A Diarylheptanoid Intermediate in the Biosynthesis of Phenylphenalenones in *Anigozanthos preissii*

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The incorporation of 1-phenyl-7-(3,4-dihydroxyphenyl)hepta-1,3-dien-5-one and two molecules of cinnamic acid, respectively, into anigorufone by cultured roots of *Anigozanthos preissii* provides the first experimental evidence for the biosynthesis of a phenylphenalenone from two C_6–C_3 units *via* an open chain type diarylheptanoid.

The 9-phenyl-1*H*-phenalen-1-ones represent a group of plant pigments mainly occurring in the family *Haemodoraceae* including the genus *Anigozanthos* which is only found in southwestern Australia.[1–3] The occurrence of compounds of this type has been used to clarify doubtful chemotaxonomic relationships.[4] However, phenylphenalenone type compounds have recently been found in *Eichhornia crassipes* and two species of *Musa* also.[5–8]

As demonstrated previously, both [1-^{14}C]- and [2-^{14}C]-phenylalanine as well as [2-^{14}C]tyrosine are effectively incorporated into haemodorine in *H. corymbosum*.[9] Specific incorporation of a label of [2-^{14}C]tyrosine into C-5 of lachnanthoside aglycone has been demonstrated in *Lachnanthes tinctoria*. [1-^{14}C]- and [3-^{14}C]-phenylalanine were effectively utilized to an approximately equal extent by *L. tinctoria* to form lachnanthoside aglycone.[10] ^{13}C NMR analysis of haemocorine from a feeding experiment with [1-^{13}C]phenylalanine indicated specific incorporation into C-7.[11] Thus, it has been postulated that the 9-phenylphenalenones are biosynthesised from two C_6–C_3 units *via* a diarylheptanoid intermediate.[12,13] In this 1,7-diarylsubstituted C_7 chain, the central carbon atom (C-4) is probably provided by C-2 of acetate. However, the possibility of incorporation of phenylalanine and tyrosine into the same C_6–C_3 unit in the above experiments has not been finally excluded and there has been no experimental evidence for a diarylheptanoid intermediate until now.

2-Hydroxy-9-phenyl-1*H*-phenalen-1-one (anigorufone) **1** is the simplest naturally occurring member of the phenylphenalenone family yet known. For the first time, compound **1** has been reported as a constituent of the roots of *Anigozanthos rufus*.[3] Beside a multitude of further pigments of the phenylphenalenone type, cultured roots of *Anigozanthos preissii* are producing **1** in amounts of about 0.2 mg g^{-1} fresh mass. For isolation, the fresh roots were frozen with liquid nitrogen, ground, and extracted with methanol. The methanol extract was evaporated to the aqueous concentrate followed by partition with *n*-hexane. TLC of the *n*-hexane fraction (silica gel, toluene : acetone 4 : 1) and HPLC (RP 18, 85% MeOH) yielded pure **1**. The identity of **1** was established by EI-MS [*m/z* = 271 (100), 272 (M$^+$, 44), 271 (8)] and NMR analysis. The ^{1}H NMR spectrum (500 MHz, [$^2H_6$]acetone) exhibited six signals, each integrating for one proton, and the signals of the phenyl ring protons in the range between δ 7.38 and 7.47 (5 H). The doublet signal at δ 8.41 (*J* 8.2 Hz) was attributed to H-7 on the basis of the chemical environment and literature data.[4] The ^{1}H,^{1}H COSY spectrum indicated correlation of H-7 with H-8 (δ 7.63, *J* 8.2 Hz). The protons H-4 (δ 7.88, d, *J* 7.1 Hz), H-5 (δ 7.70, dd, *J* 8.2, 7.1 Hz) and H-6 (δ 8.09, d, *J* 8.2 Hz) were assigned on the basis of ^{1}H,^{1}H COSY and ^{1}H,^{1}H long range COSY experiments. The assignments were confirmed by a decoupling experiment with H-6 (δ 8.09) changing the multiplicity of the signal of H-5 to a normal doublet and changing also the fine structures of H-4 and H-7. The singlet at δ 7.18 was attributed to H-3 based on correlation with H-4 in the ^{1}H,^{1}H long range COSY and a decoupling experiment.

To assign the ^{13}C signals, broad band decoupled ^{13}C, APT, HMQC and HMBC spectra were used. Beside the signal at δ 180.7 which was easily assigned to the carbonyl C-atom (C-1), the APT spectrum revealed seven more nonprotonated carbon atoms. These signals were assigned by HMBC to C-2 (δ 151.3), C-3a (δ 130.0), C-6a (δ 132.5), C-9 (δ 149.1), C-9a (δ 124.7), C-9b (δ 125.7), C-1′ (δ 143.6). From the nine signals of protonated carbons (APT), C-4′ (δ 127.8), C-3′/5′ (δ 128.8), and C-2′/6′ (δ 128.9) were related to the phenyl protons (δ 7.38–7.47) by HMQC. Heteronuclear one bond and multiple bond correlations also revealed the assignments of C-4 (δ 131.0), C-6 (δ 130.2) and C-7 (δ 136.2). The most upfield signal (δ 113.1) was assigned to C-3.

Most important for the biosynthetic experiments was the unambiguous assignment of C-5 and C-8. The HMQC spectrum indicated connectivities between H-5 (δ 7.70) and the signal at δ 127.9 which was assigned to C-5, and between H-8 (δ 7.63) and δ 132.0 (C-8). The assignments of the carbon signals and, in turn, of the protons H-5 and H-8 were confirmed by the HMBC spectrum which indicated correlations of H-8 with C-9a (δ 124.7), C-6a (δ 132.5) and C-1′ (δ 143.6). Similarly, H-5 was correlated with C-3a (δ 130.0) and C-6a (δ 132.5).

