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4-Methoxycinnamic acid – An unusual phenylpropanoid involved in phenylphenalenone biosynthesis in *Anigozanthos preissii*

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ABSTRACT

In vitro root cultures of *Anigozanthos preissii* and *Wachendorfia thyrsiflora* (Haemodoraceae) are suitable biological systems for studying the biosynthesis of phenylphenalenones. Here we report how we used these root cultures to investigate precursor–product relationships between phenylpropanoids and phenylphenalenones whose phenyl rings share identical substitution patterns. Four phenylpropanoic acids, including ferulic acid and the unusual 4-methoxycinnamic acid, were used in ^{13}C-labeled form as substrates to study their incorporation into phenylphenalenones. In addition to the previously reported 2-hydroxy-9-(4′-hydroxy-3′-methoxyphenyl)-1*H*-phenalen-1-one (trivial name musanolone F), 2-hydroxy-9-(4′-methoxyphenyl)-1*H*-phenalen-1-one (proposed trivial name 4′-methoxyanigorufone) was found as a biosynthetic product in *A. preissii*. The carbon skeleton of 4′-methoxycinnamic acid was biosynthetically incorporated as an intact unit including its 4′-*O*-methyl substituent at the lateral phenyl ring. 4′-Methoxyanigorufone is reported here for the first time as a natural product.

1. Introduction

Phenylphenalenones are a characteristic class of secondary metabolites occurring in the monocotyledoneous plant families Haemodoraceae, Pontederiaceae, Strelitziaceae and Musaceae (Munde et al., 2013). They are polycyclic aromatic compounds consisting of a tricyclic phenalene core structure with a keto functional group and a lateral phenyl ring (ring D) (see structure in Table 1). Because of their fungicidal (Otálvaro et al., 2007; Hidalgo et al., 2009) and nematicidal properties (Hölscher et al., 2014) and their inducibility after pathogen/herbivore attack (Luis et al., 1994; Jitsaeng and Schneider, 2010; Otálvaro et al., 2010), phenylphenalenones play an important role as phytoalexins in the chemical defense of *Musa* plants. The physiological and/or ecological importance of these metabolites in the three other plant families has yet to be explored, although their antioxidant capacity could be one of the reasons they are produced (Blokhina et al., 2003; Duque et al., 2010; Brewer, 2011).

Previous biosynthetic studies have demonstrated that phenylphenalenone-type compounds are formed by the condensation of two phenylpropanoids merged with one malonate unit through the diarylheptanoid pathway. The variable substitution pattern of the lateral phenyl ring (ring D: unsubstituted phenyl; 4′-hydroxyphenyl; 3′,4′-dihydroxyphenyl; 3′-methoxy-4′-hydroxyphenyl) contributes to the structural diversity of the phenylphenalenones (Hölscher and Schneider, 1995a,b; Schmitt et al., 2000). As previously demonstrated in *Anigozanthos preissii*, the substitution pattern of ring D reflects the substitution pattern of the phenylpropanoid precursors (Schmitt et al., 2000). Four phenylpropanoids have been identified so far as precursors of a C-9 moiety comprising ring D and three adjacent carbon atoms C-7 to C-9 of the phenalenone tricycle (for details see Table 1). In the case of 4′-*O*-methylirenolone, which has a methoxy group in position 4′ of ring D, the actual phenylpropanoid precursor could not be determined because 4′-methoxycinnamic acid (2), when administered to *in vitro* plants of *Musa acuminata*, was demethylated and further converted to 3′,4′-methylenedioxyphenylpropanoic acid (Otálvaro et al., 2010). Hence, due to their strong demethylation capacity for exogenously supplied 4′-methoxycinnamic acid, *Musa* plants seemed to be unsuitable for studying the suggested biosynthetic precursor–product relationship between methoxyphenylpropanoids and phenylphenalenones with methoxy-substituted ring D, such as 4′-*O*-methylirenolone.

In vitro root cultures of *A. preissii* and *Wachendorfia thyrsiflora* (both belonging to the Haemodoraceae plant family), which are suitable biological systems for studying the biosynthesis of phenylphenalenones (Hölscher and Schneider, 1995b; Schmitt et al., 2000; Munde et al., 2013), produce pairs of phenylpropanoids

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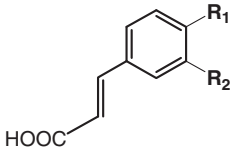
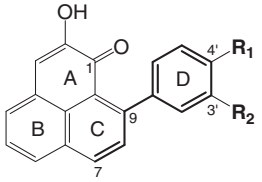
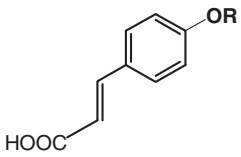
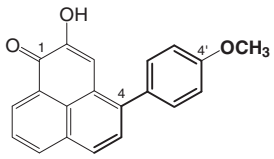
Table 1
Examples of phenylpropanoids and phenylphenalenones sharing the same substitution pattern of the phenyl ring. Part A: Moieties shown in bold are linked by confirmed biosynthetic precursor–product relationships (Schmitt et al., 2000). Part B: The precursor of 4′-*O*-methylirenolone in *M. acuminata* is uncertain. For details see text and Otálvaro et al. (2010).

