also the oxygen atoms at C-2 and C-5 of the phenalenone tricycle are suggested to be the result of monooxygenase-catalyzed reactions. From the data obtained, the "late hydroxylation"

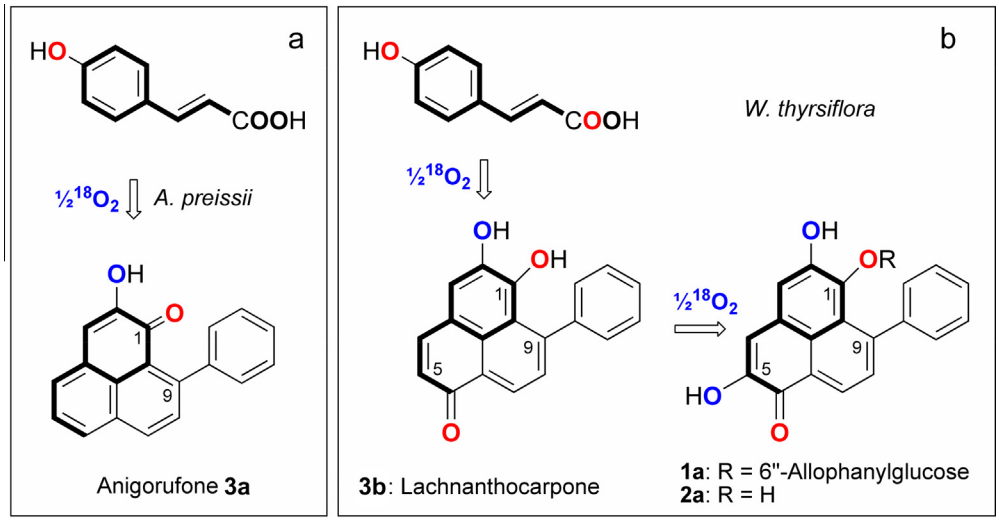

Fig. 7. Origin of oxygen atoms in anigorufone in root cultures of *A. preissii* (Munde et al., 2011) and compound **1a** in root cultures of *W. thyrsiflora*. The hydroxyl group at C-2 of **1a** is incorporated at the stage of linear diarylheptanoids (DAH-I) before the hydroxyl group at C-5, which is introduced into lachnanthocarpone (**3**). The fate of oxygen atoms is coded by color. Oxygen atoms which are retained from 4-coumaric acid are highlighted in red; oxygen atoms lost during phenylphenalenone biosynthesis are displayed in bold, and oxygen atoms incorporated from $^{18}O_2$ are highlighted in blue. The 4-coumaroyl unit is marked with bold C–C bonds.

pathway, as shown in Fig. 5, was concluded for 1,2,5,6-tetraoxygenated phenylphenalenones.

5. Experimental

5.1. General experimental procedures

^{1}H NMR and ^{13}C NMR of samples from biosynthetic incorporation experiments were measured on a Bruker AV 500 spectrometer (Bruker, Rheinstetten, Germany) equipped with a 5 mm TCI CryoProbe™. The Bruker pulse sequence zgpg30 (power-gated decoupling; 30° flip angle; relaxation delay 2 s) was used for recording ^{13}C spectra. A Bruker DRX 500 and a Bruker AV 400 NMR spectrometer were used to measure NMR spectra of synthetic phenylpropanoic acids and NAC diketide thioesters. Acetone-d_6, MeOH-d_4 and $CDCl_3$ were used as NMR solvents. Chemical shifts are given in δ values. Tetramethylsilane (TMS) was used as an internal standard for referencing ^{1}H and ^{13}C NMR spectra.

The LC-MS/MS system used for HRESIMS measurements consisted of an Ultimate3000 series RSLC (Dionex) system and an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). UPLC was performed using an Acclaim C18 column (150 × 2.1 mm, 2.2 µm, Dionex, Germany) at a constant flow rate of 300 µL min^{-1} using a binary solvent system: Solvent A, H_2O with 0.1% HCOOH and solvent B, MeCN with 0.1% HCOOH. The UPLC gradient system started with 20% B and increased linearly to 95% B in 6 min; from there it was held for 4 min, then brought back to the initial conditions and held for 4 more min in order to re-equilibrate the column for the next injection. Full-scan mass spectra were generated using 30,000 m/Δm resolving power, and for MS2 (CID) experiments the resolution power was set to 7500 m/Δm. The mass accuracy was better than 3 ppm for MS and MS2 experiments. LCESIMS for substrate incubation with WtPKS1 were obtained using a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) operating in both positive and negative ion modes. Enzymatic products were separated on a C18-RP column. Details were reported elsewhere (Brand et al., 2006).

