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O-Methylation of phenylphenalenone phytoalexins in *Musa acuminata* and *Wachendorfia thyrsiflora*

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ABSTRACT

Biosynthetic *O*-methylation at various sites along the backbone of inducible phenylphenalenones in *Musa acuminata* var. "Williams" (Musaceae) and *Wachendorfia thyrsiflora* (Haemodoraceae) was investigated using ^{13}C-labelled precursors. The inducibility of *O*-methylated metabolites was demonstrated in both species and the origin of methoxyl group from [*methyl*-^{13}C]L-methionine was confirmed. In addition to known phenylphenalenones, a methoxylated metabolite, 4-(4-hydroxy-3-methoxy-phenyl)-benzo[*de*]isochromene-1,3-dione, was detected and its structure elucidated mainly by NMR spectroscopic techniques. The experiments were used to discriminate methionine-derived and artificial methoxy groups formed during methanolic extraction. Finally, demethylation of 4′-methoxycinnamic acid and subsequent conversion to 3′,4′-methylenedioxycinnamic acid was demonstrated in *M. acuminata*.

1. Introduction

Methyl transfer reactions play a crucial role in plant secondary metabolism (Roje, 2006). *O*-Methylation occurs in early as well as in downstream steps of many biosynthetic pathways, thereby contributing to diversification of secondary metabolites and modulating their physico-chemical properties (Ferrer et al., 2008). As a consequence, in comparison to the activities of non-methylated analogues, the biological activities of *O*-methylated natural products may be altered. In the case of flavonoid and isoflavonoid derivatives, for example, regio-selective methylation modulates the *in vivo* activity by limiting the number of reactive hydroxyl groups, altering the solubility properties, and ultimately determining the extent of interaction between compounds and cell receptors (Zubieta et al., 2001). Similar methylation phenomena have been observed in cotton; there, methylated terpenoid phytoalexins are synthesized by the plant in response to invasion by pathogenic fungi (Liu et al., 1999). The process of *O*-methylation in general is catalyzed by *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferases, which have been classified according to their substrate preference and molecular architecture (Ibrahim et al., 1998; Noel et al., 2003).

Phenylphenalenones, polycyclic natural products of some monocotyledonous plant families, occur both in (poly)phenolic and partially or completely *O*-methylated form. In the Haemodoraceae, phenylphenalenone-type compounds mainly accumulate in the roots but were also detected in above-ground tissue (Opitz and Schneider, 2002). A similar distribution seems to occur in the Musaceae (Luis et al., 1996; Kamo et al., 1998). In *Musa*, phenylphenalenones have been described as phytoalexins (Luis et al., 1994; Binks et al., 1997; Kamo et al., 1998, 2000) while they seem to be constitutive plant defense compounds in the Haemodoraceae or, as preliminary reported for *Wachendorfia thyrsiflora* (Opitz, 2002; Schüler et al., 2004), inducible and constitutive phenylphenalenones co-exist in one species. Although the ecological role of non-methylated and *O*-methylated phenylphenalenones remains to be studied in detail, the two classes of compounds seem to have different domains of activities: The accumulation in secretory

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cavities (Hölscher and Schneider, 2007), which are supposed to function as reservoirs for natural products that defend the plant against chewing insects, support the assumption that *O*-permethylated phenylphenalenones are involved in herbivore deterrence rather than protection against fungal infection. In contrast, some non-methylated phenylphenalenones are more potent antifungal natural products than their methylated analogues (Otálvaro et al., 2007).

Musa acuminata and *W. thyrsiflora* were selected for this study because they belong to phytochemically related phenylphenalenone-producing plant families, the Musaceae (banana family) and Haemodoraceae (bloodroot family), which in part share identical phenylphenalenone structures. Another reason for using the two species is the availability of *in vitro*-cultures, which are suitable systems for biosynthetic precursor feeding experiments. While banana plants are economically important crop plants, some members of the bloodroot family, especially *Anigozanthos* hybrids ("kangaroo paw") are used as ornamental plants. The genus *Wachendorfia* is endemic to the cape region of South Africa. *W. thyrsiflora* is a rich source of phenylphenalenone-type compounds (Brand, 2005) and has been used to study biosynthetic enzymes of these polycyclic natural products (Brand et al., 2006).

The biosynthetic origin of the carbon skeleton of phenylphenalenones from two phenylpropane units and C-2 of acetate or malonate has been demonstrated to be basically the same in Haemodoraceae (Thomas, 1973; Hölscher and Schneider, 1995a,b) and Musaceae (Kamo et al., 2000). However, many details of the biosynthetic pathways such as the cyclization mechanism, the origin and sequence of the oxygenation and *O*-methylation still have to be established. Two different possibilities for the formation of *O*-methylphenylphenalenone derivatives are illustrated in Fig. 1. The versions indicated by the single arrows (path *i*) directly methylate a phenolic hydroxyl group of a pre-formed phenylphenalenone; this process is thought to be the hypothetical final biosynthetic step in the pathway to *O*-methylated phenylphenalenones. Alternatively, intact incorporation, as indicated by reversed arrows (path *ii*), of methoxyphenylpropanoids would result in the same methoxyphenylphenalenones to which path *i* leads.

Biosynthetic incorporation of [2-^{13}C]ferulic acid into rings C and D of musanolone F (Fig. 1, panel b, path *ii*) but not into rings A and B of methoxyanigorufone (panel a, path *ii*) was reported in *Anigozanthos preissii* (Schmitt et al., 2000). Therefore, it seems reasonable that methoxyanigorufone is formed from anigorufone by *O*-methylation of the hydroxyl group at C-2 (Fig. 1, panel a, path *i*), and musanolone F via incorporation of ferulic acid-CoA ester during condensation (Fig. 1, panel b, path *ii*).

The last reaction was surprising because condensation with the diketide to form a linear diarylheptanoid requires an activated intermediate, which is thought to be the phenylpropanoyl-CoA ester. However, a specific ferulate-CoA ligase has not yet been reported from plants and the feruloyl-CoA ester is considered to be formed by caffeoyl-CoA-*O*-methyltransferase-catalyzed *O*-methylation of caffeoyl-CoA. Ferulate-CoA ligase activity would explain the observed incorporation of ferulic acid in *Anigozanthos*. However, the alternative formation of musanolone F from dihydroxyanigorufone cannot be ruled out because caffeic acid-*O*-methyltransferases have been shown to methylate the 3-position of catechol moieties of various phenylpropanoids (Parvathi et al., 2001; Do et al., 2007; Lin et al., 2006).