Phenylpropanoic acids				Phenylphenalenones	
Part A					
R_1	R_2				
H	H	Cinnamic acid		Anigorufone	
OH	H	4-Coumaric acid		Hydroxyanigorufone	
OH	OH	Caffeic acid		Dihydroxyanigorufone	
OH	OCH_3	Ferulic acid (**1**)		Musanolone F (**3**)	
Part B					
R					
H	4-Coumaric acid (+SAM)			4′-*O*-Methylirenolone	
CH_3	4′-Methoxy-cinnamic acid (**2**)				

SAM = *S*-adenosyl-L-methionine.

and phenylphenalenones with corresponding methoxy-substituted phenyl ring substitutions. Here we report the ^{1}H NMR and HPLC-guided identification of phenylpropanoic acids using an isotope dilution approach. In the second part of this work, we offer evidence for a precursor–product relationship by administering the ^{13}C-labeled phenylpropanoids then isolating the resulting phenylphenalenones and analyzing its ^{13}C enrichment by ^{13}C NMR and HRMS.

2. Results and discussion

2.1. Detection of phenylpropanoic acids in plant root cultures

In vitro root cultures of *A. preissii* and *W. thyrsiflora* are rich sources of phenylphenalenones (Hölscher and Schneider, 1997; Opitz et al., 2002; Opitz and Schneider, 2003; Fang et al., 2011) and therefore were selected for the present study. However, only one compound of that type, 2-hydroxy-9-(4′-hydroxy-3′-methoxyphenyl)-1*H*-phenalen-1-one (musanolone F (3)), which has a methoxylated lateral phenyl ring D, was reported from *A. preissii* (Schmitt et al., 2000). Musanolone F (**3**) is biosynthetically derived from ferulic acid (**1**), which is an abundant phenylpropanoic acid in *A. preissii* (Schmitt and Schneider, 2001). Most of the phenylphenalenones occurring in *W. thyrsiflora* have an unsubstituted ring D (Fang et al., 2011, 2012), and no compound with a methoxy substituent in ring D has yet been reported from this plant. Also no data about the occurrence of phenylpropanoic acids in *W. thyrsiflora* are available.

We synthesized [2-^{13}C]ferulic acid ([2-^{13}C]-**1**), [2-^{13}C]isoferulic acid, [2-^{13}C]4′-methoxycinnamic acid ([2-^{13}C]-**2**), and [2-^{13}C]3′,4′-methylenedioxycinnamic acid and used an isotope dilution approach to analyze the two plant systems for the occurrence of these phenylpropanoic acids. Although compound **1** is widely distributed in plants (Kroon and Williamson, 1999) including *A. preissii* (Schmitt and Schneider, 2001), isoferulic acid and 4′-methoxycinnamic acid (**2**) are rarely found in plants (Liu et al., 1999; Sobolev et al., 2006; Kuddus et al., 2010). Free 3′,4′-methylenedioxycinnamic acid has to our knowledge been identified only in *Piper philippinum* (Chen et al., 2007) and *M. acuminata* (Otálvaro et al., 2010). The four [2-^{13}C]-labeled phenylpropanoids were separately administered to the two *in vitro*-root cultures. ^{1}H NMR and HPLC-guided isolation was used to identify ^{13}C-labeled phenylpropanoic acids and their unlabeled native counterparts in the crude root extracts of both cultures.

The fingerprint signals of H-2 of the *E*-configured double bonds ($J_{H2\text{-}H3}$ = 16 Hz) and their ^{13}C satellite signals ($J_{C2\text{-}H2}$ = 161 Hz) were used to detect isotopologue mixtures of non-metabolized [2-^{13}C]-phenylpropanoids together with the unlabeled counterparts in the plant extracts. Using this approach, the natural occurrence of free ferulic acid (**1**) was confirmed in root cultures of *A. preissii* and detected for the first time in root cultures of *W. thyrsiflora* (Fig. 1A, B). 4′-Methoxycinnamic acid (**2**) was found in *A. preissii* but not in *W. thyrsiflora* (data not shown). Isoferulic acid and 3′,4′-methylenedioxy phenylpropanoic acid were not detectable in either of the two root cultures.

