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Variability of phenylpropanoid precursors in the biosynthesis of phenylphenalenones in *Anigozanthos preissii*

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Dedicated to Professor H.-R. Schütte on the occasion of his 70 birthday.

Abstract

Feeding experiments using ^{13}C labelled precursors and NMR spectroscopic studies revealed general biosynthetic incorporation of phenylalanine and variable incorporation of cinnamic acid, *p*-coumaric acid, caffeic acid and ferulic acid into phenylphenalenones in root cultures of *Anigozanthos preissii*. Evidence was obtained for parallel pathways of phenylphenalenone biosynthesis, with respect to the left phenylpropanoid unit, and a sequence involving utilisation of *p*-coumaric acid with late generation of an intermediate catechol moiety in the right phenylpropanoid unit. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Phenylphenalenones are characteristic constituents of the Haemodoraceae plant family (Cooke & Edwards, 1980). They accumulate in roots of *Anigozanthos preissii* and have also been found in root cultures of that species (Hölscher & Schneider, 1997). Recent biosynthetic studies demonstrated the symmetrical incorporation of two molecules of cinnamic acid and *p*-coumaric acid into anigorufone (**1**) (Hölscher & Schneider, 1995a) and hydroxyanigorufone (**2**) (Hölscher & Schneider, 1995b), respectively. The involvement of diarylheptanoids in the phenylphenalenone biosynthesis was shown by feeding a labelled precursor of that type, 1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-dien-5-one, carrying a catechol moiety (Hölscher & Schneider, 1995a). It has been suggested that the catechol portion undergoes oxidation to an *o*-quinone, generating an excellent dienophile capable of involvement in a proposed Diels–Alder cycloaddition, resulting in the phenylphenalenone ring system (Bazan, Edwards & Weiss, 1978; Schmitt & Schneider, 1999). However, details of the biosynthetic pathway are still hypothetical. Until now, for example, it was uncertain whether the second hydroxyl group of the catechol moiety in the diarylheptanoid intermediate, originated from caffeic acid or, alternatively, whether it was introduced by hydroxylation at a later stage of the biosynthesis. In this paper, we describe in vivo feeding experiments using labelled phenylpropanoids, having different substitution patterns in the phenyl ring, and how these precursors are biosynthetically incorporated into phenylphenalenones.

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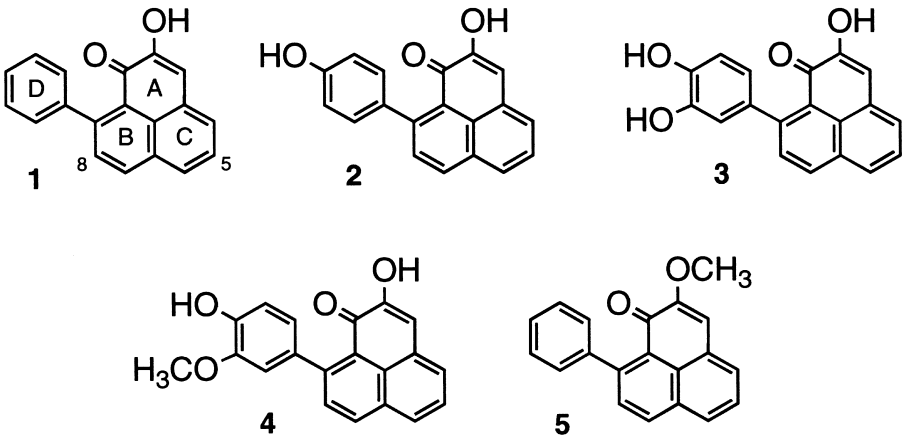

Fig. 1. Structure of phenylphenalenones isolated from root cultures of *Anigozanthos preissii* upon administration of labelled precursors: anigorufone (**1**), hydroxyanigorufone (**2**), dihydroxyanigorufone (**3**), musanolone F (**4**), and methoxyanigorufone (**5**).

