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Dihydrocinnamic acids are involved in the biosynthesis of phenylphenalenones in *Anigozanthos preissii*

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Abstract

Feeding experiments using root cultures of *Anigozanthos preissii* and NMR spectroscopical studies revealed reversible interconversion between phenylpropanoids and dihydrophenylpropanoids, and their incorporation into phenylphenalenones. Multiply labelled dihydrocinnamic acid was transformed to coumaric acid and further metabolized to 2-hydroxy-9-(4-hydroxyphenyl)-*1H*-phenalen-1-one (hydroxyanigorufone). ^{13}C NMR spectroscopy and HPLC-^{1}H NMR coupling were employed to detect trace amounts of multiply labelled biosynthetic intermediates and further metabolites. The pathway of the phenylphenalenone biosynthesis is discussed. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Anigozanthos preissii*; Haemodoraceae; Biosynthesis; Diels–Alder reaction; Diarylheptanoids; Dihydrophenylpropanoids; HPLC-NMR; Hydroxyanigorufone; Phenylphenalenones; Phenylpropanoids

1. Introduction

Phenylphenalenones represent a group of plant pigments that were first isolated from several species of the Haemodoraceae plant family, especially the genus *Anigozanthos* (Cooke & Edwards, 1980). Previous biosynthetic studies have demonstrated the incorporation of tyrosine and phenylalanine into the phenylphenalenones (Thomas, 1971; Edwards, Schmitt, & Weiss, 1972; Harmon, Edwards, & Highet, 1977). Root cultures of *Anigozanthos preissii* containing a number of phenylphenalenones (Hölscher & Schneider, 1997) have been employed to prove the utilization of two phenylpropanoic acid units and C-2 of acetate in the synthesis of phenylphenalenone molecule (Hölscher & Schneider, 1995a). Moreover, the highly efficient incorporation of 1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-dien-5-one suggested that phenylphenalenone biosynthesis is related to that of the diarylheptanoids (Hölscher & Schneider, 1995b). However, details of the phenylphenalenone biosynthesis are still hypothetical. Until now, for example, it was uncertain whether the saturated phenylpropanoic moiety in the diarylheptanoid intermediate, 1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-dien-5-one, was incorporated as a dihydrocinnamic acid precursor or, alternatively, originated from a cinnamic acid precursor followed by hydrogenation after condensation with a second cinnamate unit. The fact that dihydrocinnamoyl–CoA was accepted as a substrate by chalcone synthases (CHS) in vitro (Tropf, Lanz, Rensing, Schröder, & Schröder, 1994) was another motivation for the experiments presented here. In this paper we describe in vivo feeding experiments using multiply labelled dihydrophenylpropanoids and how these precursors are involved in phenylphenalenone biosynthesis.

2. Results

In order to study the involvement of dihydrophenylpropanoids in the biosynthesis of phenylphenalenones, [2-^{13}C]dihydrocinnamic acid ([2-^{13}C]-**4**) was adminis-