No information is available about the origin of the methoxy group of 4′-*O*-methylirenolone (Fig. 1, panel c). It is unclear whether this compound is formed by direct *O*-methylation of irenolone via path *i* or by incorporation of 4′-methoxycinnamic acid (path *ii*). Taking into account that 4′-methoxycinnamic acid is not a member of the established phenylpropanoid pathway, 4′-*O*-methylirenolone might be formed by 4′-*O*-methylation of irenolone rather than by incorporation of 4′-methoxycinnamic acid as a unit. On the other hand, 4′-methoxycinnamic acid recently was found as a natural product in *Aquilegia vulgaris* (Ranunculaceae) (Bylka, 2004) and may have been overlooked in other plants. If present in *Musa*, its SCoA ester could function as a putative precursor for the biosynthesis of 4′-*O*-methylirenolone. The occurrence of further *O*-methylated phenylphenalenones in various species of the Haemodoraceae and Musaceae also raised the question whether the methoxy groups were introduced before or after the formation of the skeleton and prompted us to study the biosynthesis of such compounds in the two families.

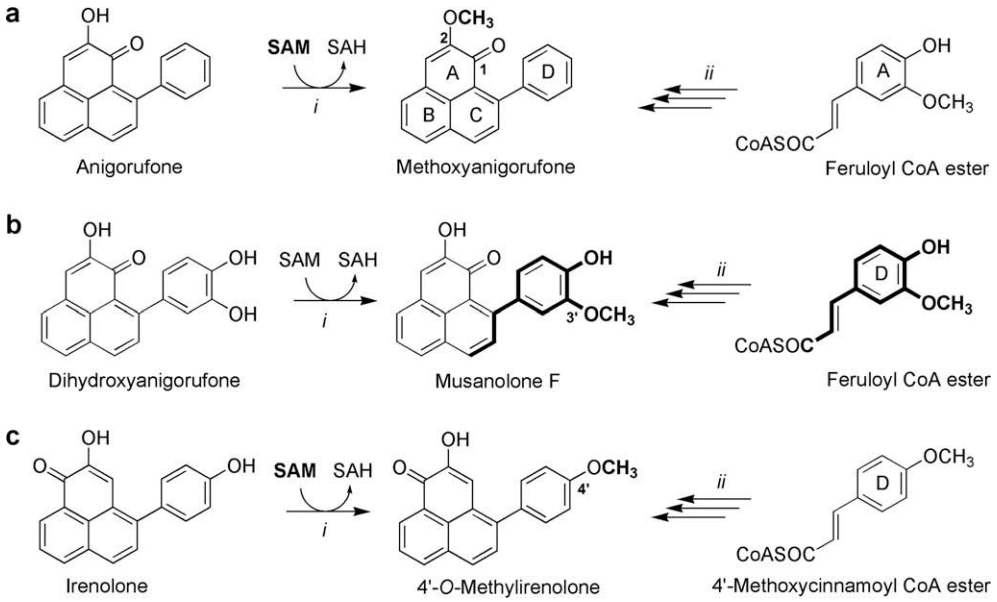

Fig. 1. Hypothetical formation of methoxyphenylphenalenones by *O*-methylation of hydroxyl groups of phenylphenalenones (i) or incorporation of intact methoxyphenylpropanoids (ii) in form of their SCoA esters. More likely conversions are marked in bold (for details see text). SAM: *S*-adenosyl-L-methionine, SAH: *S*-adenosylhomocysteine.

In this paper, we report the existence of regio-specific *O*-methyl-phenylphenalenone-type natural products including a new compound in a member of the Musaceae (*M. acuminata* cv. Williams) and Haemodoraceae (*W. thyrsiflora*), discuss the possibility of *O*-methylation occurring at different steps of the phenylphenalenone biosynthetic pathway, and provide evidence for the inducibility of methoxy-phenylphenalenone biosynthesis in the two species. In addition, *O*-methyl groups formed enzymatically by methyl transfer from SAM and solvent-derived *O*-methyl artifacts are discriminated by isotopic labelling.

2. Results and discussion

2.1. ^{13}C NMR-guided isolation of methoxy-phenylphenalenones in Musa and Wachendorfia

The *O*-methylation patterns of phenylphenalenones in *M. acuminata* cv. "Williams" (Musaceae) and *W. thyrsiflora* (Haemodoraceae) were previously reported to be different. *W. thyrsiflora* produces phenylphenalenones bearing *O*-methyl groups at the tricyclic nucleus but not at the lateral phenyl ring (Opitz et al., 2002). 4′-Methoxyphenylphenalenones such as 4′-*O*-methylirenolone are typical for *Musa* species, which also produce phenylphenalenones showing a 3′-methoxy-4′-hydroxy substitution pattern in the phenyl ring (Fig. 1) (Luis et al., 1996; Kamo et al., 1998).

In order to detect methoxyphenylphenalenones in *Musa* selectively, [*methyl*-^{13}C]L-methionine was administered to *in vitro* plantlets, which simultaneously were treated with $CuCl_2$ for elicitation. After 6 days, the roots of the plants were cut, frozen in liquid nitrogen, macerated and extracted with acetone. Extraction with methanol was omitted in this case in order to avoid non-enzymatic *O*-methylation. The crude acetone extract was submitted to ^{1}H and ^{13}C NMR spectroscopic analysis. Greatly enhanced ^{13}C NMR signals, in comparison to a control without [*methyl*-^{13}C]L-methionine, appeared in the region between 55 and 60 ppm, suggesting *O*-$^{13}CH_3$ groups from the labelled precursor have been incorporated (Fig. S1). Chromatographic separation (semipreparative HPLC method 3) of the extract gave two relevant fractions, which showed a total of four intense ^{13}C NMR peaks around 55 ppm in the region of interest. Further ^{13}C NMR-guided isolation (TLC; HPLC method 1) resulted in methoxy-phenylphenalenones **1**–**4** (Fig. 2). ^{1}H and ^{13}C NMR spectroscopic data of known compounds **1**–**3** matched those of 4′-*O*-methylirenolone (**1**) (Luis et al., 1994), (2*S*,3*S*)-(+)-2,3-dihydro-2,3-dihydroxy-4-(4′-methoxyphenyl)-phenalen-1-one (**2**) (Luis et al., 1994), and musanolone F (**3**) (Schmitt et al., 2000). 2-(4-hydroxy-3-methoxyphenyl)-naphthalene-1,8-dicarboxylic anhydride (**4**) is a new natural product. For details of structure elucidation, Section 4.5. This experiment showed that the *O*-methyl groups of compounds **1**–**4** originated from L-methionine via SAM but did not reveal at which biosynthetic step the *O*-methylation occurred.