2. Results

Previous feeding experiments using ^{14}C labelled phenylalanine have established the ready incorporation into phenylphenalenones in *Haemodorum corymbosum* (Thomas, 1971) and *Lachnanthes tinctoria* (Edwards, Schmitt & Weiss, 1972). Specific incorporation of label from [1-^{13}C]phenylalanine into C-7 but not C-6 of lachnanthoside aglycone was observed in *L. tinctoria* (Harmon, Edwards & Highet, 1977). In our biosynthetic studies, two molecules of [1-^{13}C]phenylalanine were shown to be specifically incorporated into the phenylphenalenones of *Anigozanthos preissii*. This was indicated by enhancement of the ^{13}C resonances of C-6 and C-7, measured directly in crude extracts obtained from fed root cultures of *A. preissii* containing anigorufone (**1**), hydroxyanigorufone (**2**), dihydroxyanigorufone (**3**), musanolone F (**4**), and methoxyanigorufone (**5**) (Hölscher & Schneider, 1997) (Fig. 1). Enlarged ^{13}C-NMR signals C-6 and C-7 in isolated **1** (δ 130.2, C-6; δ 136.2, C-7) (Fig. 2), **2** (δ 130.1, C-6; δ 136.1, C-7), and **5** (δ 129.6, C-6; δ 135.0, C-7) confirmed this result. The high quantity of specific incorporation of ^{13}C into C-6 and C-7 was indicated by comparison of integral ratios between non-labelled references (integral ratio C-7:C-8:C-6 = 1:1:1) and labelled phenylphenalenones. In compound **1**, for example, the integral ratio C-7:C-8:C-6 is 11:1:11 (Fig. 2) corresponding to 11.2% enhancement of both C-6 and C-7 above natural abundance. This finding also indicated symmetric incorporation of two molecules phenylalanine into one phenylphenalenone molecule. Due to C–C coupling, the resonances of C-6 and C-7 appear as pseudo triplets (see extensions in Fig. 2(a)), indicating the occurrence of singly labelled isotopomers (singlet, central resonances of the pseudo triplets) and relatively high amounts of isotopomers containing two labelled carbons in these positions (doublets, $^2J_{C\text{-}6\text{-}C\text{-}7} = 2.7$ Hz).

Another series of feeding experiments using [2-^{13}C]cinnamic acid, [2-^{13}C]*p*-coumaric acid, [2-^{13}C]caffeic ic acid, and [2-^{13}C]ferulic acid were carried out, employing the same root culture line of *A. preissii*. Compounds **1**, **2** and **3** were isolated from the cultures upon administration of cinnamic acid and *p*-coumaric acid. ^{13}C-NMR spectra were measured in order to find out whether incorporation into both parts or into only one half of the phenylphenalenone molecule had occurred. Enhanced ^{13}C-NMR signals were expected for either C-5 or C-8, or both of these carbon atoms. The results are summarised in Table 1. As previously demonstrated (Hölscher & Schneider, 1995a), anigorufone (**1**) was symmetrically labelled at C-5 (δ 127.9) and C-8 (δ 132.0) following administration of [2-^{13}C]cinnamic acid. Symmetrical incorporation was also shown for compounds **2** (δ 127.7, C-5; δ 132.5, C-8) and **3** (δ 127.7, C-5; δ 132.5, C-8), isolated from analogous feeding experiments. [2-^{13}C]*p*-Coumaric acid was symmetrically incorporated into hydroxyanigorufone (**2**), as previously published (Hölscher & Schneider, 1995b), and into dihydroxyanigorufone (**3**). Incorporation of *p*-coumaric acid exclusively into the right part of **1** was a trivial finding. High incorporation rates of phenylpropanoic acids into phenylphenalenones were observed in all feeding experiments. The values, determined from EI-MS data, in this series of experiments varied between 7.1% ^{13}C in singly labelled (C-5 or C-8) and 2.3% in doubly labelled molecules (both C-5 and C-8) of anigorufone **1** biosynthesised from [2-^{13}C]cinnamic acid and 31.7%/3.6% ^{13}C in hydroxyanigorufone (**2**) biosynthesised from [2-^{13}C]*p*-coumaric acid. Unfortunately, mass spectral fragmentation did not allow discrimination between incorporation into