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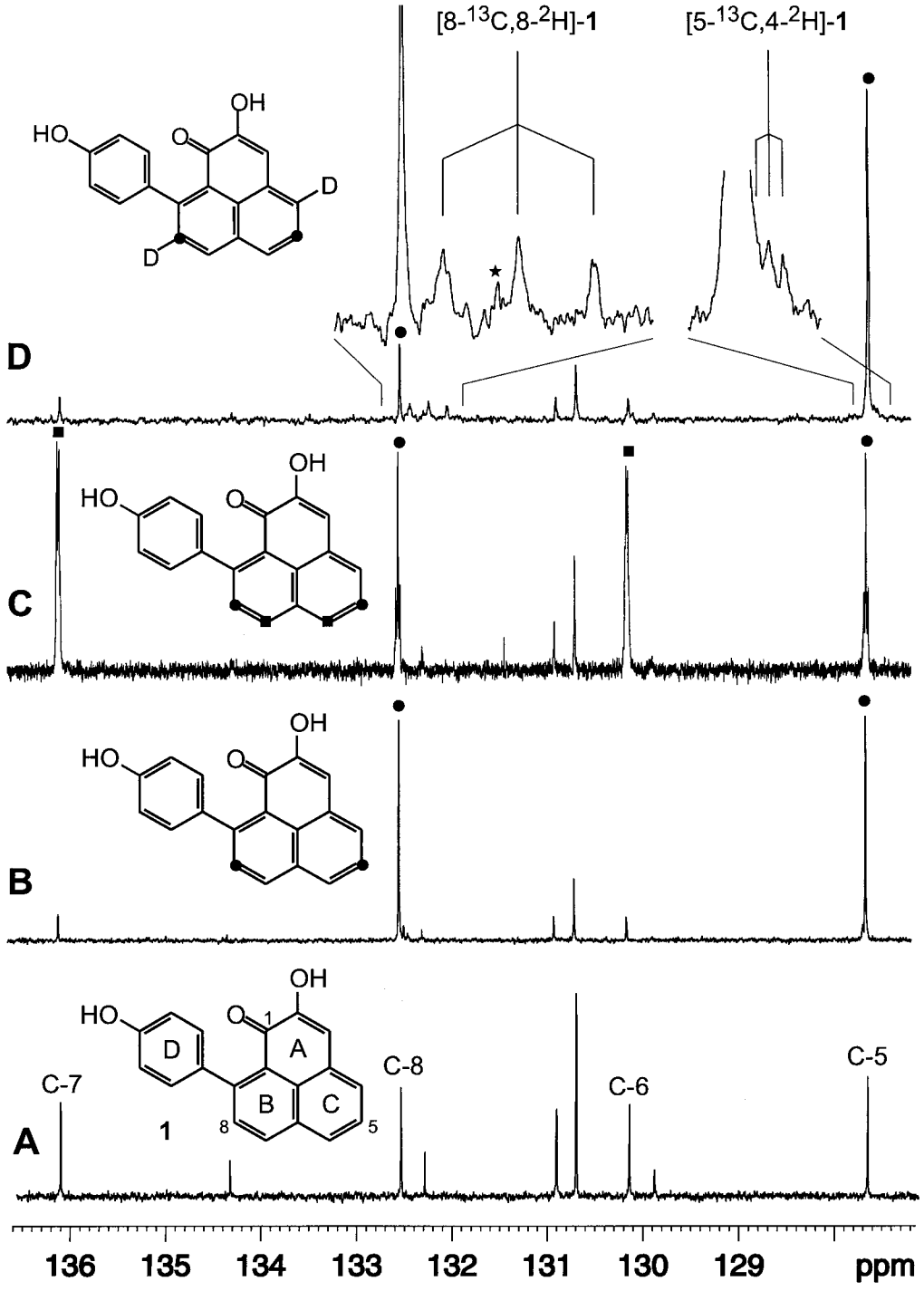

Fig. 1. ^{13}C-NMR spectra of hydroxyanigorufone (**1**) isolated from root cultures of *Anigozanthos preissii*. (a) Nonlabelled reference. (b) After administration of [2-^{13}C]dihydrocinnamic acid ([2-^{13}C]-**4**). Enhanced signals marked by a full circle (●) are due to isotopomers labelled by ^{13}C. (c) After administration of a mixture of [2-^{13}C]-**4** and [1-^{13}C]cinnamic acid ([1-^{13}C]-**3**). Enhanced signals marked by a full circle (●) are due to isotopomers labelled by ^{13}C from [2-^{13}C]-**4**. Enhanced signals marked by a full square (■) are due to isotopomers labelled by ^{13}C from [1-^{13}C]-**3**. (d) After administration of [2-^{13}C,2,2,3–$^{2}H_3$]-**4**. Enhanced signals marked by a full circle (●) are due to isotopomers labelled by ^{13}C. The extensions show an α-shifted triplet of an isotopomer of **1** doubly labelled with deuterium directly bound to ^{13}C-8 ([8-^{13}C,8-^{2}H]-**1**) and a β-shifted triplet of ^{13}C-**5** due to a doubly labelled isotopomer of **1** carrying deuterium at C-4 ([5-^{13}C,4-^{2}H]-**1**). The asterix (★) indicates the natural abundance signal of C-6a of **1**.

tered to intact root cultures of *A. preissii*. After an incubation time of 10 days, hydroxyanigorufone (**1**) was isolated and subjected to ^{13}C NMR spectroscopy. Incorporation of one molecule of dihydrophenylpropa-