Preparative HPLC was performed on a Merck–Hitachi LiChrograph chromatography system (L-6200A gradient pump, L-4250 UV/Vis detector). Flow rate 3.5 ml min^{-1}; UV detection at 254 nm. Semipreparative and analytical HPLC was performed on an Agilent (Palo Alto, CA, USA) series HP1100 (binary pump G1312A; auto sampler G1313A; diode array detector G1315B, 200–700 nm). Flow rate 0.8 ml min^{-1}; monitoring wavelength 254 nm.

5.2. Plant material and culture conditions

Root cultures of *W. thyrsiflora* were established from seeds as previously described for root cultures of *A. preissii* (Hölscher and Schneider, 1997). Sterile cultures were grown in liquid M3 (Murashige and Skoog, 1962) medium (100 ml) in conical flasks (volume 250 ml) on a gyratory shaker (85 rpm) at 23 °C under permanent diffuse light (4.4 µmol m^{-2} s^{-1}) until they reached a fresh mass of up to 40 g. Three days before labelled precursors were administered, the *in vitro*-cultured roots were transferred into fresh medium.

5.3. Synthetic procedures and analytical data

5.3.1. Synthesis of activated ester derivatives

Phenylpropanoyl–CoA esters were prepared according to the method described by Wang et al. (2000), using RP-18 cartridges (LiChrolut®, VWR Darmstadt, Germany) in a modified purification procedure (Beuerle and Pichersky, 2002). 3-(4-Hydroxyphenyl) pyruvic acid and (*R*)- and (*S*)-3-(4-hydroxyphenyl)lactic acids were prepared as described below for the ^{13}C-labelled compounds. The same procedure was used to prepare phenylpyruvic acid, and (*R*)- and (*S*)-phenyl)lactic acids. To synthesize the *N*-acetyl cysteamine derivatives, the corresponding acid (cinnamic acid, phenylpropionic acid, phenylpyruvic acid, 3-(4-hydroxyphenyl)pyruvic acid, (*R*)- and (*S*)-phenyllactic acid, (*R*)- and (*S*)-3-(4-hydroxyphenyl)lactic acid) was dissolved in CH_3CN. Dicyclohexyl-carbodiimide, *N*-hydroxy-1,2,3-benzotriazole (both equimolar) and *N*-acetylcysteamine (in threefold molar excess) were added and the mixture was stirred overnight. The yield after purification by preparative HPLC was 82% for phenylpropionyl–NAC and up to 67% for *R*- and *S*-phenyl lactoyl–NAC's and phenylpyruvoyl–NAC. The 4-hydroxyphenyllactic and -pyruvic acids were protected using the tetrahydropyranyl group (Parham and Anderson, 1948). Pyridinium 4-toluenesulfonate (5 mg, 4 d, 5 ml THF) was used for deprotection. In the latter

case the total yields were very low (1%) but still more than sufficient for the enzyme assays.

5.3.2. Analytical data of N-acetyl cysteamine derivatives

Cinnamoyl-*N*-acetylcysteamine. ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.63 (1H, d, J = 15.9 Hz), 7.63 (2H, m, H-2/6), 7.41 (3H, m, H-3/5, H-4), 6.87 (1H, d, J = 15.9 Hz, H-8), 3.39 (2H, t, J = 6.7 Hz, H-12), 3.13 (2H, t, J = 6.7 Hz, H-11), 1.93 (3H, s, C-15).

Phenylpropionyl-*N*-acetylcysteamine. ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.25 (2H, m, H-3/5), 7.18 (3H, m, H-2/6, H-4), 3.29 (2H, t, J = 6.7 Hz; H-12), 2.98 (2H, t, J = 6.7 Hz, H-11), 2.94 (2H, m, H-8), 2.88 (2H, m, H-7), 1.90 (3H, s, H-15).