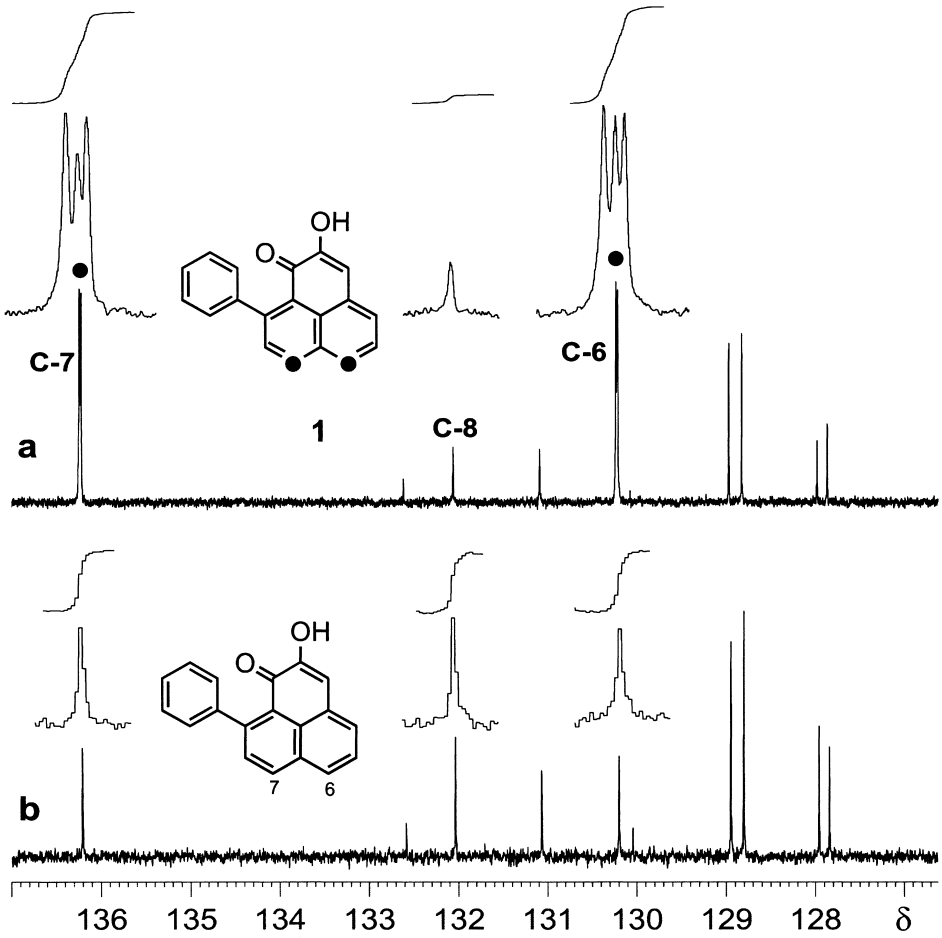

Fig. 2. Partial ^{13}C-NMR spectrum of anigorufone (**1**) isolated from root cultures of *Anigozanthos preissii* upon (a) feeding [1-^{13}C]phenylalanine. Compared with the spectrum (b) of the non-labelled reference (integral ratio C-7:C-8:C-6 = 1:1:1), the resonances of C-6 and C-7 are enhanced (integral ratio C-7:C-8:C-6 = 11:1:11) and, due to $^{2}J_{C\text{-}C} = 2.7$ Hz, appear as pseudo triplets (for details, see text).

C-5 and C-8. Consequently, individual incorporation rates of doubly labelled compounds were not accessible from our MS data. Actually this was the reason for employing ^{13}C-NMR spectroscopy to prove specific incorporation unambiguously. In contrast, incorporation rates of singly labelled compounds were readily determined from the mass spectral data. 15.4% ^{13}C were found in anigorufone (**1**) biosynthesised from [2-^{13}C]*p*-coumaric acid.