Moreover, the proportion of [2-^{13}C]-labeled and unlabeled phenylpropanoids was determined from the integral ratio of the doublets of H-2 and the ^{13}C satellite signals in the ^{1}H NMR spectra (Schneider et al., 2003). Since the feeding experiments with *A. preissii* and *W. thyrsiflora* were performed under identical conditions, the integral ratios suggested a lower endogenous level of free ferulic acid (**1**) in *W. thyrsiflora* (ratio ^{13}C-labeled: unlabeled 8.0: 1) compared to *A. preissii* (2.7: 1). The isotopologue ratio of [2-^{13}C]-**2** to unlabeled **2** (natural abundance ^{13}C contents) in *A. preissii* was found to be 8.1: 1 under the experimental conditions.

As ferulic acid (**1**) is a common cell wall lignin component (Buanafina, 2009), the proportion of this phenylpropanoic acid potentially available for the biosynthesis of phenylphenalenones is difficult to estimate. Unlike ferulic acid (**1**), 4′-methoxycinnamic acid (**2**) seems not to be used as a precursor in major plant metabolic pathways such as lignin formation. Therefore, all of the endogenous pool found in *A. preissii* should be available for the biosynthesis of phenylphenalenones. Hence, our search for methoxyphenylphenalenones and their biosynthesis was focused on *A. preissii*.

2.2. Detection and biosynthesis of methoxyphenylphenalenones in plant root cultures

The administration of [2-^{13}C]ferulic acid (**1**) and [2-^{13}C]4′-methoxycinnamic acid (**2**) resulted in their successful incorporation into the corresponding phenylphenalenones from *A. preissii*,