Phenylpyruvoyl-*N*-acetylcysteamine. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.97 (2H, dd, J = 8.0 Hz, 1.5 Hz, H-2/6), 7.60 (1H, dt, J = 8.0 Hz, 1.5 Hz, H-4), 7.47 (2H, t, J = 8.0 Hz, H-3/5), 3.84 (0.8 H, s, H-7, signal intensity reduced due to H/D exchange), 3.54 (2 H, q, H-12), 3.24 (2H, t, J = 6.5 Hz, H-11), 1.98 (3H, s, H-15).

3-(4-Hydroxyphenyl)pyruvoyl-*N*-acetylcysteamine. ^{1}H NMR (500 MHz, acetone-d_6): δ 7.62 (2H, d, J = 8.8 Hz, H-2/6), 6.92 (2H, d, J = 8.8 Hz, H-3/5), 3.36 (2H, dt, J = 6.7, 5.9 Hz, H-12), 3.09 (2H, t, J = 6.7 Hz, H-11), 1.87 (3H, s, H-15). H-7 not detected due to H/D exchange.

(*R*)-Phenyllactoyl-*N*-acetylcysteamine. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.32 (2H, m, H-3/5), 7.28 (1H, m, H-4), 7.25 (2H, m, H-2/6), 4.49 (1H, dd, J = 7.7, 4.3 Hz, H-8), 3.42 (2H, m, H-12), 3.18 (1H, dd, J = 14.2, 4.3 Hz, H-7a), 3.04 (2H, dt, J = 6.3, 1.5 Hz, H-11), 2.98 (1H, dd, J = 14.2, 7.7 Hz, H-7b) 1.96 (3H, s, H-15).

(*S*)-Phenyllactoyl-*N*-acetylcysteamine. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.32 (2H, m, H-3/5), 7.28 (1H, m, H-4), 7.25 (2H, m, H-2/6), 4.49 (1H, dd, J = 7.7, 4.3 Hz, H-8), 3.42 (2H, m, H-12), 3.18 (1H, dd, J = 14.2, 4.3 Hz, H-7a), 3.04 (2H, m, H-11), 2.98 (1H, dd, J = 14.2, 7.7 Hz, H-7b) 1.96 (3H, s, H-15).

(*R*)-4-Hydroxyphenyllactoyl-*N*-acetylcysteamine. ^{1}H NMR (500 MHz, acetone-d_6): δ 7.24 (2H, d, J = 8.5 Hz, H-2/6), 6.92 (2H, d, J = 8.5 Hz, H-3/5), 5.45 (1H, dd, J = 8.7, 4.4 Hz, H-8), 3.44 (2H, dt, J = 6.8, 5.8 Hz, H-12), 3.24 (1H, dd, J = 14.2, 4.4 Hz, H-7a), 3.15 (2H, dt, J = 6.8, 2.9 Hz, H-11), 3.11 (1H, dd, J = 14.2, 8.7 Hz, H-7b), 1.86 (3H, s, H-15).

(*S*)-4-Hydroxyphenyllactoyl-*N*-acetylcysteamine. ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.05 (2H, d, J = 8.3 Hz, H-2/6), 6.69 (2H, d, J = 8.3 Hz, H-3/5), 4.25 (1H, dd, J = 8.7, 4.0 Hz, H-8), 3.29 (2H, overlap, H-12), 2.98 (1H, dd, J = 14.2 Hz, 4.0 Hz, H-7a), 2.95 (2H, dt, J = 6.7, 1.9 Hz, H-11), 2.73 (1H, dd, J = 14.2, 8.7 Hz, H-7b), 1.92 (3H, s, H-15).