Anigorufone (**1**), hydroxyanigorufone (**2**), and dihydroxyanigorufone (**3**) were also isolated upon administration of [2-^{13}C]caffeic acid and [2-^{13}C]ferulic acid. Additionally, two phenylphenalenones bearing a methoxyl group, musanolone F (**4**) and methoxyanigoru-

Table 1
Incorporation of [2-^{13}C]labelled phenylpropanoids into phenylphenalenones determined by means of ^{13}C-NMR spectroscopy of phenylphenalenones **1**–**5**

[2-^{13}C]labelled precursor	Enhancement of ^{13}C signals									
	1		**2**		**3**		**4**		**5**	
	C-8	C-5	C-8	C-5	C-8	C-5	C-8	C-5	C-8	C-5
Cinnamic acid	+	+	+	+	+	+	n.d.[a]		n.d	
p-Coumaric acid	–	+	+	+	+	+	n.d		n.d	
Caffeic acid	–	–	–	–	+	(+)	+	–	–	–
Ferulic acid	–	–	–	–	–	–	+	–	–	–

[a] n.d. = not determined.

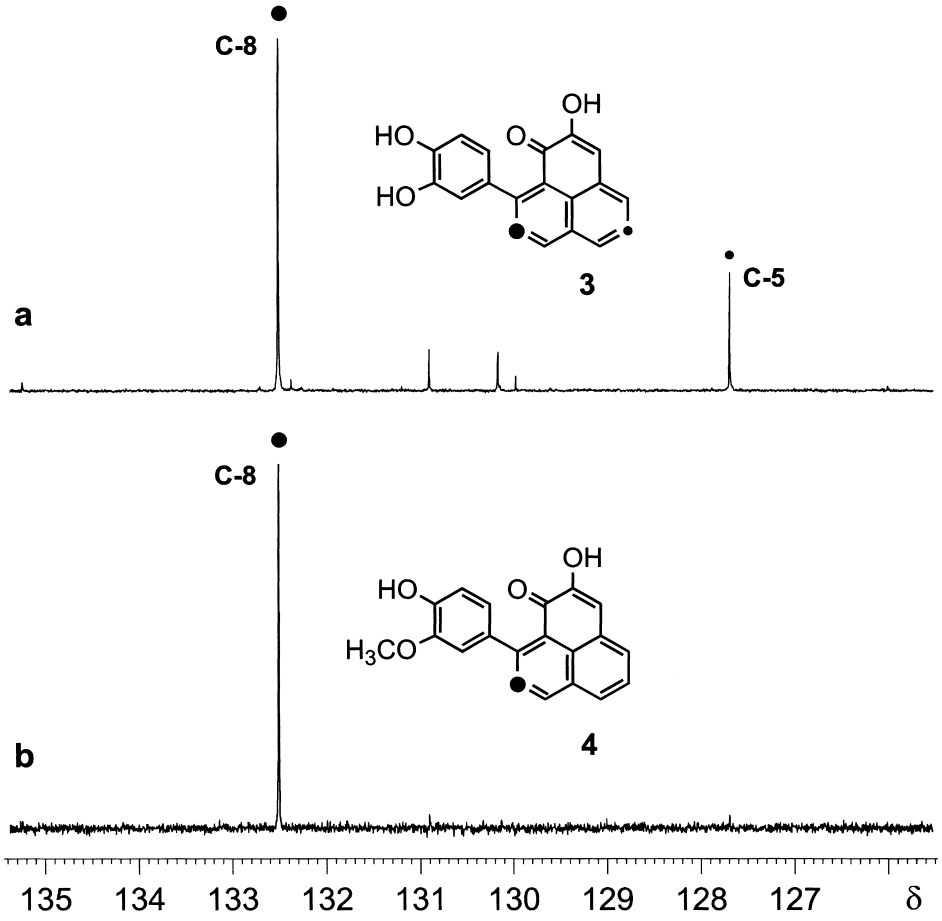

Fig. 3. Partial ^{13}C-NMR spectra of phenylphenalenones isolated from root cultures of *Anigozanthos preissii* upon administration of ^{13}C labelled phenylpropanoids. (a) Dihydroxyanigorufone (**3**) biosynthesised from [2-^{13}C]caffeic acid, (b) musanolone F (**4**) biosynthesised from [2-^{13}C]ferulic acid.