Administering [*methyl*-^{13}C]L-methionine to root cultures of *W. thyrsiflora* and ^{13}C NMR-guided fractionation (MeOH extraction; semipreparative HPLC method 4; TLC; final purification by HPLC method 1) resulted in isolation of three methoxyphenylphenalenone derivatives **5**–**7**. ^{1}H and ^{13}C NMR spectroscopic data of compounds **5** and **6** matched those of previously isolated methoxyanigorufone (**5**) (Hölscher and Schneider, 1997), and 5-hydroxy-2-methoxy-6-oxa-benzo[*def*]chrysen-1-one (**6**) (Opitz et al., 2002). No analytical data for compound **7** were available. Enrichment of ^{13}C was inferred from enhanced *O*-methyl signals in the ^{13}C NMR and cross peaks in the HSQC spectra. In addition, ^{13}C labelling was detected by means of the large ^{1}H–^{13}C coupling constant (J_{H-C} = 144.5 Hz) of the O$^{13}CH_3$ resonances in the ^{1}H NMR spectra (Schneider et al., 2003; Schneider, 2007). The ^{1}H NMR signals of the *O*-methyl groups appear as pseudo triplets, which are composed of the central singlet of the [O$^{12}CH_3$]-isotopomer and a doublet indicating the [O$^{13}CH_3$]-isotopomer.

For example, the partial ^{1}H NMR spectrum of compound **3** (Fig. 3) from *in vitro* plants of *M. acuminata* displays the signal of the *O*-methyl group. The integral ratio of the O$^{13}CH_3$ doublet (a + a′) to the O$^{12}CH_3$ singlet (b) were used to determine the proportions of ^{13}C-labelled molecules derived from the ^{13}C-labelled precursor, [*methyl*-^{13}C]L-methionine (99% ^{13}C), and from unlabelled endogenous L-methionine. The incorporation levels for compounds **1**–**7** were determined to be as follows: **1**: 13%, **2**: 50%, **3**: 30%, **4**: 33%, **5**: 12%, **6**: 10%, **7**, 3-OCH$_3$: natural abundance, 6-OCH$_3$: 12%. Isotope dilution from the 99% ^{13}C of the precursor to

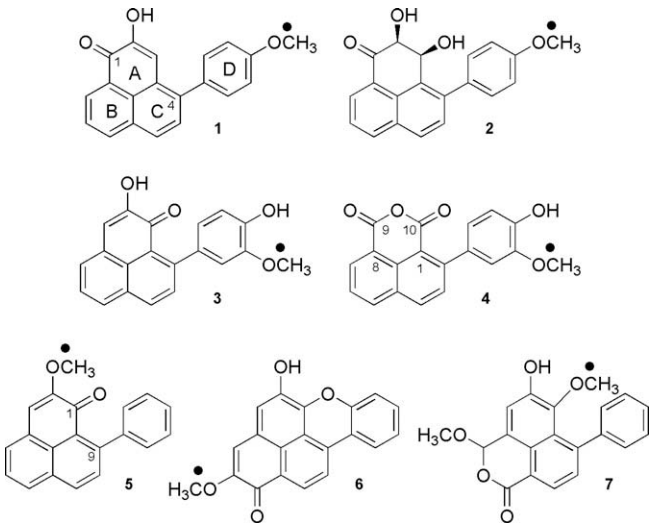

Fig. 2. Structures of methoxy-phenylphenalenones isolated in this study. Compounds **1**–**4** were isolated by ^{13}C NMR-guided fractionation from *in vitro* plants of *M. acuminata* after administration of [*methyl*-^{13}C]L-methionine. Compounds **5**–**7** were obtained from root cultures of *Wachendorfia thysiflora*. •, ^{13}C incorporated from [*methyl*-^{13}C]L-methionine.

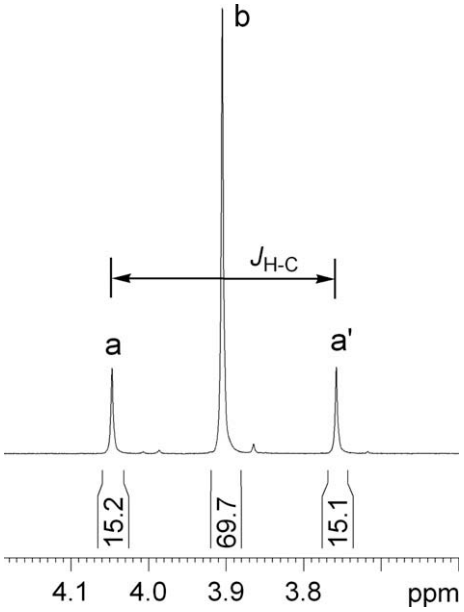

Fig. 3. Partial ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of musanolone F (**3**) from *M. acuminata* displaying the 3′-*O*-methyl signal. The integral ratio of the O$^{13}CH_3$ doublet (a + a′; J_{C-H} = 144.5 Hz) to the O$^{12}CH_3$ singlet (b) were used to determine the proportions of isotopomers derived from the ^{13}C-labelled precursor, [*methyl*-^{13}C]L-methionine (99% ^{13}C), and from unlabelled endogenous L-methionine.

the specified percentages demonstrated that compounds **1**–**6** are natural products of *M. acuminata* and *W. thyrsiflora*, respectively. Interestingly, although compound **7** possesses two *O*-methyl groups, 3-OCH_3 and 6-OCH_3, only the last one was enriched with ^{13}C (Fig. 4). Obviously, the *O*-methyl group in position 3 has not been provided by [*methyl*-^{13}C]L-methionine via SAM but must be of artificial origin, presumably due to the extraction with MeOH.

This result paralleled data previously reported by Opitz et al. (2002), who found that extraction of *Xiphidium caeruleum* with MeOH resulted, among other phenylphenalenone-type compounds, in 3-methoxy-5,6-dihydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one. This compound was not detectable after isolation using acetone as a solvent. It was concluded, therefore, that *O*-methyl groups in position 3 of 7-phenyl-benzoisochromenones seem to be artifacts of the extraction procedure.

2.2. Inducibility of methoxy-phenylphenalenone biosynthesis

The biosynthesis of phenylphenalenones in *Musa* was shown to be stimulated by biotic (Luis et al., 1994; Binks et al., 1997; Kamo et al., 1998) and chemical elicitors (Luis et al., 1996). This has been confirmed by the present experiments with *M. acuminata* var. Williams, which produced only minute amounts of phenylphenalenones when grown *in vitro* under sterile conditions (Fig. 5, panel B). Treatment of *in vitro* plants with $CuCl_2$ (Fig. 5, panel A) dramatically increased phenylphenalenone production. Highest increase was observed for the peak of anigorufone, a non-*O*-methylated phenylphenalenone (AR, R_t 40.1 min). Additional unidentified compounds were detected between 43 and 53 min. Their ^1H NMR spectra did not display any signal from phenolic OCH_3 groups between 3.6 and 4.0 ppm. 4′-*O*-Methylirenolone (**1**, R_t 42.0 min) was also identified but it was not the major metabolite in these experiments. Hence, although there was a clear $CuCl_2$-dependent stimulation of phenylphenalenone biosynthesis, specific elicitation of *O*-methylated phenylphenalenones was not observed in these experiments. Treating *in vitro* plants of *M. acuminata* with jasmonic acid (JA) as an elicitor instead of $CuCl_2$ also enhanced levels of phenylphenalenones, including 4′-*O*-methylirenolone. However, results largely diverged even in parallel experiments and were not always reproducible.