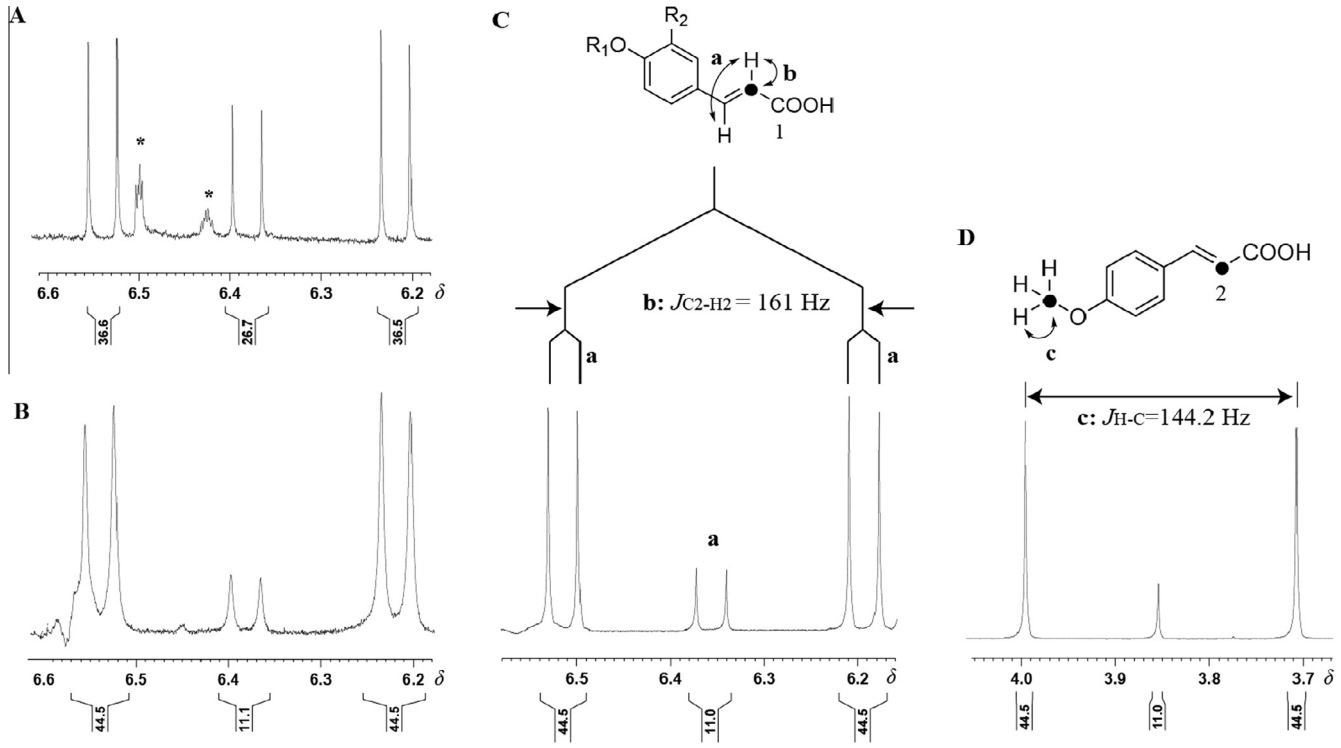


Fig. 1. Partial ^{1}H NMR spectra (500 MHz, MeOH-d_4) displaying signals of isotopologue mixtures of unlabeled (natural abundance ^{13}C) and ^{13}C-labeled phenylpropanoic acids: Panels (A) and (B): H-2-signals of ferulic acid (**1**) (R_1 = H, R_2 = OCH_3) from *in vitro* root cultures of *A. preissii* and *W. thyrsiflora*, respectively, incubated with [2-^{13}C]-**1**. Panel (C): H-2-signal 4′-methoxycinnamic acid (**2**) (R_1 = CH_3, R_2 = H) from root cultures of *A. preissii* incubated with [2-^{13}C,4′-O$^{13}CH_3$]-**2**). Panel (D): *O*-Methyl signal of **2** (R_1 = CH_3, R_2 = H) from root cultures of *A. preissii* incubated with [2-^{13}C,4′-O$^{13}CH_3$]-**2**). Integral values are given at the bottom of each spectrum. The structure drawing and splitting lines in panels (C) and (D) explain the spin–spin coupling pattern of the signals of ^{13}C-labeled and unlabeled isotopologues. For details, see text. ● = ^{13}C. * = signals of contaminations.

whereas no incorporation of **1** into any phenylphenalenone was detected in the case of *W. thyrsiflora*. Musanolone F (**3**) was the only phenylphenalenone isolated after *A. preissii* was incubated with [2-^{13}C]-**1**, and its ^{13}C NMR spectra as well as the isotopologue analysis by HRESIMS (data not shown) are in accordance with the data previously reported (Schmitt et al., 2000). This leads us to conclude that the characteristic 3′-OMe,4′-OH substitution pattern found in ring D of **3** is derived from **1**.

The incubation of *A. preissii* root cultures with [2-^{13}C]4′-methoxycinnamic acid (**2**) and ^{13}C NMR-guided fractionation indicated that an unknown metabolite had been enriched with ^{13}C (Fig. S1). This metabolite, which was subjected to HPLC-guided isolation, was finally identified by 1D and 2D NMR and mass spectrometry as 2-hydroxy-9-(4′-methoxyphenyl)-1*H*-phenale-1-one (**4**). This compound, for which we propose the trivial name 4′-methoxyanigorufone, was previously synthesized and characterized by spectroscopic methods (Cooke and Dagley, 1978) and also found in *Musella lasiocarpa* (D. Hölscher, personal communication). Here **4** is reported for the first time as a naturally occurring phenylphenalenone. The ^{13}C NMR spectrum of **4** showed an enhanced signal at δ 132.6 of C-8 in the phenylphenalenone skeleton (Fig. 2, panel b), and the isotopologue analysis carried out by HRESIMS indicated the enrichment of 53.7% ^{13}C singly labeled molecule of [8-^{13}C]2-hydroxy-9-(4′-methoxyphenyl)-1*H*-phenale-1-one (**4**) biosynthesized from the administered [2-^{13}C]-labeled precursor **2** (Table 2).

This result clearly indicates a precursor–product relationship between 4′-methoxycinnamic acid (**2**) and the new phenylphenalenone (**4**). However, it opens the question whether **2** was incorporated (after being activated to the SCoA ester) as an intact unit into 4′-methoxydiarylheptanoid and, further, whether **2** was incorporated into the phenylphenalenone **4** (early *O*-methylation; route *i* in Fig. 3). Alternatively an *O*-demethylation of 4′-methoxycinnamic acid (**2**) could have been involved (route *ii* in Fig. 3); such an *O*-demethylation could have led to 4-coumaric acid which has been reported for being a precursor of the lateral phenyl ring in the phenylphenalenone skeleton (Funk and Brodelius, 1990; Hölscher and Schneider, 1995a). Route *ii* would require re-methylation of the 4′-OH group, probably at the stage of hydroxyanigorufone (late *O*-methylation) or the 4′-hydroxydiarylheptanoid (Fig. 3). This option was considered because in *M. acuminata*, the *O*-demethylation of 4′-methoxycinnamic acid (**2**) was reported; however, no conclusive evidence was provided about whether the 4′-*O*-methyl group of 4′-*O*-methylirenolone, an isomer of **4**, is the result of an early or a late *O*-methylation (Otálvaro et al., 2010).