fone (**5**), were isolated from these feeding experiments and analysed by ^{13}C-NMR. Both **4** (Luis et al., 1996) and **5** (Luis, Fletcher, Echeverri, Abad, Kishi & Perales, 1995) are known from *Musa acuminata*. Compound **4** is described here for the first time as a constituent of a Haemodoraceae species. Compounds **1**, **2** and **5**, isolated upon feeding [2-^{13}C]caffeic acid, did not show any incorporation of the labelled precursor (Table 1). This is, again, trivial for the left part of the phenylphenalenone molecule, because the phenyl ring carries less substituents than the precursor. Non-incorporation into the right part of the molecule indicates that ring A and C-4 to C-6 of the phenylphenalenones are not formed from caffeic acid. The ^{13}C-NMR spectra of dihydroxyanigorufone (**3**) (Fig. 3(a)) and musanolone F (**4**), isolated from the same experiment, exhibited strong enhancement of C-8. EI-MS data indicated 5.7% ^{13}C in singly labelled (C-5 or C-8) and 1.7% in doubly labelled molecules (both C-5 and C-8) of compound **3**, and 16.0% ^{13}C for compound **4** (singly labelled). These findings clearly indicated that the left part of both **3** and **4** was formed from caffeic acid. In contrast, the right part must be derived from *p*-coumaric acid as the immediate precursor, which undergoes condensation with a second (variably substituted) phenylpropanoid via a central C-1 unit (Schmitt & Schneider, 1999). Subsequent *o*-hydroxylation, instead of caffeic acid incorporation, is suggested to form the catechol moiety involved in the pathway. In addition to the enhanced ^{13}C signal of C-8 in **3**, slight enhancement of the C-5 resonance was detected after feeding of [2-^{13}C]caffeic acid. The small incorporation of caffeic acid into the right part of the ring system could be observed only for **3** and not for other phenylphenalenones, suggesting the occurrence of an alternative subpathway of minor significance.

A crude extract obtained from root cultures of *A. preissii* upon feeding [2-^{13}C]ferulic acid exhibited only a single strongly enhanced signal at δ 132.5, which was assigned to musanolone F (**4**). This was confirmed after isolation of **4** by additional NMR (Fig. 3(b)) and MS data. Compounds **1**–**3** and **5** were also isolated but enhanced signals were not detectable in the spectra of these compounds. This result substantiates the pro-