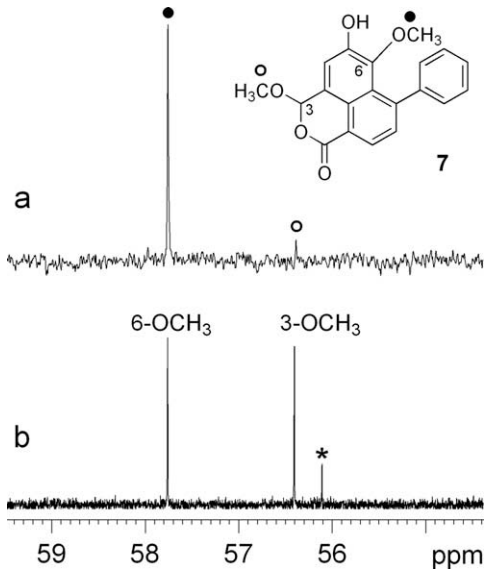

Fig. 4. Partial ^{13}C NMR spectra of compound **7** isolated from *W. thyrsiflora*. (a) after incorporation of [*methyl*-^{13}C]L-methionine, •, signal of ^{13}C-enriched methyl group; ○, naturally abundant ^{13}C signal; (b) unlabelled reference. The asterisk indicates an impurity signal.

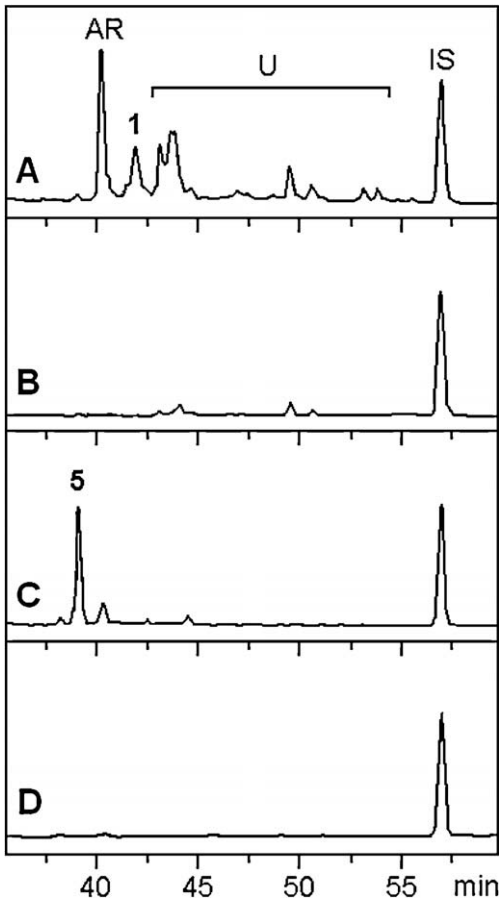

Fig. 5. Partial HPLC traces (HPLC method 2, UV 254 nm) of extracts from *Musa acuminata* (roots of *in vitro* plants; A and B) and *Wachendorfia thyrsiflora* (cultured roots; C and D). (A) *M. acuminata* treated with $CuCl_2$; (C) *Wachendorfia thyrsiflora* treated with jasmonic acid (JA); (B and D) Untreated controls. **1**, 4′-*O*-methylirenolone (R_t 42.0 min); **5**, methoxyanigorufone (R_t 38.9 min); AR, anigorufone (R_t 40.1 min); U, unidentified compounds showing no *O*-methyl signals in the ^1H NMR spectrum; IS, internal standard (0.5 μg pyrene per injection; R_t 57.0 min). For details of HPLC methods and sample preparation, see Section 4.

Anigorufone, a 1,2-oxygenated phenylphenalenone, which is a major secondary compound in most Haemodoraceae, was detected in *W. thyrsiflora* only in traces (Opitz, 2002; Brand, 2005). However, preliminary experiments indicated an eliciting effect of JA (Opitz, 2002) and coronalone (Schüler et al., 2004) on the formation of anigorufone. In addition, methoxyanigorufone (**5**), which was not detectable in extracts of untreated plant roots, was formed in high levels upon elicitation (Fig. 5, panel C, R_t 38.9 min).

In order to evaluate how the availability of L-methionine affects the level of *O*-methylated phenylphenalenones, the media of *in vitro* plants of *M. acuminata* and root cultures of *W. thyrsiflora* were supplemented with [*methyl*-^{13}C]L-methionine alone and with [*methyl*-^{13}C]L-methionine together with elicitors ($CuCl_2$, JA). Although the ^{13}C label was incorporated into methoxyphenylphenalenone methyl groups, there was no significant increase in the level of methoxyphenylphenalenones compared to the untreated controls and the experiments with elicitors, respectively (data not shown).

2.3. Metabolism of [2-^{13}C,4′-$O^{13}CH_3$]4′-methoxycinnamic acid in *M. acuminata*

Labelling experiments are not only useful for NMR-guided isolation of natural products (Section 2.1) but also to establish precursor – product relationships and infer biosynthetic pathways

(Schneider, 2007). In this context, the results presented above (Section 2.1) did not only indicate the occurrence of *O*-^{13}C-methylated natural products in extracts and fractions but, although not surprising, also demonstrated that the *S*-methyl group of L-methionine is the source of the *O*-methyl groups of the methoxyphenylphenalenones **1**–**6** and one of the two *O*-methyl groups of **7**. However, *O*-methyl groups of phenylphenalenones are supposed to become labelled from [*methyl*-^{13}C]L-methionine via *S*-adenosylmethionine regardless of the mode of synthesis, *O*-methylation of phenylphenalenones (Fig. 1, path *i*) or incorporation of intact methoxyphenylpropanoids (Fig. 1, path *ii*). Two biosynthetic origins of methoxyphenylphenalenones bearing the *O*-methyl group at C-4′ of the lateral phenyl ring are possible. One option is the 4′-*O*-methylation of irenolone to **1** (path *i* in Fig. 1). Alternatively, hypothesis *ii*, similar to the formation of musanolone F (**3**) in *A. preissii* (Schmitt et al., 2000), suggested *O*-methylation of *p*-coumaric acid (or the SCoA ester) followed by incorporation of 4′-methoxycinnamoyl-CoA into methoxyphenylphenalenones.