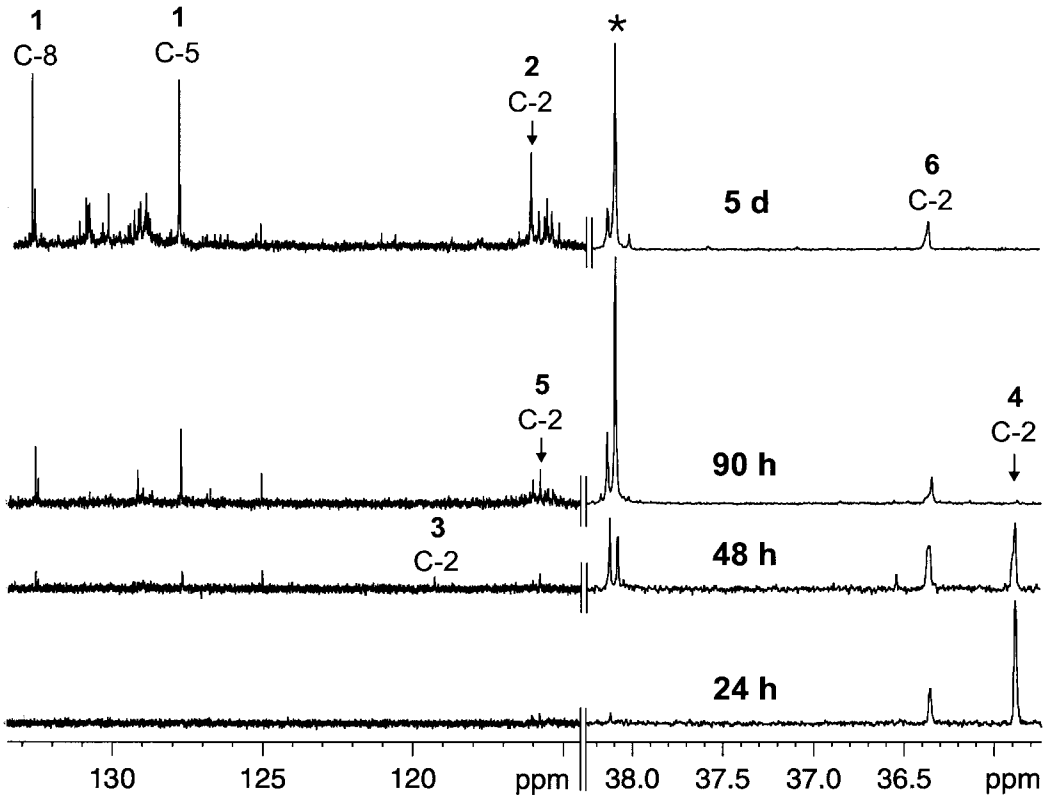

Fig. 2. ^{13}C NMR spectra of CH_2Cl_2 soluble fractions prepared from root cultures of *Anigozanthos preissii* in a time course experiment. The root cultures were incubated with [2-^{13}C]dihydrocinnamic acid ([2-^{13}C]-**4**) for 24 h to 5 days. The flux of the label from [2-^{13}C]-**4** through dihydrocoumaric acid (**6**) or cinnamic acid (**3**) into coumaric acid (**5**) and further into hydroxyanigorufone (**1**) was observed.

noic acid (**4**) into the moiety of a hypothetical diarylheptanoid intermediate forming ring A and C-4 to C-6 of ring C of the phenylphenalenone molecule was expected, with the label ending up in C-5 of hydroxyanigorufone (**1**). Surprisingly, in comparison with the ^{13}C NMR spectrum of the nonlabelled reference (Fig. 1a), the spectrum of **1** (Fig. 1b) exhibited enlarged carbon signals of C-5 and C-8 indicating incorporation of two molecules of the precursor **4**. Three scenarios could be responsible for this finding, each involving condensation of two precursor molecules via a central C-1 unit derived from C-2 of acetate: (1) joining of two [2-^{13}C]dihydrophenylpropanoids, (2) coupling of a [2-^{13}C]dihydrophenylpropanoid with a nonlabelled phenylpropanoid followed by evenly distribution of the label to both halves of the intermediary diarylheptanoid via an additional symmetrical intermediate, and (**3**) coupling of two [2-^{13}C]phenylpropanoid units as indicated by previous feeding experiments (Hölscher & Schneider, 1995a,b). Variant (3) requires conversion of dihydrocinnamic acid (**4**) to cinnamic acid (**3**) prior to incorporation. Moreover, since phenylpropanoids are precursors of dihydrophenylpropanoids, this implies reversible interconversion between both pools of compounds in *A. preissii*.

Another feeding experiment employing a mixture of [1-^{13}C]-**3** and [2-^{13}C]-**4** indicated symmetrical incorporation of both precursors into hydroxyanigorufone (**1**) (Fig. 1c). This is shown by enhancement of the signals of C-5 and C-8, originating from [2-^{13}C]-**4**, as well as C-6 and C-7, coming from [1-^{13}C]-**3**, in the ^{13}C NMR spectrum of **1**. In comparison with C-5 and C-8 the stronger signals of C-6 and C-7 (integral ratio 1:2) revealed a higher degree of incorporation of [1-^{13}C]-**3** than of [2-^{13}C]-**4**. This finding indicates direct incorporation of phenylpropanoid type precursors into phenylphenalenones while dihydrophenylpropanoids are incorporated probably after conversion to phenylpropanoids. The occurrence of isotopomers of **1** formed from both [1-^{13}C]-**3** and [2-^{13}C]-**4** could be demonstrated by analysis of the C–C coupling patterns in the ^{13}C NMR spectrum (Fig. 1c), indicating close relation between both pools of precursors. Additionally, isotopomers singly or doubly labelled from either [2-^{13}C]-**4** or [1-^{13}C]-**3** were found. The pseudo triplet signals of **1** at δ 127.7 and δ 132.5 represented two isotopomers singly labelled at C-5 and C-8, one isotopomer doubly labelled at both of these carbon atoms, and two further isotopomers doubly labelled at C-5/C-7 and C-8/C-6. The coupling constants $^3J_{C\text{-}5\text{–}C\text{-}7}$ and $^3J_{C\text{-}6\text{–}C\text{-}8}$ were 5.3 Hz. The pseudo quintet signals of C-6 (δ 130.1) and C-7 (δ 136.1) were composed of singlets of