In order to investigate the demethylation of 4′-methoxycinnamic acid (**2**) and to check which of the two routes, *i* or *ii*, is operating in *A. preissii*, [2-^{13}C,4′-O$^{13}CH_3$]-**2** was synthesized; this compound contains 99% ^{13}C enrichment in both positions, at C-2 of the carbon skeleton and in the 4′-*O*-methyl group. The specific ^{13}C enrichment at C-2 was employed to verify that the C-9 phenylpropanoid skeleton had been incorporated through the established general biosynthetic pathway; the label of the 4′-*O*-methyl group was used to check for a possible 4′-*O*-demethylation step. Thus, after the incubation of *A. preissii* root cultures with [2-^{13}C,4′-O$^{13}CH_3$]-**2**, a part of the substrate was isolated from this feeding experiment again and analyzed by ^{1}H NMR and HRESIMS to see what part of it was labeled. The integrals of the central and ^{13}C satellite signals of H-2 (δ 6.36) and the OCH_3 signal (δ 3.85) in the ^{1}H NMR spectrum (Fig. 1C, D) showed 89% ^{13}C in

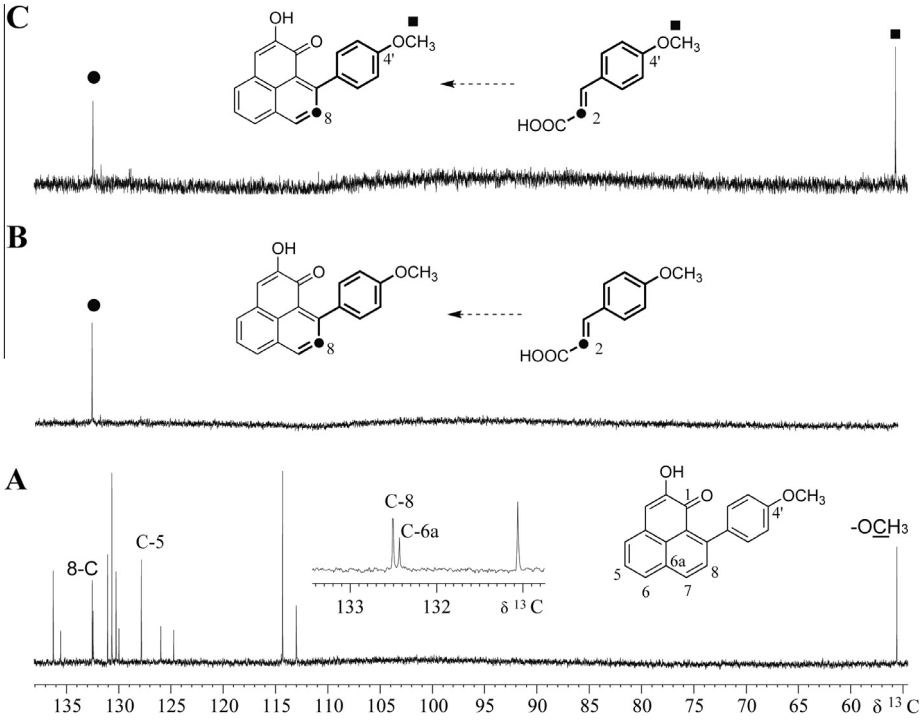


Fig. 2. Partial ^{13}C NMR spectra (125 MHz, acetone-d_6) of 2-hydroxy-9-(4′-methoxyphenyl)-1*H*-phenalen-1-one (**4**) from root cultures of *A. preissii*. (a) Spectrum of unlabeled reference. (b) Isotope enrichment of **4** in position C-8 upon the incorporation of [2-^{13}C]4′-methoxycinnamic acid ([2-^{13}C]-**2**). (c) Isotope enrichment of **4** in position C-8 and in the *O*-methyl carbon atom upon the incorporation of [2-^{13}C,O$^{13}CH_3$]4′-methoxycinnamic acid ([2-^{13}C,O$^{13}CH_3$]-**2**). ●, ■ = Signal of the ^{13}C-enriched C-8 and the 4′-*O*-methyl group, respectively.

Table 2
Isotopologue composition of 2-hydroxy-9-(4′-methoxyphenyl)-1*H*-phenalen-1-one (**4**) in root cultures of *A. preissii*, determined by HRESIMS after incubation with [2-^{13}C]-**2** and [2-^{13}C,O$^{13}CH_3$]-**2**, respectively.