To investigate the latter hypothesis, [2-^{13}C,4′-O^{13}CH$_3$]4′-methoxycinnamic acid (99% ^{13}C) was synthesized and administered to *in vitro* plants of *M. acuminata*. The doubly labelled precursor, which is ^{13}C-enriched in the side chain of the phenylpropanoid skeleton and the *O*-methyl group, was used to distinguish between intact incorporation of the precursor and demethylation before incorporation. The crude root extract obtained after incubation with [2-^{13}C,4′-O^{13}CH$_3$]4′-methoxycinnamic acid did not show signals of the parent compound. Therefore it was subjected to ^{13}C NMR-guided fractionation. However, the ^{13}C NMR spectra of phenylphenalenone-containing *n*-hexane and dichloromethane fractions did not exhibit the enhanced ^{13}C NMR signals of the carbon atoms C-5 and C-8 (128 and 132 ppm) that would be expected from incorporation of two phenylpropanoid units into the aromatic tricycle. Nonetheless, small amounts of two non-methylated compounds, anigorufone and hydroxyanigorufone, were purified from this feeding experiment but no label was detected by ^{13}C NMR in these compounds. In addition, musanolone F (**3**), bearing an *O*-methyl group at C3′, was purified and subjected to NMR and mass spectral analysis. Again, no ^{13}C enrichment in positions **5** and **8** of the phenalenone skeleton and in the 3′-OCH$_3$ group was detected.

A dichloromethane-soluble fraction was obtained in the same experiment, which surprisingly showed a strong ^{13}C NMR signal at δ 116.6. The labelled compound was isolated and identified by NMR and mass spectroscopic analysis as 3′,4′-methylenedioxycinnamic acid. The enhanced ^{13}C signal at δ 116.6 was attributed to C-2 (Fig. 6), indicating formation from the precursor, [2-^{13}C,4′-O^{13}CH$_3$]4′-methoxycinnamic acid. No label was detected in the methylenendioxy group. This observation clearly proved loss of the O^{13}CH$_3$ group of [2-^{13}C,4′-O^{13}CH$_3$]4′-methoxycinnamic acid. The extensive demethylation of 4′-methoxycinnamic acid upon feeding to *M. acuminata* may prevent intact incorporation into 4′-methoxyphenylphenalenones, even if the putative PKS-type condensing enzyme would accept 4′-methoxycinnamoyl-CoA acid as a starter unit. *O*-Demethylation, a common step in many biosynthetic pathways, is frequently involved in xenobiotic metabolism (Schuler, 1997). Hence, in the present feeding experiment, 4′-*O*-demethylation might detoxify exogenously applied 4′-methoxycinnamic acid to form *p*-coumaric acid, which then is transformed to 3′,4′-methylenedioxycinnamic acid. This conversion requires 3′-hydroxylation, 3′-*O*-methylation and formation of the methylenedioxy bridge. The last reaction could be catalyzed by a cytochrome P450, as reported for the formation of methylenedioxy groups in lignan biosynthesis (Ono et al., 2006). The original [2-^{13}C]-content of the precursor, 4′-methoxycinnamic acid, of 99% was reduced to 8% in [2-^{13}CH$_3$]3′,4′-methylenedioxycinnamic acid, which indicated relatively high endogenous levels of this compound in *M. acuminata*.

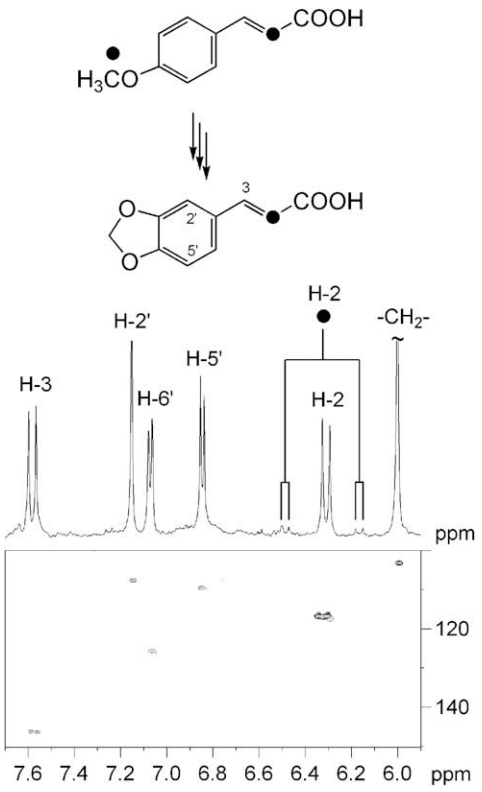

Fig. 6. HSQC spectrum (500 MHz, MeOH-d_4) of 3′,4′-methylenedioxycinnamic acid obtained from *in vitro*-cultures of *Musa acuminata* upon administration of [2-^{13}C,4′-O^{13}CH$_3$]4′-methoxycinnamic acid. The intensity-enhanced cross signal of C-2/H-2 (δ_H 6.31/δ_C 116.7) and the satellite signals of H-2 ($^1J_{C-H}$ = 161 Hz) in the ^{1}H NMR spectrum shown on top of the 2D spectrum indicate enrichment of ^{13}C in this position. No ^{13}C enrichment in the methylenedioxy group (δ_H 6.00/δ_C 103.1) was observed. The sequence of steps indicated by the triple arrows is presumably 4′-*O*-demethylation, 3′-hydroxylation, 3′-*O*-methylation, and finally formation of the methylenedioxy ring.

The question why musanolone F (**3**) was not labelled upon incubation of *Musa* plantlets with [2-^{13}C,4′-O^{13}C]4′-methoxycinnamic acid cannot conclusively answered from the present experiments. However, according to the generally accepted phenylpropanoic pathway (Ferrer et al., 2008), 4′-coumaric acid (which here may come from 4′-methoxycinnamic acid) is converted to activated esters (SCoA ester, quinate, shikimate) before 3′-hydroxylation and 3′-*O*-methylation occurs. Subsequent PKS III-catalyzed condensations to form diarylheptanoids (which are intermediates in phenylphenalenone biosynthesis) also require SCoA esters as substrates (Brand et al., 2006). Hence, free ferulic acid probably is not an intermediate in phenylphenalenone biosynthesis in *Musa*. According to such pathway considerations and previous results (Schmitt et al., 2000), details of the musanolone F formation between *Anigozanthos* and *Musa* might be different.