Table 1
HPLC–^{1}H NMR data of phenylpropanoids and phenolics from root cultures of *Anigozanthos preissii* (500 MHz, MeCN–D_2O)

H	Coumaric acid 5	Phloretic acid 6	Ferulic acid 2	H	4-Hydroxy-benzoic acid	4-Hydroxy-benzaldehyde	Vanillic acid
2	6.32 d (16.1)	2.60 t (7.3)	6.36 d (16.1)				
3	7.63 d (16.1)	2.80 t (7.3)	7.62 d (16.1)	1	–	9.70 s	–
2′	7.52 d (8.8)	7.10 t (8.8)	7.23 s	2′	7.90 d (8.8)	7.82 d (8.1)	7.56 s
6′	7.52 d (8.8)	7.10 t (8.8)	7.14 d (8.1)	6′	7.90 d (8.8)	7.82 d (8.1)	7.57 d (8.1)
3′	6.87 d (8.8)	6.77 t (8.8)	–	3′	6.91 d (8.8)	6.98 d (8.1)	–
5′	6.87 d (8.8)	6.77 t (8.8)	6.88 d (8.1)	5′	6.91 d (8.8)	6.98 d (8.1)	6.93 d (8.1)
MeO			3.84 s	MeO			3.85 s

singly labelled isotopomers, doublets with vicinal coupling constants $^3J_{C\text{-}6\text{–}C\text{-}8}$ and $^3J_{C\text{-}5\text{–}C\text{-}7}$ of 5.3 Hz, and further doublets due to couplings via two bonds, $^2J_{C\text{-}6\text{–}C\text{-}7} = 2.7$ Hz. The size of this geminal coupling constant of 2.7 Hz was confirmed both for compound **1** and its dimer (3,3′-bis-hydroxyanigorufone (Hölscher & Schneider, 1997) by a separate feeding experiment using [1-^{13}C]-**3** as the only labelled precursor (data not shown).

To gain further insights into the early steps of the phenylpropanoid metabolism in *A. preissii*, a time course experiment was carried out. [2-^{13}C]-**4** was administered to root cultures and portions of the roots were harvested after 24, 48, 90 h and 5 days. Dichloromethane soluble fractions (see Experimental) were subjected to ^{13}C NMR spectroscopy (Fig. 2). The aliphatic part of the spectrum obtained from the 24-h extract exhibited a dominant signal for C-2 of [2-^{13}C]-**4** (δ 35.9) and a minor signal at δ 36.4 owing to the C-2 of [2-^{13}C]phloretic acid (dihydrocoumaric acid, **6**). Dihydrocinnamic acid (**4**) disappeared completely within 90 h whereas the signal of **6** was visible throughout the entire incubation period. The rapid appearance of **6** suggested hydroxylation of **4** directly rather than indirectly via cinnamic (**3**) and coumaric acid (**5**). 4-Hydroxylation of **4** is assumed to be involved in lignan biosynthesis (Neish, 1956) and has been reported to occur in cell cultures of *Dioscoreophyllum cumminsii* (Ushiyama, Kumagai, & Furuya, 1989). However, since cinnamate 4-hydroxylase (CA4H) was shown to be highly substrate specific (Pierrel et al., 1994), more detailed studies are required to provide further evidence for this unusual hydroxylation step. The peaks around δ 38.1 are assumed to represent further labelled intermediates of phenylpropanoid metabolism, but unfortunately could not be assigned to specific compounds. The aromatic parts of the spectra showed tiny C-2 signals of **5** (δ 115.8) and ferulic acid (**2**) (δ 116.0) from which only the latter compound accumulated to substantial concentrations after 5 days. Cinnamic acid (**3**) (δ 119.3) was detectable only in the 48 h extract. Several phenylphenalenones, including **1** (δ 127.7, C-5; δ 132.5, C-8) as the major compound of that type, first appeared in the 24-h spectrum and increased continuously over the entire experimental time period. In summary, this time course experiment indicated the flux of the label from **4**–**6** or **3**–**5** and further into the phenylphenalenones. Thus, indirect incorporation of phenylpropanoids (scenario 3, see above) seems to be the preferred pathway. Although it cannot be finally excluded, conclusive evidence of direct incorporation of dihydrophenylpropanoids into phenylphenalenones could not be obtained in either the time course experiment or after administration of a mixture of [1-^{13}C]-**3** and [2-^{13}C]-**4**.