Root cultures of *Anigozanthos preissii* were grown in liquid LS medium[14] (140 ml in 300 ml Erlenmeyer flasks) at 20 °C on a gyratory shaker (100 rpm) under permanent light (600 lux). Three days before administration of the precursors the cultured roots (*ca.* 5 g) were transferred to fresh medium.

[2-^{13}C]Cinnamic acid (99% ^{13}C atom excess) was used to prepare [2-^{13}C]1-phenyl-7-(3,4-dihydroxyphenyl)hepta-1,3-dien-5-one **2** [EI-MS: *m/z* = 295 (M$^+$, 74)] following the procedure of Bazan *et al.*[15] with slight modifications. The

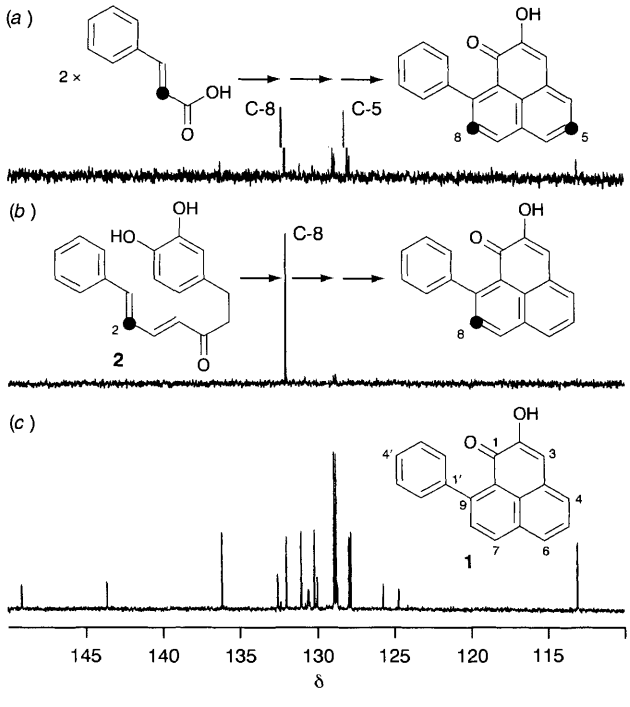

Fig. 1 Proton-decoupled ^{13}C NMR spectra of anigorufone **1**. (*a*) Biosynthesized from [2-^{13}C]cinnamic acid; (*b*) biosynthesized from [2-^{13}C]1-phenyl-7-(3,4-dihydroxyphenyl)]hepta-1,3-dien-5-one **2**; (*c*) non-labelled anigorufone **1**.

structure and the correct position of the label at C-2 were verified by analysis of the crystalline diacetyl derivative of compound **2**, [2-^{13}C]1-phenyl-7-(3,4-diacetoxyphenyl)hepta-1,3-dien-5-one [EI-MS: *m/z* = 379 (M^+, 58); ^{13}C NMR (enhanced signal of C-2, δ 126.6)].

[2-^{13}C]Cinnamic acid (28 μmol) was administered to root cultures as a solution in EtOH (1 ml) through a membrane filter. Anigorufone **1**, isolated from cultured roots of *Anigozanthos preissii* 24 h after administration of [2-^{13}C]cinnamic acid, was subjected to NMR analysis. In comparison with non-labelled **1** [Fig. 1(*c*)] the ^{13}C NMR spectrum (125.70 MHz, [$^2H_6$]acetone) demonstrated two enhanced signals at δ 127.9 and 132.0 corresponding to C-5 and C-8 [Fig. 1(*a*)]. This result clearly proved the biosynthesis of **1** from two C_6–C_3 units. Furthermore, incorporation of label into these positions of **1** is due to the diarylheptanoid pathway hypothesis.[12,13] Incorporation of 7.1% ^{13}C in singly labelled (C-5 or C-8) and 2.3% in doubly labelled molecules of **1** (both C-5 and C-8) was calculated from EI-MS data [*m/z* = 271 (100), 272 (53), 273 (14), 274 (3)].

[2-^{13}C]**2** (32 μmol) was administered to cultured roots under identical conditions. Anigorufone **1** again was isolated from the roots as described above and subjected to NMR analysis. The ^{13}C NMR spectrum of **1** [Fig. 1(*b*)] demonstrated a dramatically enlarged signal at δ 132.0 which corresponded to C-8. This signal unequivocally indicated the utilization of **2** by the cultured roots of *A. preissii* to produce **1**. EI-MS analysis of **1** [*m/z* = 271 (87), 272 (100), 273 (133), 274 (5)] showed 41.4% enrichment of one ^{13}C. No displacement of the label to other positions was observed.

The cyclisation of **2** obviously represents an intramolecular [4 + 2]cycloaddition of the Diels–Alder type. This reaction should give lachnanthocarpone as an intermediate which by subsequent reduction is transformed to anigorufone **1**. As previously reported, compound **2** after periodate oxidation to the 1,2-diketone spontanously resulted in the formation of lachnanthocarpone.[15] This observation may give rise to discussions on the possibility of non-enzymatic character of this transformation step which remains to be studied. The involvement of Diels–Alder reactions in the natural product biosynthesis has been supposed for several metabolic pathways.[16–18]

The extraordinary high rate of incorporation within 24 h demonstrated that compound **2** is a direct precursor of anigorufone **1**. Furthermore, it is conclusive that the cyclisation and the subsequent steps are proceeding very rapidly. Thus, now it is understood why there is no report on the occurrence of diarylheptanoids of the open chain type in *Anigozanthos* and, in general, in the *Haemodoraceae*.

Conclusively, this investigation provides the first experimental evidence for a diarylheptanoid to be an intermediate in the biosynthesis of the phenylpenalenones.

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