3. Conclusion

NMR-guided isolation of methoxyphenylphenalenones in *M. acuminata* and *W. thyrsiflora* using [*methyl*-^{13}C]L-methionine as a ^{13}C source for labelling *O*-methyl groups resulted in identification of *O*-methylated phenylphenalenones of various structural types. ^{13}C-Enrichment of *O*-methyl groups simultaneously indicates their origin from [*methyl*-^{13}C]L-methionine via a *S*-adenosylmethionine-dependent process. The obtained compounds include a new natural product, 2-(4-hydroxy-3-methoxyphenyl)-naphthalene-1,8-dicarboxylic anhydride from *M. acuminata* and known compounds

decorated with *O*-methyl groups in various positions, both in the tricycle and the lateral phenyl ring. The *O*-methyl groups, in principle can be introduced early in biosynthesis as a substituent of the phenylpropanoid precursor or at a late step, e.g. by *O*-methylation of hydroxyl groups of phenylphenalenones. Discrimination between early and late *O*-methylation is not trivial, especially in cases where arguments exist for either of the two options. An interesting case of that category is 4′-*O*-methylirenolone (**2**), which in *M. acuminata* could be formed by late *O*-methylation of the non-methylated analogue, irenolone, or by incorporation of intact 4′-methoxycinnamic acid. Since ^{13}C-enrichment of the *O*-methyl group from labelled [*methyl*-^{13}C]L-methionine does not allow to discriminate between early and late methoxylation, [2-^{13}C,4′-O^{13}CH$_3$]4′-methoxycinnamic acid as an advanced precursor was administered to *Musa*. Demethylation of the labelled 4′-*O*-methyl group (and unexpected formation of 3′,4′-methylenedioxycinnamic acid) suggests that 4′-*O*-methylirenolone is not formed from a 4′-methoxycinnamoyl precursor but more likely from the 4′-*O*-methylation of irenolone. Molecular and biochemical studies are required in Musaceae and Haemodoraceae to shed light on methoxylation of various positions of the phenylphenalenone skeleton and characterize their putative *O*-methyltransferases. Enhanced formation of phenylphenalenones including their *O*-methylated analogues in response to elicitation with $CuCl_2$ and jasmonic acid, respectively, in *M. acuminata* and *W. thyrsiflora* suggest that these natural products help defend plants against pathogens and/or herbivores.

4. Experimental

4.1. Plant material and administration of elicitors and labelled precursors

M. acuminata: Sterile *in vitro* plants of *M. acuminata* var. Williams (group AAA) were provided by Universidad Católica de Oriente (Rionegro-Antioquia, Colombia). They were maintained in MS liquid medium (Murashige and Skoog, 1962) at 23 °C under a dark/light cycle of 12 h. Plants with both average root lengths of 5 cm and minimum heights of 5 cm were used for feeding experiments.

Administration of [*methyl*-^{13}C]L-methionine to *M. acuminata* for ^{13}C NMR-guided fractionation (Section 2.1): In a large-scale experiment, 600 *in vitro* plants were transferred into conical flasks (volume 250 ml) containing six plants each and a total volume of 30 ml of liquid MS medium per flask. An aqueous solution (500 ml) containing of [*methyl*-^{13}C]L-methionine (100 mg) and $CuCl_2$ (10 mg) was prepared. A volume of 5 ml of this solution was added through a membrane filter (0.2 μm) into each flask and then incubated at 23 °C for 6 days. Fifty plants without [*methyl*-^{13}C]L-methionine and $CuCl_2$ treatment were used as control. The extracts were prepared in identical procedures from treated and untreated plants and equal amounts subjected to analysis. An analogous experimental setup was used for feeding [2-^{13}C,4′-O^{13}CH$_3$]4′-methoxycinnamic acid (30 mg, 99% ^{13}C) to *M. acuminata* (Section 2.3). To study the inducibility of methoxy-phenylphenalenone biosynthesis in *M. acuminata* (Section 2.2), *in vitro* plants of *M. acuminata* were transferred individually to 30 ml MS liquid medium in 250 ml Erlenmeyer flasks and maintained at 23 °C for one week before the experiment started. Aqueous solutions of [*methyl*-^{13}C]L-methionine (0.3 ml, 1 mg ml^{-1}), $CuCl_2$ (0.1 ml, 1 mg ml^{-1}) and JA (0.3 ml of a 10 mM solution to obtain a concentration of 100 μM in the medium), respectively, were added to the cultures under sterile conditions through a membrane filter (0.2 μm) and further cultivated as described above. The following combinations of elicitors and [*methyl*-^{13}C]L-methionine (^{13}C-met) were performed in duplicate or triplicate: $CuCl_2$; $CuCl_2$ + ^{13}C-met; JA; JA + ^{13}C-met; ^{13}C-met; untreated control. The plants were extracted after 6 days.

W. thyrsiflora: Root cultures of *W. thyrsiflora* Burm. were established from seeds (Chiltern Seeds, Ulverston, Cumbria, UK) as described for *in vitro*-cultures of other Haemodoraceae (Hölscher and Schneider, 1997). The sterile cultures were maintained in liquid M3 (Murashige and Skoog, 1962) medium (100 ml; pH 5.3) in conical flasks (volume 250 ml) on a gyratory shaker (80 rpm) at 23 °C under permanent diffuse light (4.4 μmol m^{-2} s^{-1}).

Administration of [*methyl*-^{13}C]L-methionine to *W. thyrsiflora* for ^{13}C NMR-guided fractionation (Section 2.1): Root cultures of *W. thyrsiflora* were transferred to fresh medium 4 days before an aqueous solution of [*methyl*-^{13}C]L-methionine (4 mg) was added through a membrane filter (0.2 μm) to give a final concentration of 270 μM. Then JA was added in the same manner to obtain a concentration of 80 μM in the medium. The same procedure was used to study the inducibility of methoxy-phenylphenalenone biosynthesis in *W. thyrsiflora* (Section 2.2). The following combinations of JA and [*methyl*-^{13}C]L-methionine (^{13}C-met) were performed in duplicate or triplicate: JA; JA + ^{13}C-met; ^{13}C-met; untreated control. The plants were extracted after 6 days.

4.2. Extraction of plant material and isolation of phenylphenalenone-type compounds and 3′,4′-methylenedioxycinnamic acid

After the incubation, roots of *in vitro* plants of *M. acuminata* (Section 2.1) were cut, frozen in liquid N_2, macerated and extracted with acetone at room temperature. After evaporation (<30 °C) the extract was dissolved in 1 ml MeOH, and phenylphenalenone-related compounds **1**–**4** were separated by semipreparative reversed-phase HPLC (HPLC method 3). Compound **1**: R_t 33.3 min, **2**: R_t 18.7 min, **3**: R_t 24.4 min, **4**: R_t 18.7 min. Further purification of compounds **2** and **4** was achieved by preparative TLC on precoated Si gel 60 F_{254} plates (Merck, layer thickness 0.25 mm) using *n*-hexane–diethyl ether (1:2) as an eluent. HPLC method 1 was used for final purification.