Formula	*m/z* [M+H]$^+$ (calcd.)	Isotopologue analysis of compound **4** after incorporation of					
		Unlabeled **2** (reference)		[2-^{13}C]-**2**		[2-^{13}C,O$^{13}CH_3$]-**2**	
		m/z [M+H]$^+$ (found)	Relative abundance (%)	*m/z* [M+H]$^+$ (found)	Relative abundance (%)	*m/z* [M+H]$^+$ (found)	Relative abundance (%)
$C_{20}H_{15}O_3^+$	303.1021	303.1016	**80.3**	303.1018	33.6	303.1019	35.9
C_{19}**^{13}C**$H_{15}O_3^+$	304.1055	304.1051	17.6	304.1053	**53.7**	304.1051	7.5
C_{18}**$^{13}C_2$**$H_{15}O_3^+$	305.1088	305.1084	1.9	305.1085	12.3	305.1086	**45.9**
$C_{17}{}^{13}C_3H_{15}O_3^+$	306.1122	306.1118	0.2	306.1120	0.4	306.1118	10.7

The bold numbers and symbols highlight the major isotopologues detected after incubation with unlabeled, singly, and doubly labeled precursor **2** and confirm incorporation into **4**.

each of the two labeled positions, thus ruling out the possibility that the label in the OCH_3 group was lost through a hypothetical demethylation/remethylation mechanism (Fig. 3). The isotope enrichment determined by ^1H NMR was in good agreement with that found for the abundance of the double-labeled isotopologue [2-^{13}C,4′-O$^{13}CH_3$]-**2** of 84.4% found by HRESIMS (Table 3), although no single labeled isotopologues [2-^{13}C]-**2** and [4′-O$^{13}CH_3$]-**2** were detected.

As expected, the administration of [2-^{13}C,4′-O$^{13}CH_3$]-**2** to the root cultures of *A. preissii* also resulted in the formation of metabolite **4**, which was isolated and analyzed by NMR and HRESIMS. The ^{13}C NMR spectrum of [2-^{13}C,4′-O$^{13}CH_3$]-**4** obtained from this experiment displayed two equally enhanced signals at δ 132.6 and δ 55.6, corresponding to an identical degree of ^{13}C enrichment at C-8 and 4′-O<u>C</u>H$_3$ (Fig. 2, panel c); this enrichment proved that the intact 4′-methoxycinnamic acid was incorporated as a unit into phenylphenalenone **4**. Additionally, the isotopologue analysis by HRESIMS showed 45.9% relative abundance of the doubly ^{13}C-labeled isotopologue (Table 2). Hence, route *i* in Fig. 3 represents the actual biosynthetic pathway of this metabolite.

3. Conclusions

Biosynthetic precursor–product relationships have been demonstrated in *A. preissii* between ferulic acid (**1**) and musanolone F (**3**) and between 4′-methoxycinnamic acid (**2**), which is a rare phenylpropanoid, and 4′-methoxyanigorufone (**4**). This finding clearly shows that in root cultures of this plant the substitution pattern at the lateral phenyl ring (ring D) of phenylphenalenones reflects the existence of the corresponding phenylpropanoids in the plant tissue. 4′-Methoxyanigorufone (**4**) is reported here for the first time as a natural product. The involvement of 4′-methoxycinnamic acid (**2**) in the phenylphenalenone biosynthesis represents an extension of the general phenylpropanoid pathway (Noel et al., 2005; Vogt, 2010). Thus, route *i* (Fig. 3) requires a new CoA ligase or an additional function for a promiscuous