5.4. Labelled compounds

5.4.1. Origin and synthesis of labelled compounds

[1-^{13}C]L-Phenylalanine (99% ^{13}C) and [2-^{13}C]malonic acid (99% ^{13}C) were purchased from Deutero GmbH (Castellaun, Germany). $^{18}O_2$ (95%) was from EurisoTop (Saarbrücken, Germany). [2,3,4,5,6-^{3}H]L-Phenylalanine, [U–^{14}C]4-coumaric acid, and [2-^{14}C]malonyl–CoA were from Hartmann Analytic (Braunschweig, Germany). [2-^{13}C]4-Coumaric acid (99% ^{13}C) was synthesized from 4-hydroxybenzaldehyde and [2-^{13}C]malonic acid using the Knoevenagel condensation. [2-^{13}C]3-(4-Hydroxyphenyl)pyruvic acid was synthesized under an Ar atmosphere from [2-^{13}C]tyrosine (99% ^{13}C, 0.3 g, 1.6 mmol), dimethylaminopyridine (DMAP, 9.9 mg) and $(CF_3CO)_2O$ TFA, (4 ml, 28.8 mmol) by refluxing for 20 h. After evaporation, the residue was reconstituted with HCl (10 ml, 1%) and stirred for 12 h at room temperature. After 12 h at 4 °C the precipitate was filtered, washed with cold H_2O, and dried over (P_4O_{10}) to obtain the product (0.24 g, 81%). [2-^{13}C](*R*)- and [2-^{13}C](*S*)-3-(4-hydroxyphenyl)lactic acids were prepared from [2-^{13}C]3-(4-hydroxyphenyl)pyruvic acid using (–)-diisopinocampheyl chloroborane [(–)DIP-Cl] and (+)-diisopinocampheyl chloroborane [(+)DIP-Cl], respectively. Et_3N (77 μl, 0.55 mmol) was added at –20 °C to a solution of [2-^{13}C](4-hydroxyphenyl)pyruvic acid (100 mg, 0.55 mmol) in THF (10 ml). After 5 min, a solution of (–)DIP-Cl or (+)DIP-Cl (0.3 g, 0.94 mmol) in THF was added dropwise to the mixture. The solution was stirred for 8 h at room temperature and then quenched with 2 N NaOH (1.2 ml) and H_2O (0.6 ml). The combined aqueous fractions obtained from extraction of the aqueous phase with *t*-butylmethyl ether (2×) and the organic phase with H_20 (2×) were acidified to pH 1 with 2 N HCl and extracted with EtOAc. Evaporation to dryness yielded [2-^{13}C](*R*)-3-(4-hydroxyphenyl)lactic acid (65%) and [2-^{13}C](*S*)-3-(4-hydroxyphenyl)lactic acid (65%), respectively. [6-^{13}C]1-(4-Hydroxyphenyl)-7-phenylhepta-4,6-dien-1-one ([6-^{13}C]DAH-I) and [6-^{13}C]1-(3,4-dihydroxyphenyl)-7-phenylhepta-4,6-dien-1-one ([6-^{13}C]DAH-II) were synthesized according to a previously reported procedure (Baranovsky et al., 2003; Munde et al., 2011). The synthesis of [*phenyl*–$^{13}C_6$]lachnanthocarpone **3b** (99% ^{13}C) was reported previously (Otálvaro et al., 2004).

5.4.2. Analytical data of synthetic labelled compounds

[2-^{13}C]3-(4-Hydroxyphenyl)pyruvic acid. ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.63 (d, J = 8.8 Hz, 2H, H-2'/6'), 6.75 (d, J = 8.8 Hz, 2H, H-3'/5'), 6.44 (d, $^2J_{H\text{-}C}$ = 2.5 Hz, 1H, H-3); ^{13}C NMR (125 MHz, MeOH-d_4): δ 169.1 (C-1), 158.3 (C-4'), 140.1 (C-2, signal enhanced due to ^{13}C enrichment), 132.4 (C-2'/6'), 128.0 (C-1'), 116.2 (C-3'/5'), 112.6 (C-3). EIMS *m/z* (rel. int.): 181 [M$^+$] (16), 135 (20), 107 (100), 77 (17).

[2-^{13}C](*R*)-3-(4-Hydroxyphenyl)lactic acid. ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.08 (d, J = 8.5 Hz, 2H; H-2'/6'), 6.70 (d, J = 8.5 Hz, 2H; H-3'/5'), 4.26 (dd, $^3J_{H\text{-}2\text{–}H\text{-}3b}$ = 7.8 Hz, $^3J_{H\text{-}2\text{–}H\text{-}3a}$ = 4.0 Hz, $^1J_{H\text{-}2\text{–}C\text{-}2}$ = 145.5 Hz, 1H; H-2), 3.01 (dd, $^3J_{H\text{-}3a\text{–}H\text{-}2}$ = 4.0 Hz, $^2J_{H\text{-}3a\text{–}H\text{-}3b}$ = 14.0 Hz, 1H; H-3a), 2.80 (dd, $^3J_{H\text{-}3b\text{–}H\text{-}2}$ = 7.8 Hz, $^2J_{H\text{-}3b\text{–}H\text{-}3a}$ = 14.0 Hz, 1H; H-3b); ^{13}C NMR (125 MHz, MeOH-d_4): δ 174.1 (C-1), 157.1 (C-4'), 131.7 (C-2'/6'), 129.9 (C-1'), 116.1 (C-3'/5'), 74.1 (C-2, signal enhanced due to ^{13}C enrichment), 40.9 (C-3). EIMS *m/z* (rel. int.): 183 [M$^+$] (9), 107 (100), 77 (10).