The procedure used for *M. acuminata* was also employed to extract cultured roots of *W. thyrsiflora* (Section 2.1) and isolate phenylphenalenones except that MeOH was used instead of acetone in order to also extract some hydrophilic phenylphenalenone glucosides occurring in this plant. After evaporation, the MeOH extract was partitioned between *n*-hexane–H_2O and CH_2Cl_2–H_2O, and compounds **5**–**7** were purified from the CH_2Cl_2 fraction using semipreparative reversed-phase HPLC. Compound **5**: R_t 30.6 min, **6**: R_t 20.4 min, **7**: R_t 26.0 min, HPLC method 4.

Roots of *in vitro* plants from "induction" experiments with *M. acuminata* (Section 2.2) were lyophilized and extracted with EtOH, and the extracts evaporated to dryness. The residue was partitioned between *n*-hexane–H_2O, CH_2Cl_2–H_2O and EtOAc–H_2O. The *n*-hexane and CH_2Cl_2 phases were pooled and fractionated using semipreparative HPLC method 3. The fraction eluting with approximately 60–70% MeCN in H_2O was again evaporated to dryness and a sample dissolved in MeOH to a concentration of 1 mg ml^{-1}. Then pyrene was added to an amount of 0.5 μg per injection as an internal standard from a stock solution (0.1 mg pyrene per ml MeOH). A volume of 10 μl was injected to the analytical reversed-phase HPLC method 2. Cultured roots of *W. thyrsiflora* from "induction" experiments (Section 2.2) were extracted with MeOH and evaporated to dryness. A sample of the residue was dissolved in MeOH to a concentration of 2.5 mg ml^{-1}. A volume of 50 μl of this solution was supplemented with 0.5 μg pyrene as an internal standard and analyzed using HPLC method 2.

Isolation of 3′,4′-methylenedioxycinnamic acid and phenylphenalenones (Section 2.3): 6 days after administration of [2-^{13}C,4′-O^{13}CH$_3$]4′-methoxycinnamic acid to *M. acuminata*, the roots were extracted with acetone using the above-mentioned procedure

and the residue partitioned between *n*-hexane–H_2O and CH_2Cl_2–H_2O. The organic phases were evaporated and subjected to ^{13}C NMR analysis. 3′,4′-Methylenedioxycinnamic acid was isolated from the CH_2Cl_2 fraction by HPLC method 2. Anigorufone (R_t 40.1 min), hydroxyanigorufone (R_t 24.8 min) and musanolone F (**3**, R_t 25.3 min) were also purified by HPLC method 2.

4.3. Synthesis of [2-^{13}C,4′-O$^{13}CH_3$]4′-methoxycinnamic acid

[2-^{13}C]*p*-Coumaric acid was synthesized from 4-hydroxybenzaldehyde and [2-^{13}C]malonic acid (99% ^{13}C) (Deutero GmbH, Kastellaun, Germany) using the Knoevenagel condensation. The product (100 mg, 0.6 mmol) was methylated using a total of 3 ml of an ethanol-containing ethereal solution of [^{13}C]diazomethane, which was prepared from *N*-[^{13}C]methyl-*N*-nitroso-*p*-toluenesulfonamide (Cambridge Isotope Laboratories) using a standard procedure. The product, [2-^{13}C,4′-O$^{13}CH_3$]4′-methoxycinnamic acid methyl ester (70 mg, 0.4 mmol), was refluxed with 8 ml of an aqueous solution of sodium hydroxide (25%) for 2 h. The solution was acidified to pH ~1 and extracted with two portions of 10 ml of diethyl ether. Evaporation of the solvent gave [2-^{13}C,4′-O$^{13}CH_3$]4′-methoxycinnamic acid. ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.62 (1H, *dd*, $^2J_{H\text{-}3\text{–}C\text{-}2}$ = 3.0 Hz, $^3J_{H\text{-}3\text{–}H\text{-}2}$ = 16.0 Hz, H-3), 7.54 (2H, *d*, *J* = 8.8 Hz, H-2′/6′), 6.95 (2H, *d*, *J* = 8.8 Hz, H-3′/H-5′), 6.36 (1H, *dd*, $^1J_{H\text{-}2\text{–}C\text{-}2}$ = 160.9 Hz, $^3J_{H\text{-}2\text{–}H\text{-}3}$ = 16.0 Hz, H-2), 3.80 (3H, *d*, $^1J_{H\text{–}C}$ = 144.2, OCH_3); ^{13}C NMR (100 MHz, MeOH-d_4): δ 146.5 (C-4′), 145.8 (C-3), 130.9 (C-2′/C-6′, *d*, $^3J_{C\text{-}2'/6'\text{–}C\text{-}2}$ = 4.5 Hz), 128.4 (C-1′), 116.6 (C-2), 115.4 (C-2′/C-6′, *d*, $^3J_{C\text{-}3'/C\text{-}5'\text{–}OCH3}$ = 4.2 Hz), 55.9 (OCH_3), the carbonyl signal (C-1) was not detected due to poor signal-to-noise ratio.

4.4. Spectroscopic and chromatographic methods

NMR spectroscopic analyses were performed on a Bruker AV 500 NMR spectrometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C) or a Bruker AV 400 NMR spectrometer operating at 400.13 MHz (^{1}H) and 100.75 MHz (^{13}C) (Bruker-Biospin, Karlsruhe, Germany). Chemical shifts are reported relative to tetramethylsilane (TMS). ^{13}C NMR spectra of crude extracts were run using 5 mm broadband probes. ^{1}H NMR, ^{13}C NMR and 2D NMR spectra of isolated compounds were recorded using a 2.5 mm broadband microprobe (500 MHz), a 5 mm TXI cryoprobe (500 MHz), or a 5 mm broadband inverse probe (400 MHz). ESI-MS and ESI-HRMS were measured in the positive ion mode on a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass Ltd., Manchester, UK).

Analytical reversed-phase HPLC was carried out on an Agilent 1100 chromatography system (binary pump G1312A, DAD G1315B, autosampler G1313A). HPLC method 1: LiChrospher 100 RP-18 column (5 μm; 250 × 5 mm); MeCN–H_2O gradient (0.1% TFA) 5%:95% → 65%:35% in 50 min → 90:10% in 5 min and 90%:10% for another 5 min → 5%:95% in 5 min (total time 65 min); flow rate 0.8 ml min^{-1}; DAD detection 200–620 nm, monitoring wavelength at 254 nm. HPLC method 2: Nucleosil 100-3 C18 (3 μm; 250 × 3 mm); MeCN–H_2O gradient (0.1% TFA) 35%:65% → 70%:30% in 60 min → 35%:65% in 5 min (total time 65 min); flow rate 0.4 ml min^{-1}; DAD detection 200–620 nm, monitoring wavelength at 254 nm.