The occurrence of dihydrophenylpropanoids in many plants (Snook, Csinos, & Chortyk, 1992; Williamson, Obee, & Weidenhamer, 1992; Kraus & Spiteller, 1997) indicates that double bond reduction of cinnamic acid and coumaric acid is a common reaction. In contrast, the reverse conversion of **4** to **3** seems to be rather unusual. An equilibrium between coumaric acid (**5**) and phloretic acid (**6**) has been assumed to be involved in the biosynthesis of mesembrine alkaloids (Jeffs, Karle, & Martin, 1978).

In order to demonstrate the reduction of dihydrophenylpropanoids in *A. preissii* more directly further feeding experiments were carried out. [2-^{13}C]Dihydrocinnamic acid ([2-^{13}C]-**4**) was administered to intact root cultures of *A. preissii* and corresponding extracts were investigated for phenylpropanoids. A fraction of the 90 h CH_2Cl_2 extract, obtained by passing a solution in MeCN: H_2O 1:4 through a RP18 cartridge, was subjected to HPLC analysis. Coumaric acid (**5**, R_t 17.5 min) and ferulic acid (**2**, R_t 19.5 min) were detected by HPLC–^{1}H NMR (Table 1). Moreover, four further compounds were identified as 4-hydroxybenzoic acid (R_t 10.5 min), vanillic acid (R_t 12.0 min), 4-hydroxybenzaldehyde (R_t 14.2 min) and phloretic acid (**6**, R_t 15.1 min). The enhanced satellite signals of H-2 in the HPLC–^{1}H NMR spectra of **5** ($J_{C\text{-}2\text{–}H\text{-}2} = 161$ Hz), **2** ($J_{C\text{-}2\text{–}H\text{-}2} = 162$ Hz) and **6** ($J_{C\text{-}2\text{–}H\text{-}2} = 129$ Hz) clearly indicated ^{13}C label at C-2. The spectrum of 2 is shown in Fig. 3a as an example. The integrals of the signal areas of H-2 attached to ^{12}C (nonlabelled isotopomer) and ^{13}C (labelled isotopomer), respectively, afforded ^{13}C enrichment of about 95% of **6**, 80% of **5** and 25%