[2-^{13}C](*S*)-3-(4-Hydroxyphenyl)lactic acid. ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.08 (d, J = 8.5 Hz, 2H; H-2'/6'), 6.70 (d, J = 8.5 Hz, 2H; H-3'/5'), 4.26 (dd, $^3J_{H\text{-}2\text{–}H\text{-}3b}$ = 7.8 Hz, $^3J_{H\text{-}2\text{–}H\text{-}3a}$ = 4.4 Hz, $^1J_{H\text{-}2\text{–}C\text{-}2}$ = 145.5 Hz, 1H; H-2), 3.01 (dd, $^3J_{H\text{-}3a\text{–}H\text{-}2}$ = 4.0 Hz, $^2J_{H\text{-}3a\text{–}C\text{-}3b}$ = 14.0 Hz, 1H; H-3a), 2.80 (dd, $^3J_{H\text{-}3b\text{–}H\text{-}2}$ = 7.8 Hz, $^2J_{H\text{-}3b\text{–}H\text{-}a}$ = 14.0 Hz, 1H; H-3b); ^{13}C NMR (100 MHz, MeOH-d_4): δ 174.9 (C-1), 155.8 (C-4'), 130.3 (C-2'/6'), 128.3 (C-1'), 114.7 (C-3'/5'), 71.8 (C-2, signal enhanced due to ^{13}C enrichment), 39.7 (C-3). EIMS *m/z* (rel. int.): 183 [M$^+$] (13), 107 (100), 77 (5).

5.5. Enzyme assay

Assay conditions were previously reported (Brand et al., 2006). In brief, the phenylpropanoyl starter substrates (50 μM), [2-^{14}C]malonyl–CoA (80 μM, 0.17 kBq nmol^{-1}) and the recombinant WtPKS1 protein (20 μg) (EMBL/DDBJ/GenBank™ database accession number AY727928) were added to the buffer (0.1 M Hepes, pH 6.85; assay volume 100 μl) and incubated for 1 h at 37 °C. Products were extracted with (2 × 200 μl) EtOAc. The combined EtOAc extracts were evaporated, reconstituted in MeOH (20 μl), and analyzed by reversed-phase HPLC using a Supelcosil LC18 column, 250 × 2.1 mm i.d. (Sigma–Aldrich, Taufkirchen, Germany), solvent A: H_2O containing 0.1% TFA, solvent B: MeCN, gradient: 10% to 50% solvent B over 40 min at a flow rate of 0.25 ml min^{-1}. Enzyme products were detected at 280 nm and, after adding scintillation liquid (Ultima-Flo AP, 1 ml min^{-1}, Perkin Elmer), by radioactivity monitoring using a flow scintillation analyzer (Canberra-Packard, Dreieich, Germany). For NMR and MS analysis, the assays were scaled up 10 to 18-fold, with the appropriate amount of unlabelled malonyl–CoA.