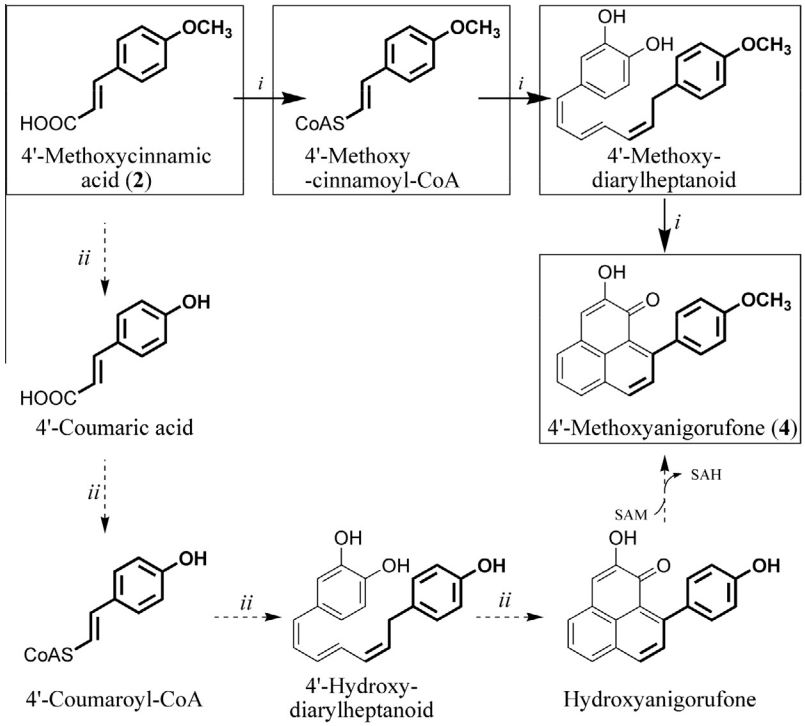


Fig. 3. Hypothetical biosynthetic pathways of 2-hydroxy-9-(4′-methoxyphenyl)-1*H*-phenalen-1-one (**4**). *i*: early 4′-*O*-methylation, *ii*: late 4′-*O*-methylation. The phenylpropanoid unit is highlighted in bold. SAM: *S*-adenosyl-L-methionine; SAH: *S*-adenosylhomocysteine.

Table 3
Isotopologue composition of 4′-methoxycinnamic acid (**2**) determined by HRESIMS. The isotopologue mixture of **2** was isolated after feeding experiments to *in vitro* root cultures of *A. preissii* and compared with the data from untreated root cultures of the same plant (control).

Formula	*m/z* $[M+H]^+$ (calcd.)	Isotopologue analysis of compound **2** isolated from			
		Control plant		After incubation with [2-^{13}C,O^{13}CH$_3$]-**2**	
		m/z $[M+H]^+$ (found)	Relative abundance (%)	*m/z* $[M+H]^+$ (found)	Relative abundance (%)
$C_{10}H_9O_3^-$	177.0557	177.0551	96.3	177.0553	14.2
$C_9{}^{13}CH_9O_3^-$	178.0591	178.0587	3.6	178.0585	0.1
$C_8{}^{13}\mathbf{C_2}H_9O_3^-$	179.0624	179.0622	0.1	179.0619	84.4
$C_7{}^{13}C_3H_9O_3^+$	180.0658	n.d.	n.d.	180.0655	1.3

n.d.: Not detected.

4-coumarate CoA-ligase. This work also demonstrates the potential power of isotopically labeled compounds as a tool, not only in the elucidation of biosynthetic pathways but also in the detection and quantification of naturally occurring minor metabolites.

4. Experimental

4.1. General experimental procedures

^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, HMBC, and HSQC spectra were measured on a Bruker Avance 500 NMR spectrometer (Bruker Biospin, Karlsruhe, Germany), operating at 500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C. A TCI cryoprobe (5 mm) was used to measure spectra at 300 K. Tetramethyl silane was used as an internal standard for referencing ^{1}H and ^{13}C NMR spectra. Electrospray ionization mass spectra (ESIMS) and LC-ESIMS were recorded on a Bruker Esquire 3000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). HRESIMS was recorded on a UPLC–MS/MS system consisting of an Ultimate 3000 series RSLC (Dionex, Idstein, Germany) system, and an Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Analytical and semipreparative HPLC was performed on an Agilent series HP1100 (binary pump G1312A; auto sampler G1367A; diode array detector G1315A; connected to fraction collector Advantec CHF122SB) using a LiChrospher RP18e column (5 µm, 250 × 10 mm) and a linear gradient MeOH-0.1% aq. trifluoroacetic acid from 35% to 100% in 25 min (flow rate 0.8 mL/min; monitoring wavelength 254 nm). The UV spectra were recorded by the DAD during analytical HPLC.

4.2. Plant material

Sterile root cultures of *A. preissii* L. and *W. thyrsiflora* Burm. were established as previously described (Hölscher and Schneider, 1997) and maintained in liquid MS medium (100 mL) in conical flasks (300 mL) on a gyratory shaker (85 rpm) at 23 °C under permanent light conditions (4.4 µmol m^{-2} s^{-1}). Three days before the precursor was administered, the cultured roots were transferred to fresh medium.