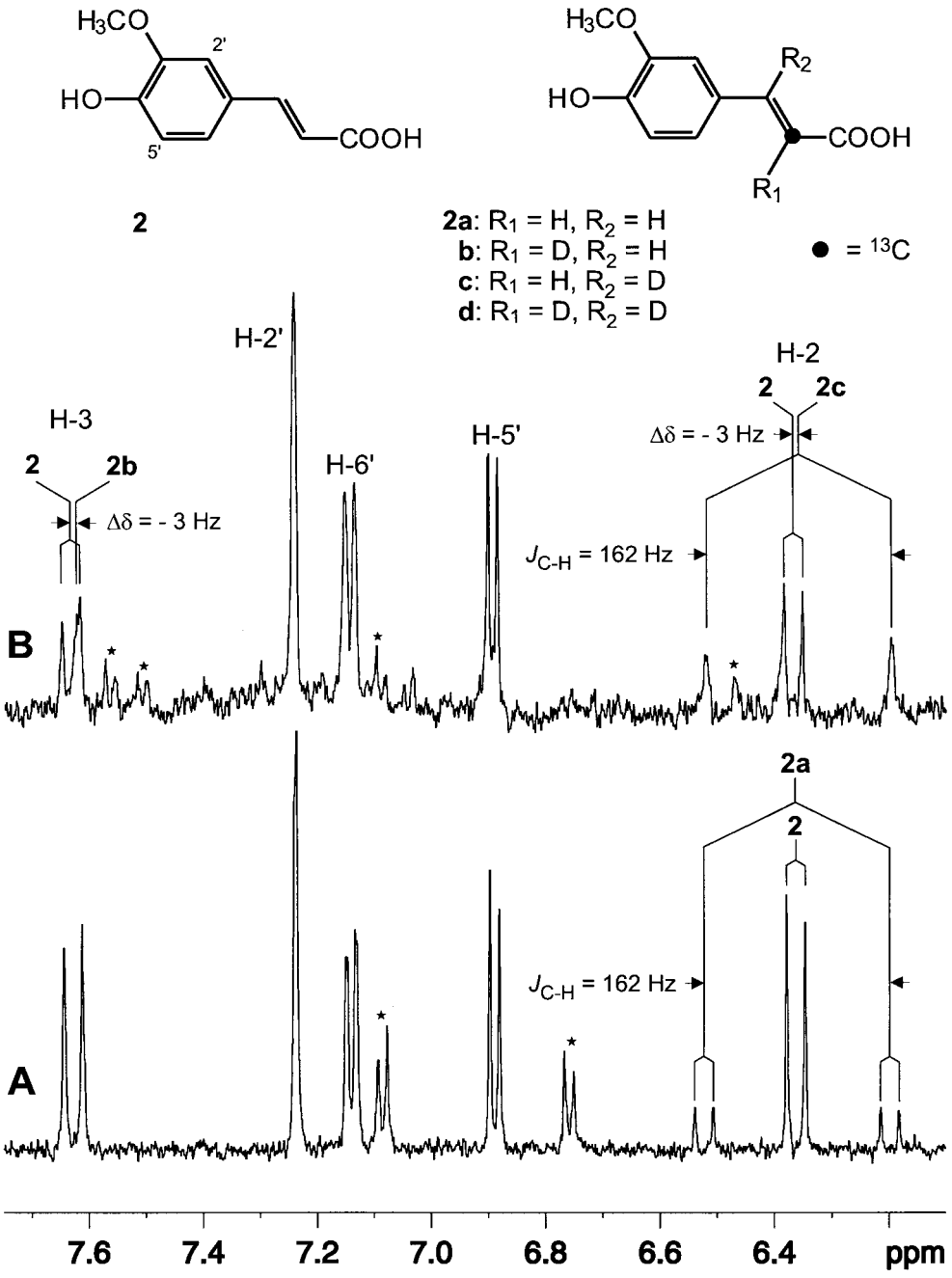

Fig. 3. HPLC–^{1}H NMR spectra of ferulic acid (**2**) isolated from root cultures of *Anigozanthos preissii* upon feeding (a) [2-^{13}C]dihydrocinnamic acid ([2-^{13}C]-**4**) and (b) [2-^{13}C,2,2,3–$^2H_3$]-**4**. The abundance of the individual isotopomers was estimated from integral ratios of spectrum a (76% of **2**; 24% of **2a**) and spectrum b (approximately 40% **2**; 0% **2a**; 25% **2b**; 25% **2c**; 10% **2d**). The asterixes (★) indicate impurities.

of **2**. These data were confirmed by GC–MS of TMS derivatives of **2**, **5** and **6** recorded from the samples immediately collected after HPLC–^{1}H NMR analysis. The decreasing labeling degrees within the sequence of compounds **6** → **5** → caffeic acid (not detected) →**2** were due to the increasing number of conversion steps between the precursor and the analysed metabolite during which mixing with nonlabelled endogenous phenylpropanoids takes place. Moreover, the higher labelling of **6** in comparison with **5** may be considered as an argument for direct 4-hydroxylation of **4**. Cinnamic acid (**3**) and caffeic acid were not found in that analysed fraction. 4-Hydroxybenzaldehyde, 4-hydroxybenzoic acid and vanillic acid might be also derived from phenylpropanoids but did not carry labels due to loss of the side chain. These simple phenolics (and compounds **2**, and **5** as well) occurred also upon administration of cinnamic acid (**3**) and lower concentrations were found in nontreated root cultures.