Semipreparative reversed-phase HPLC runs were performed on a Merck-Hitachi HPLC system composed of an L-4250 UV–Vis detector and an L-6200A pump. HPLC method 3: LiChrospher 100 RP18 column (10 μm; 250 × 10 mm); MeCN (0.1% TFA)–H_2O (0.1% TFA) gradient 30:70% → 90:10% in 50 min → 30:70% in 5 min (total time 55 min); flow rate 3.5 ml min^{-1}; UV detection at 254 nm. HPLC method 4: LiChrospher 100 RP18 column (10 μm; 250 × 10 mm); MeCN-H_2O gradient 30:70% → 65:35% in 30 min → 90:10% in 5 min → 30:70% in 5 min (total time 40 min); flow rate 3.5 ml min^{-1}; UV detection at 254 nm.

4.5. Identification of methoxy-phenylphenalenones and 3′,4′-methylenedioxycinnamic acid

Compounds **1** and **2** were identified by comparing their ^{1}H and ^{13}C NMR spectra with literature data. Musanolone F (**3**), 5-hydroxy-2-methoxy-6-oxa-benzo[*def*]chrysen-1-one (**6**), methoxyanigorufone (**5**), anigorufone, and hydroxyanigorufone were identified by comparing their retention times and ^{1}H NMR spectra with those of authentic references obtained in previous studies (Schmitt et al., 2000; Opitz et al., 2002; Hölscher and Schneider, 1997).

2-(4-Hydroxy-3-methoxyphenyl)-naphthalene-1,8-dicarboxylic anhydride (**4**) is a new natural product. ^{1}H NMR and ^{1}H,^{1}H COSY spectra showed signals of an AB spin system of H-3 and H-4, and two AMX spin systems, one of which is due to H-5/H-6/H-7 and the other one to H-2′/H-5′/H-6′ of the lateral 3′,4′-disubstituted phenyl ring. An exchangeable broad singlet (δ 8.00) indicated a phenolic hydroxyl group. The methoxy signal at δ 3.98, typical of an *O*-methyl group, appears as a singlet of an [O$^{12}CH_3$]-isotopomer and, due to ^{13}C–^{1}H coupling, a doublet ($J_{H\text{–}C}$ = 144.5 Hz) of the labelled [O$^{13}CH_3$]-isotopomer. The missing singlet of a ring A methine, together with an also missing carbon (only 18 instead of usual 19 C atoms of the phenylphenalenone skeleton were detected, not counted the additional *O*-methyl carbon) and two carbonyl resonances, suggested an anhydride structure of ring A. The downfield resonance of H-7 at δ 8.63 was in agreement with a *peri* position of this proton relative to a carbonyl group. A HMBC cross signal supporting this suggestion was detected between H-7 and the carbonyl signal at δ 159.8 (C-9). The anhydride structure was further confirmed by a weak HMBC w-correlation through four bonds between H-3 (δ 7.79) and the other carbonyl group (C-10, δ 162.1). The location of the methoxy group at C-3′ was established by a HMBC correlation of the corresponding signal (δ 3.89) with the carbon at δ 147.2. HREIMS obs. *m*/*z* 320.06918 (calc. for $C_{19}H_{12}O_5$, 320.06847). ^{1}H NMR (500 MHz, acetone-d_6): δ 8.63 (1H, *dd*, J_1 = 7.6 Hz; J_2 = 1.3 Hz, H-7), 8.54 (1H, *dd*, J_1 = 8.0 Hz; J_2 = 1.0 Hz, H-5), 8.48 (1H, *d*, *J* = 8.5 Hz, H-4), 8.00 (1H, *s*, –OH), 7.94 (1H, *dd*, J_1 = 7.6 Hz; J_2 = 7.5 Hz, H-6), 7.79 (1H, *d*, *J* = 8.5 Hz, H-3), 7.18 (1H, *d*, *J* = 2.0 Hz, H-2′), 7.01 (1H, *dd*, J_1 = 8.0 Hz; J_2 = 2.0 Hz, H-6′), 6.95 (1H, *d*, *J* = 8.0 Hz, H-5′), 3.89 (3H, *s*, –OCH_3). ^{13}C NMR (125 MHz, acetone-d_6): δ 162.1 (C-9), 159.8 (C-10), 150.6 (C-1′), 150.5 (C-2), 147.7 (C-4′), 147.2 (C-3′), 135.9 (C-5), 134.5 (C-4), 133.3 (C-7), 132.6 (C-3), 132.3 (C-4a), 132.2 (C-8a), 127.5 (C-6), 122.2 (C-6′), 120.4 (C-8), 116.6 (C-1), 115.2 (C-5′), 113.3 (C-2′), 55.9 (–OCH_3).

5-Hydroxy-3,6-dimethoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**7**). ^{1}H NMR (500 MHz, MeOH-d_4): δ 8.22 (1H, *d*, *J* = 7.1 Hz, H-9), 7.58 (1H, *s*, H-4), 7.34 (5H, *m*, H-1′–H-5′), 7.32 (1H, *d*, *J* = 7.1 Hz, H-8), 6.47 (1H, *s*, H-3), 3.96 (3H, *s*, 6-OCH_3), 3.67 (3H, *s*, 3-OCH_3); ^{13}C NMR (125 MHz, MeOH-d_4): δ 166.3 (C-1), 147.2 (C-7), 145.2 (C-5), 145.0 (6-OCH_3), 144.7 (C-1′), 129.8 (C-8), 128.7 (C-9), 128.0 (C-2′/6′), 127.8 (C-3′/C-5′ and C-4′), 125.6 (C-9b), 122.0 (C-6a), 119.3 (C-9a), 119.0 (C-3a), 115.4 (C-4), 103.9 (C-3), 57.8 (6-OCH_3), 56.4 (3-OCH_3).

[2-^{13}C]3′,4′-Methylenedioxycinnamic acid: EIMS *m*/*z* (rel. int.): 193 [M$^+$ of 2-^{13}C-isotopomer] (15), 192 [M$^+$ of natural abundance isotopomer] (100). ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.58 (1H, *d*, *J* = 15.9 Hz, H-3), 7.15 (1H, *d*, *J* = 1.8 Hz, H-2′), 7.07 (1H, *dd*, *J* = 8.1, 1.8 Hz, H-6′), 6.84 (1H, *d*, *J* = 8.1 Hz, H-5′), 6.31 (1H, *d*, *J* = 15.9 Hz, H-2), 6.00 (2H, *s*, –CH_2–); ^{13}C NMR (125 MHz, MeOH-d_4): δ 170.5 (C-1), 150.3 (C-4′), 149.3 (C-3′), 146.4 (C-3), 130.4 (C-1′), 125.8 (C-6′), 116.7 (C-2), 109.6 (C-5′), 107.6 (C-2′), 103.2 (–CH_2–).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.10.019.

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