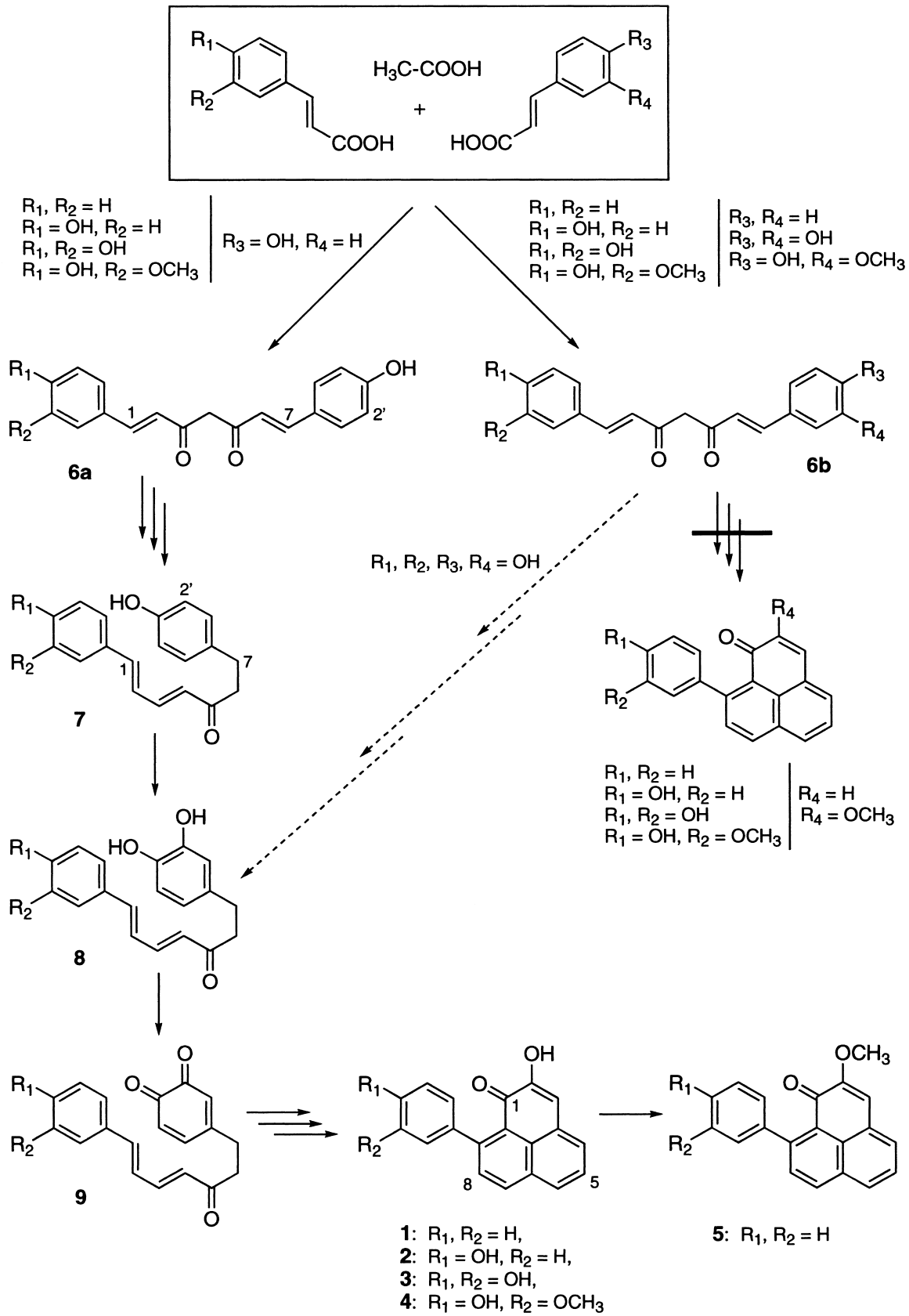

Fig. 4. Pathway of phenylphenalenone biosynthesis, proposed on the basis of precursor feeding experiments using ^{13}C labelled phenylpropanoids. Intermediates **6a**, **6b**, **7**, and **9** are putative. Incorporation of **8** (R_1, R_2 = H, 1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-diene-5-one) into anigorufone (**1**) has been demonstrated previously (Hölscher & Schneider, 1995a).

posed variability of precursors forming the left part of the phenylphenalenone molecule and the restriction of the right-half-precursor to *p*-coumaric acid. Moreover, the methoxy group at C-2 of **5** must have been formed by hydroxylation and subsequent methylation in later biosynthetic steps.