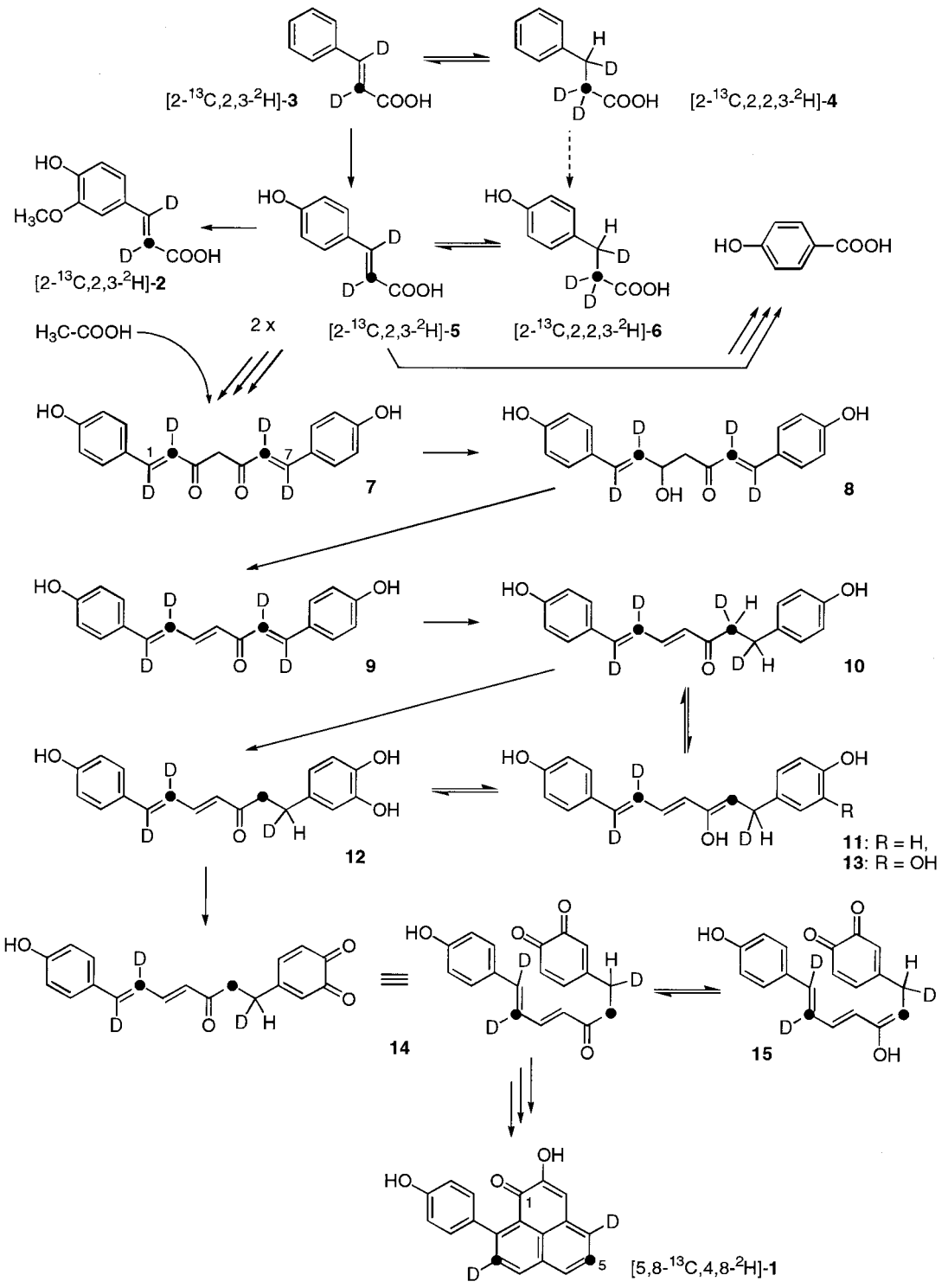

Fig. 4. Proposed pathway of phenylphenalenone biosynthesis as deduced from *in vivo* feeding experiments of labelled [2-^{13}C,2,2,3–^{2}H$_3$]dihydrocinnamic acid ([2-^{13}C,2,2,3–^{2}H$_3$]-**4**) and considering related biogenetic conversions (see Discussion). Intermediates **7**–**11** and **13**–**15** are putative. Incorporation of a synthetic analogue of **12**, 1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-diene-5-one, into phenylphenalenones (anigorufone) has been demonstrated previously (Hölscher & Schneider, 1995).

Administration of [2-^{13}C]phloretic acid ([2-^{13}C]-**6**) afforded similar results. Enrichment of ^{13}C at C-2 of **5** and **2** was detected by ^{13}C NMR of the CH_2Cl_2 extract and, indirectly, by HPLC–^{1}H NMR spectroscopy. Incorporation of ^{13}C into C-5 and C-7 of **1** was found as well (data not shown). This finding indicates that conversion not only occurs between **3** and **4** but seems to be a common reaction also between other members

of both phenylpropanoid and dihydrophenylpropanoid pools.

Further feeding experiments using multiply labelled [2-^{13}C,2,2,3-$^{2}H_3$]-**4** were carried out with intact root cultures to get information on the mechanism of the phenylphenalenone biosynthesis. After 4 days of incubation, enhanced C-2 signals at δ 115.8 and δ 116.0 in the ^{13}C NMR spectrum of the CH_2Cl_2 soluble fraction again confirmed conversion to **5** (R_t 17.5 min) and **2** (R_t 19.5 min). Detailed inspection of the HPLC–^{1}H NMR spectrum of **2** revealed the occurrence of four isotopomers **2** and **2b**–**d** (Fig. 3b). Approximate ratios of these isotopomers were obtained on the basis of the peak integrals. The signals of the aromatic protons of H-2' (δ 7.23, br s), H-5′ (δ 6.88, d) and H-6′ (δ 7.13, br d) were common of all isotopomers (100%). Nonlabelled **2**, best represented by the doublet of H-2 (δ 6.36, $J_{H\text{-}2\text{–}H\text{-}3}$ = 16.1 Hz) made up about 40% of the isotopomeric mixture. About 25% of each monodeuterated isotopomers **2b** and **2c** were found. The signals of **2c** (δ 6.35, $J_{C\text{-}2\text{–}H\text{-}2}$ = 162 Hz) and **2b** (δ 7.62) exhibited a highfield isotopic shift of 3 Hz as compared with nonlabelled ferulic acid (**2**). A double doublet of H-2 ($J_{H\text{-}2\text{–}H\text{-}3}$ = 16 Hz, $J_{C\text{-}2\text{-}H\text{-}2}$ = 162 Hz) of **2a**, which was supposed to have completely lost both deuterium atoms, was missed in the spectrum, indicating that isotopomer **2a** has not been formed in substantial amounts. Finally, the remaining 10% were attributed to isotopomer **2d** which, due to substitution by ^{2}H at C-2 and C-3, did not exhibit ^{1}H signals of the propanoic acid moiety but contributes only to H-2′, H-5′ and H-6′ of the aromatic ring. This isotopomer pattern implies partial loss of ^{2}H both at positions C-2 and C-3. Mass spectral data confirmed incorporation of both ^{13}C and ^{2}H into **2**. Coumaric acid (**5**) was also found although its concentration was insufficient to allow detection of ^{2}H labelling by means of coupling patterns and integral ratios in the HPLC–^{1}H NMR spectrum.