4.3. Reagents and the chemical synthesis of the ^{13}C-labeled precursors

The [2-^{13}C]hydroxycinnamic acids ([2-^{13}C]ferulic acid ([2-^{13}C]-**1**), [2-^{13}C]isoferulic acid, [2-^{13}C]4-methoxycinnamic acid ([2-^{13}C]-**2**), [2-^{13}C]4-coumaric acid and [2-^{13}C]3,4-methylenedioxy cinnamic acid) were prepared from the corresponding substituted benzaldehyde (0.33 mmol; Sigma–Aldrich, ⩾95.0%) (vanillin, isovanillin, anisaldehyde, 4-hydroxybenzaldehyde, piperonal) with [2-^{13}C]malonic acid (99% ^{13}C; 0.72 mmol; Deutero GmbH, Kastellaun, Germany) in a Knoevenagel reaction. The ^{13}C-methylated [2-^{13}C,4′-O^{13}CH$_3$]4-methoxycinnamic acid ([2-^{13}C,4′-

$O^{13}CH_3$]-**2**) was synthesized by refluxing [2-^{13}C]4-coumaric acid (10 mg, 0.061 mmol), K_2CO_3 (10 mg), and $^{13}CH_3I$ (20 μL, 0.32 mmol, 99 atom%^{13}C Sigma–Aldrich) in acetone (5 mL) for 4 h in order to yield 8.7 mg (79%) of the desired compound.

4.4. Feeding experiments, extraction and isolation of phenylphenalenones

Each ^{13}C-labeled compound (10 μmol) was dissolved in an EtOH:H_2O 4:1 solution (1 mL) and passed through a membrane filter (0.1 μm diameter) before being administered to the root culture. The incubation time was 4 d; afterwards, the roots (ca. 20 g fresh weigh) were frozen in liquid N_2, ground and extracted with methanol at room temperature for 1 h (3 × 100 mL). The solvent was removed by evaporation under vacuum pressure (<40 °C), and the crude extract was fractionated by partitioning between *n*-hexane–H_2O, CH_2Cl_2–H_2O and EtOAc–H_2O. The ^{13}C NMR labeling experiment was used to identify metabolites specifically enriched by the incorporation of the labeled precursor. Musanolone F (**3**, R_t 20.9 min) and 2-hydroxy-9-(4′-methoxyphenyl)-1*H*-phenalen-1-one (**4**, R_t 23.9 min), the products derived from successful incorporation, were isolated by semipreparative HPLC as described above. The analytical data of compound **3** exactly matched those of musanolone F available in our in-house database.

4.5. 2-Hydroxy-9-(4′-methoxyphenyl)-1H-phenalen-1-one (4)

The ^{1}H- and ^{13}C NMR spectra of compound **4** resembled that of hydroxyanigorufone, which was previously reported from *A. preissii* root cultures (Hölscher and Schneider, 1997). The only exception was an additional singlet in the ^{1}H NMR spectrum (δ 3.89) and a corresponding signal in the ^{13}C NMR spectrum (δ 55.6), suggesting an *O*-methyl group was attached to one of the hydroxyl groups at 2-OH or 4′-OH. An HMBC cross-peak between the ^{1}H NMR signal of the *O*-methyl group and C-4′ (δ 160.3) of the lateral phenyl ring clearly indicated the methoxy group was attached to the *p*-position of ring D. ^{1}H,^{1}H-COSY and HSQC spectra, in addition to HMBC data, allowed all ^{1}H and ^{13}C chemical shifts to be assigned. Based on the above NMR data and combined with HRESIMS (Table 2), the structure of compound **4** was elucidated as 2-hydroxy-9-(4′-methoxyphenyl)-1*H*-phenalen-1-one. ^{1}H NMR (500 MHz, acetone-d_6): δ 8.39 (1H, d, *J* = 8.3 Hz, H-7), 8.08 (1H, d, *J* = 8.2 Hz, H-4), 7.87 (1H, d, *J* = 7.2 Hz, H-6), 7.68 (1H, dd, *J* = 8.2, 7.2 Hz, H-5), 7.64 (1H, d, *J* = 8.3 Hz, H-8), 7.34 (2H, d-like, *J* = 8.8 Hz, H-2′/6′), 7.18 (1H, s, H-3), 7.02 (2H, d-like, *J* = 8.8 Hz, H-3′/5′), 3.89 (3H, s, 4′-OCH_3); ^{13}C NMR (125 MHz, acetone-d_6): δ 180.9 (C-1), 160.3 (C-4′), 151.5 (C-2), 149.3 (C-9), 136.3 (C-7), 135.6 (C-1′), 132.6 (C-8), 132.5 (C-6a), 131.1 (C-6), 130.7 (C-2′/6′), 130.3 (C-4), 130.0 (C-3a), 127.9 (C-5), 126.0 (C-9b), 124.8 (C-9a), 114.4 (C-3′/5′), 113.1 (C-3), 55.6 (4′-OCH_3). UV (MeOH–H_2O): λ_{max} 265, 315, 371, 420 nm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.07.017.

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