3. Discussion

The experiments described here clearly demonstrate that in *Anigozanthos preissii* two phenylpropanoids are being utilized to form the phenylphenalenone skeleton. Equal distribution of labelling from [1-^{13}C]phenylnylalanine into C-6 and C-7 of the phenylphenalenones provided evidence for symmetric incorporation of early precursors into both halves of the molecule. Symmetric incorporation of cinnamic acid and *p*-coumaric acid into anigorufone (**1**) and hydroxyanigorufone (**2**), representing the major phenylphenalenones of *A. preissii*, has also been demonstrated. Experiments employing caffeic and ferulic acid as precursors indicated non-symmetric incorporation only into dihydroxyanigorufone (**3**) and musanolone F (**4**), but not into **1**, **2**, and methoxyanigorufone (**5**). This variability of incorporation was used to deduce the mechanism of phenylphenalenone biosynthesis. Thus, the right portion (ring A and C-4 to C-6) of the phenylphenalenones is predominantly formed by incorporation of *p*-coumaric acid and not (or to a minor extent) caffeic acid. By analogy with chalcone biosynthesis (Schüz, Heller & Hahlbrock, 1983; Schröder, 1997), *p*-coumaric acid seems to serve as the preferred substrate in the first step of phenylphenalenone biosynthesis. Obviously a mechanism is operating that enables discrimination between two halves of intermediary diarylheptanoids of type **6a** and **6b** (Fig. 4), which are symmetrical with respect to the heptanoid chain.

Since all phenylphenalenones studied here possess an α-ketohydroxyl (or α-ketomethoxyl) functionality in ring A, hydroxylation at C-2′ (ortho with respect to the hydroxyl group already present in position 1′) of intermediate **7** must take place to form the catechol moiety of intermediate **8**. It was suggested that the catechol undergoes oxidation to an *o*-quinone (**9**) providing an excellent dienophile capable of involvement in a hypothetical Diels–Alder cycloaddition yielding the phenylphenalenone ring system (Bazan et al., 1978; Schmitt & Schneider, 1999). Thus, intermediary diarylheptanoids (**6a**, **7**) containing a *p*-hydroxyl group are required to form a catechol moiety, as in compound **8** and, further, an *o*-quinone (**9**). Diarylheptanoids **6b** having an unsubstituted phenyl or vanillyl ring in the right part of the molecule are expected to be formed from cinnamic acid or ferulic acid, respectively. However, these postulated intermediates are not suitable to undergo the biosynthetic sequence proposed in Fig. 4, since formation of catechol and *o*-quinone groups, and subsequent cyclisation to phenylphenalenones, seems to be impossible. As further demonstrated in our experiments, methoxyanigorufone (**5**) was not formed from ferulic acid but must be a product of anigorufone 2-*O*-methylation. One might also speculate that an incompatible substitution pattern in one of the phenyl rings of open-chain diarylheptanoids, like **6**, occurring in the Zingiberaceae and other plants could be the reason for the lack of phenylphenalenones in these families.

In contrast to specific precursor requirements in the right part of the phenylphenalenone molecule, less specific incorporation of various ring substituted phenylpropanoic acids into the left part (C-7 to C-9 and ring D) was observed (Fig. 4). The left part is not involved as closely as the right part in various biosynthetic steps and, therefore, the structural requirements are much less specific. The suggestions presented here are in complete accordance with detailed pathways recently proposed for early steps of the phenylphenalenone biosynthesis (Hölscher, 1996; Schmitt & Schneider, 1999). In addition, biosynthesis of phenylphenalenones exhibiting diverse substitution patterns in the phenyl ring is now explicable.

4. Experimental

4.1. Plant material, synthesis and administration of labelled compounds

Root cultures of *Anigozanthos preissii* (L.) were initiated as previously described (Hölscher & Schneider, 1997) and grown in liquid MS medium (Murashige & Skoog, 1962) (100 ml in 300 ml conical flasks) at 22°C on a gyratory shaker (100 rpm) under permanent light (4.4 μmol m^{-2} s^{-1}). Three days before administration of the precursors the cultured roots (about 15 g) were transferred to fresh medium. [1-^{13}C]Phenylalanine (99% ^{13}C) was from Deutero GmbH, Kastellaun, Germany. [2-^{13}C]Phenylnylpropanoic acids were synthesized by an Erlenmeyer type reaction from [2-^{13}C]malonic acid (99% ^{13}C) following standard procedures. For administration to root cultures, labelled phenylpropanoic acids (33 μmol in each experiment) were dissolved in EtOH:H_2O 7:3 (1 ml) and passed through a membrane filter. [1-^{13}C]Phenylalanine (100 μmol) was dissolved in 1 ml H_2O and administered in the same manner. The incubation times were 1 day (cinnamic acid, coumaric acid) or 3 days (phenylalanine, caffeic acid and ferulic acid).