5.6. Administration of labelled precursors

The administered amounts of labelled compounds were as follows: [2-^{13}C](*S*)-3-(4-hydroxyphenyl)lactic acid (3.0 mg), [2-^{13}C](*R*)-3-(4-hydroxyphenyl)lactic acid (3.0 mg), [2-^{13}C]-3-(4-hydroxyphenyl)pyruvic acid (3.0 mg), [2-^{13}C]Phe (3.5 mg), [2-^{13}C]CA (2.7 mg), [6-^{13}C]DAH-I (3.5 mg), [6-^{13}C]DAH-II (3.5 mg), and [*phenyl*–$^{13}C_6$]lachnanthocarpone **3b** (2.2 mg). Compounds were administered to *W. thyrsiflora* root cultures (100 ml medium) as an ethanolic solution (1 ml) through a sterile membrane filter. For incubation with $^{18}O_2$, the conical flask containing the root culture was connected through a three-way cock to a vacuum pump and an $^{18}O_2$ reservoir. The normal atmosphere was removed from the incubation flask under vacuum (72 mbar) and $^{18}O_2$ filled from the scaled reservoir. The incubation flask was also connected to a tube containing NaOH for trapping metabolically formed CO_2 from cultured roots. The incubation time was 4-5 days except in the $^{18}O_2$ incubation experiments (7 days) under continuous shaking (85 rpm). The administration of radio-labelled [2,3,4, 5,6-^{3}H]Phe (1.5 mg, 0.37 kBq) and [U–^{14}C]CA (1.5 mg, 0.34 kBq) in absorption experiments was carried out under conditions otherwise identical to experiments using stable isotope labelling.

5.7. Extraction, purification and derivatization of compounds **1a** and **2a**

Cultured roots were taken from the medium, shock-frozen by liquid nitrogen, ground, and extracted with MeOH at room temperature. The extract was evaporated to dryness (<40 °C), and the residue subjected to liquid–liquid separation between *n*-hexane-H_2O, CH_2Cl_2–H_2O and EtOAc–H_2O. The organic phases were subjected to preparative HPLC on a LiChrospher RP18 column (10 μm, 250 × 10 mm). A linear gradient MeCN–H_2O from 10% to 50% MeCN in 40 min (method a) and MeCN–H_2O (0.1% TFA in H_2O), 30% to 75% MeCN in 35 min (method b) was used. Pure **1a** and **2a** were obtained from the CH_2Cl_2 and ethyl acetate extracts at R_t 13.0 min (method a) and R_t 16.0 min (method b), respectively. The pure products **1a** and **2a** were analyzed by NMR and HRESIMS. Compounds obtained from $^{12}C/^{18}O$ double-labelling experiments were methylated using an ethereal solution of [^{13}C]diazomethane, which was prepared from *N*-[^{13}C]methyl-*N*-nitroso-*p*-toluenesulfonamide using a standard procedure. Semipreparative HPLC on a LiChrospher RP18 column (5 μm, 250 × 10 mm) was used to purify the *O*-methyl derivatives **1b** and **2b**. Method c: linear gradient MeCN–H_2O (0.1% TFA) from 40% to 60% MeCN in 20 min and method d: linear gradient MeCN–H_2O (0.1% TFA) from 40% to 75% MeCN in 30 min were used. Pure **1b** was obtained with method c at R_t 15.7 min and two *O*-methyl derivatives **2b** with method d at R_t 13.4 and 13.8 min.

5.8. Analytical data of biosynthetic products and their O-methyl derivatives

6-*O*-[(6''-*O*-Allophanyl)-β-D-glucopyranosyl]-2,5-dihydroxy-7-phenylphenalen-1-one (**1a**): HPLC (method a): UV (MeCN–H_2O) λ_{max} 211, 279, 378, 477 nm; ^{1}H NMR (500 MHz, MeOH-d_4): δ 2.36 (dd, *J* = 7.9, 9.0 Hz, 1H, H-2''), 3.04 (dd, *J* = 9.0, 9.3 Hz, 1H, H-4''), 3.09 (ddd, *J* = 2.2, 6.5, 9.0 Hz, 1H, H-5''), 3.19 (dd, *J* = 9.0, 9.3 Hz, 1H, H-3''), 3.96 (dd, *J* = 2.2, 11.8 Hz, 1H, H-6''b), 4.18 (dd, *J* = 6.5, 11.8 Hz, 1H, H-6''a), 4.67 (d, *J* = 7.9 Hz, 1H, H-1''), 7.04 (s, 1H, H-3), 7.22 (br, 1H, H-5'), 7.33 (br, 2H, H-4', H-2''), 7.39 (br, 1H, H-6'), 7.43 (br, 1H, H-3'), 7.46 (s, 1H, H-4), 7.54 (d, *J* = 7.6 Hz, 1H, H-8), 8.47 (d, *J* = 7.6 Hz, 1H, H-9); ^{13}C NMR (125 MHz, MeOH-d_4): δ 64.9 (C-6''), 71.1 (C-4''), 74.6 (C-2''), 75.2 (C-5''), 77.6 (C-3''), 104.1 (C-1'') 116.3 (C-3), 121.7 (C-9b), 124.2 (C-4), 127.3 (C-2'), 127.8 (C-6[a], C-4') 128.3 (C-6'), 128.5 (C-3a), 128.6 (C-9), 130.0 (C-9a), 130.7 (C-3'), 131.8 (C-5'), 132.3 (C-8), 141.0 (C-6), 145.3 (C-1'), 148.8 (C-7), 149.0 (C-5), 151.2 (C-2), 169.3 (C-1'''), 171.5 (C-2'''), 182.2 (C-1). Please note different carbon atom numbering of **1a** in the text and figures.

