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Biosynthesis of tetraoxygenated phenylphenalenones in *Wachendorfia thyrsiflora*

Tobias Munde, Silke Brand, William Hidalgo, Ravi K. Maddula, Aleš Svatoš, Bernd Schneider *

Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Straße 8, D-07745 Jena, Germany

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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

* Corresponding author. Tel.: +49 3641 571600: fax: +49 3641 571601.
E-mail address: schneider@ice.mpg.de (B. Schneider).

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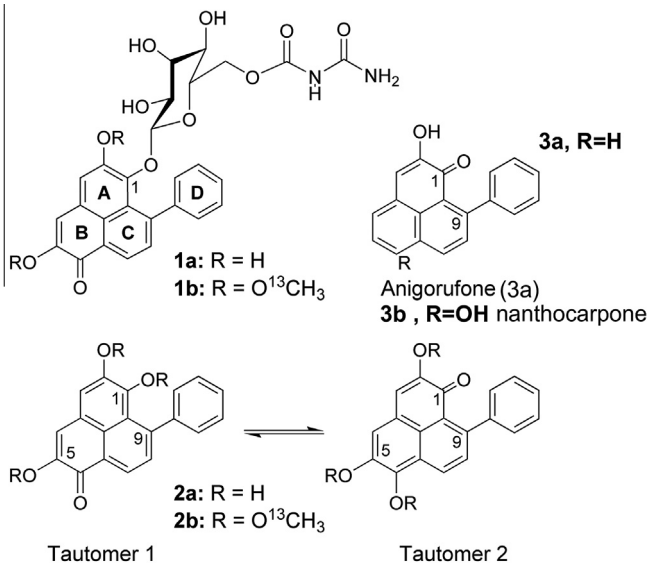

Fig. 1. Structures of 1,2,5,6-tetraoxygenated phenylphenalenones used as target compounds to study biosynthetic oxygenation in root cultures of *W. thyrsiflora* and anigorufone; the last compound exemplifies a 2-hydroxy-9-phenylphenalen-1-one. Because of tautomerism of **2a**, the carbonyl group of **2a**/**2b** 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.