4.2. Extraction of roots and isolation of phenylphenalenones

Roots were frozen in liquid N_2, ground, and extracted with MeOH at room temperature. The residue obtained by evaporation ($<40°$) of the crude MeOH extract was fractionated by partitioning between *n*-hexane–H_2O, CH_2Cl_2–H_2O, and EtOAc–H_2O. Anigorufone (**1,** R_t 24.1 min), methoxyanigorufone (**5,** R_t 22.0 min), and a fraction containing hydroxyanigorufone (**2**) and musanolone F (**4**) (R_t 13.5 min) were obtained by reversed-phase HPLC (LiChrospher 100 RP18, 250 × 4 mm, 5 μm, 0.8 ml min^{-1}, UV 254 nm) of the *n*-hexane fraction using a linear gradient MeCN–H_2O (0.1% TFA) from 45% to 75% MeCN in 30 min. Rechromatography of the fraction at R_t 13.5 min (Waters Symmetry Shield™ RP18, 250 × 4.6 mm, 5 μm, 1.0 ml min^{-1}, UV 254 nm, linear gradient MeCN–H_2O (0.1% TFA) from 53% to 63% MeCN in 30 min) afforded pure **2** (R_t 11.8 min) and **4** (R_t 10.5 min). Dihydroxyanigorufone (**3**, R_t 15.5 min) was isolated from the CH_2Cl_2 and EtOAc fractions by reversed-phase HPLC (LiChrospher 100 RP18, 250 × 4 mm, 5 μm, 0.8 ml min^{-1}, UV 254 nm) using a linear gradient MeCN–H_2O (0.1% TFA) from 35% to 65% MeCN in 30 min).

The analytical data of compounds **1**, **2**, **3**, and **5** (^{1}H- and ^{13}C-NMR, MS) exactly matched those of authentic references (Hölscher & Schneider, 1997). Compound **4** was identified by means of ^{1}H- and ^{13}C-NMR, 2D heteronuclear correlation experiments (HMQC, HMBC), and mass spectrometry. ^{1}H-NMR: δ 3.85 (3H, *s*, 3′-OCH_3), 6.89 (1H, *dd*, 8.2, 1.8 6′–H), 6.92 (1H, *d*, 8.2, 5′-H), 7.04 (1H, *d*, 1.8, 2′-H), 7.20 (1H, *s*, 3-H), 7.67 (1H, *d*, 8.2, 8-H), 7.67 (1H, *dd*, 8.1, 7.0, 5-H), 7.85 (1H, *d*, 7.0, 4-H), 8.06 (1H, *d*, 8.1, 6-H), 8.40 (1H, *d*, 8.2, 7-H). ^{13}C-NMR: δ 56.8 (3′-OCH_3), 112.9 (3-C), 113.8 (2′-C), 115.9 (5′-C), 122.4 (6′-C), 127.8 (5-C), 130.4 (6-C), 131.2 (4-C), 132.5 (8-C), 136.4 (7-C), 149.8 (9-C), 180.3 (1-C). EIMS (70 eV): *m*/*z* 318 $[M]^+$. The data of **4** are in accordance with literature values (Luis et al., 1996).

4.3. Spectroscopic methods

Bruker DRX 500 NMR spectrometer: 500.13 MHz (^{1}H), 125.75 MHz (^{13}C), Me_2CO-d_6, TMS as int. standard. ^{13}C-NMR spectra were run using a 2.5 mm broadband microprobe. ^{1}H-NMR, HMBC and HMQC spectra were measured in a 2.5 mm inverse detection microprobe. Mass spectra were recorded on a Micromass MasSpec (70 eV).

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