2,5,6-Trihydroxy-9-phenylphenalen-1-one (**2a**): HPLC (gradient b): UV (MeCN–H_2O) λ_{max} 208, 278, 374 nm; ^{1}H NMR (500 MHz, acetone-d_6): δ 7.36 (s, 1H, H-3), 7.38–7.44 (m, 5H, H-2' to H-6'), 7.54 (d, *J* = 8.2 Hz, 1H, H-8), 7.84 (s, 1H, H-4), 8.66 (d, *J* = 8.2 Hz, 1H, H-9); ^{13}C NMR (125 MHz, acetone-d_6): δ 112.9 (C-3), 118.4 (C-3a), 120.7 (C-9b), 122.7 (C-4), 125.4 (C-9a), 126.9 (C-4'), 128.2 (C-3'/5'), 129.1 (C-2'/6'), 129.2 (C-9), 129.9 (C-6a), 130.6 (C-8), 139.0 (C-6), 144.2 (C-1'), 147.3 (C-7), 149.0 (C-5), 150.7 (C-2), 178.4 (C-1).

[2,5-di-O^{13}CH$_3$]6-*O*-[(6''-*O*-Allophanyl)-β-D-glucopyranosyl]-2,5-methoxy-7-phenylphenalen-1-one (**1b**): HPLC (gradient c): UV (MeCN–H_2O) λ_{max} 201, 279, 374 nm; ^{1}H NMR (500 MHz, MeOH-d_4): δ 2.05 (dd, *J* = 7.7, 9.3 Hz, 1H, H-2''), 2.97 (ddd, *J* = 2.3, 5.6, 9.7 Hz, 1H, H-5''), 3.03 (dd, *J* = 8.7, 9.7 Hz, 1H, H-4''), 3.21 (dd, *J* = 8.7, 9.3 Hz, 1H, H-3''), 3.45 (dd, *J* = 5.6, 11.9 Hz, 1H, H-6''a), 3.60 (dd, *J* = 2.3, 11.9 Hz, 1H, H-6''b), 3.99 (s, $^1J_{H-C}$ = 145.0 Hz, 3H, 2-O^{13}C*H*$_3$), 4.05 (s, $^1J_{H-C}$ = 145.0 Hz, 3H, 5-O^{13}C*H*$_3$), 5.11 (d, *J* = 7.7 Hz, 1H, H-1''), 7.29 (s, 1H, H-3), 7.32 (br, 1H, H-5'), 7.38 (br, 2H, H-2' and H-4'), 7.44 (br, 1H, H-6'), 7.50 (br, 1H, H-3'), 7.55 (d, *J* = 7.6 Hz, 1H, H-8), 7.86 (s, 1H, H-4), 8.56 (d, *J* = 7.6 Hz, 1H, H-9); ^{13}C NMR (125 MHz, MeOH-d_4): δ 56.31 (2-^{18}O^{13}CH$_3$, $^1\Delta\delta$ ^{18}O(^{13}C)=−25 ppb), 56.34 (2-^{16}O^{13}CH$_3$), 57.73 (5-^{18}O^{13}CH$_3$, $^1\Delta\delta$ ^{18}O(^{13}C)=−29 ppb), 57.76 (5-^{16}O^{13}CH$_3$), 62.8 (C-6''), 71.5 (C-4''), 74.6 (C-2''), 77.8 (C-5''), 78.2 (C-3''), 103.0 (C-1''), 115.6 (C-3), 121.4 (C-9b), 122.3 (C-4), 126.6 (C-3a), 128.0 (C-6a, C-2', C-4'), 128.6 (C-9), 128.9 (C-6'), 129.5 (C-9a), 129.7 (C-3'), 130.3 (C-5'), 132.6 (C-8), 143.6 (C-6), 145.8 (C-1'), 149.7 (C-7), 151.4 (C-5), 153.7 (C-2), 169.3 (C-1'''), 171.5 (C-2'''), 179.2 (C-1). Please note different carbon atom numbering of **1b** in the text and figures.

[2,5,6-tri-O^{13}CH$_3$]2,5,6-Trimethoxy-7-phenylphenalen-1-one (**2b**, R_t 13.8 min): HPLC (gradient d): R_t 13.8 min, UV (MeCN–H_2O) λ_{max} 208, 279, 373 nm; ^{1}H NMR (500 MHz, acetone-d_6): δ 3.27 (s, $^1J_{H-C}$ = 144.7 Hz, 3H, 6-O^{13}C*H*$_3$), 3.93 (s, $^1J_{H-C}$ = 144.5 Hz, 3H, 2-O^{13}C*H*$_3$), 4.03 (s, $^1J_{H-C}$ = 144.6 Hz, 3H, 5-O^{13}C*H*$_3$), 7.22 (s, 1H, H-3), 7.39-7.47 (br, 5H, H-2' to H-6'), 7.56 (d, *J* = 7.5 Hz, 1H, H-8), 7.86 (s, 1H, H-4), 8.49 (d, *J* = 8.5 Hz, 1H, H-9); ^{13}C NMR (125 MHz, acetone-d_6): δ 55.7 (2-^{16}O^{13}CH$_3$), 57.2 (5-^{16}O^{13}CH$_3$), 60.64 (6-^{18}O^{13}CH$_3$, $^1\Delta\delta$ ^{18}O(^{13}C)=−28 ppb), 60.67 (6-^{16}O^{13}CH$_3$), 114.1 (C-3), 119.9 (C-4), 121.9 (C-9b), 125.9 (C-3a), 127.2 (C-9a), 127.4 (C-4'), 127.8 (C-3'/C-5'), 128.2 (C-9), 129.3 (C-2'/C-6'), 129.9 (C-6a), 131.3 (C-8), 144.7 (C-1'), 146.9 (C-7), 146.6 (C-6), 151.5 (C-5), 153.4 (C-2), 179.6 (C-1). Please note different carbon atom numbering in the text and figures.

[2,5,6-tri-O^{13}CH$_3$]2,5,6-Trimethoxy-9-phenylphenalen-1-one (**2b**, R_t 13.4 min): HPLC (gradient d): UV (MeCN–H_2O) λ_{max} 209, 277, 374 nm; ^{1}H NMR (500 MHz, acetone-d_6): δ 3.84 (s, $^1J_{H-C}$ = 144.5 Hz, 3H, 2-O^{13}C*H*$_3$), 4.10 (s, $^1J_{H-C}$ = 144.8 Hz, 3H, 6-O^{13}C*H*$_3$), 4.11 (s, $^1J_{H-C}$ = 144.7 Hz, 3H, 5-O^{13}C*H*$_3$), 7.12 (s, 1H, H-3), 7.34 - 7.45 (br, 5H, H-2' to H-6'), 7.56 (d, *J* = 8.5 Hz, 1H, H-8), 7.82 (s, 1H, H-4), 8.52 (d, *J* = 8.5 Hz, 1H, H-7); ^{13}C NMR (125 MHz, acetone-d_6): δ 55.8 (2-^{16}O^{13}CH$_3$), 57.24 (5-^{18}O^{13}CH$_3$, $^1\Delta\delta$ ^{18}O(^{13}C)=−28 ppb), 57.27 (5-^{16}O^{13}CH$_3$), 61.9 (6-^{16}O^{13}CH$_3$), 112.2 (C-3), 119.6 (C-4), 121.9 (C-6b), 126.3 (C-3a), 127.5 (C-4'), 128.4 (C-7), 128.6 (C-6a), 128.8 (C-3'/C-5'), 129.0 (C-2'/C-6'), 129.9 (C-9a), 132.2 (C-8), 144.3 (C-1'), 144.8 (C-6), 145.8 (C-5), 146.5 (C-9), 153.9 (C-2), 179.8 (C-1).

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