The further fate of intermediates derived from [2-^{13}C,2,2,3-$^{2}H_3$]-**4** was followed by ^{13}C NMR analysis of phenylphenalenones. Hydroxyanigorufone (**1**) obtained after feeding of **4** was significantly labelled by ^{13}C at C-5 and C-8 (Fig. 1d). Thus, a symmetrical intermediate like **7,** which is expected to occur also as keto-enol tautomer (not shown) must be involved in the pathway (Fig. 4). However, in contrast to previous feeding experiments using singly ^{13}C labelled precursors (Hölscher & Schneider, 1995a), the signal of C-8 (δ 132.5) was less intense than that of C-5 (δ 127.7). An isotopomer, carrying ^{2}H at C-8, was indicated by a deuterium induced α-shift of the signal of C-8 appearing as a triplet at δ 132.2. This signal provided explanation of the reduced enhancement of the C-8 singlet as compared with that of C-5. The common occurrence of [8-^{13}C,8–^{2}H]- and [8-^{13}C]-isotopomers of **1** confirmed partial loss of ^{2}H in this position during incorporation of [2-^{13}C,2,2,3–$^{2}H_3$]-**4**.

Although enhancement of the signal of C-5 (δ 127.7) also proved the incorporation of **4** into the A/C ring part of **1**, no ^{2}H was detected at C-5. An explanation could be keto-enol tautomerism of the intermediates **10** and **12**, which completely loose ^{2}H during reversible conversion to **11** and **13**, respectively (Fig. 4).

Determination of the fate of ^{2}H-3 of [2-^{13}C,2,2,3–$^{2}H_3$]-**4** was hindered by the fact that this ^{2}H was not directly bound to ^{13}C. A weak signal close to C-5 ($\Delta\delta = -0.07$ ppm) was interpreted to be due to an isotopomer of **1** carrying ^{2}H attached to C-4 ($J_{C\text{-}5\text{–}D\text{-}4}$ = 4 Hz) (Fig. 1d). This additional C-5 resonance, a consequence of a deuterium induced β-shift, in part was superimposed by the signal of nondeuterated isotopomers. In summary, ^{2}H was partly retained in positions 4 and 8 of hydroxyanigorufone (**1**), while position 5 was not labelled by ^{2}H. This pattern is in agreement with the pathway outlined in Fig. 4.

3. Discussion

As an outcome of the feeding experiments presented here, the reversible interconversion of phenylpropanoic acids into dihydrophenylpropanoic acids and their role in the biosynthesis of phenylphenalenones was clearly demonstrated. Additionally, simple phenolics have been found which are supposed to be derived from phenylpropanoid metabolism. Moreover, stilbenes were also present in *A. preissii* (Hölscher & Schneider, 1996). Thus, three different pathways competing for one substrate, phenylalanine (and cinnamic acid, respectively), could be observed. The significance of these branching pathways affording diverse secondary products is not yet clear. It might be speculated that these various types of natural products play different roles in defence against various pathogens and predators. The reversible interconversion between phenylpropanoids and dihydrophenylpropanoids could contribute to a flexible shift of precursors into one or the other pathway to afford a variable ratio between phenylphenalenones and simple phenolics that can be adapted to environmental requirements.

The common occurrence of related pathways in one species raises a question about the evolution of these pathways. This is particularly the case for the general biogenetic relation between phenylphenalenones and stilbenes. Moreover, a close relation between stilbene and chalcone biosynthesis (the last was not yet found in *Anigozanthos* species) has been discussed for various plants (Schröder, 1997) as they have the first biosynthetic step in common, namely the condensing reaction between 4-coumaroyl–CoA and the first malonyl–CoA. This reaction also seems to be the first step of

phenylphenalenone biosynthesis in the Haemodoraceae. Thus, we would expect the same enzyme family to catalyze the first step of stilbene, chalcone and phenylphenalenone biosynthesis.

Moreover, CHS was described to be coordinately induced with chalcone reductase (CHR) (Welle & Grisebach, 1989). The far-reaching structural similarity between the diketide intermediate of chalcone biosynthesis with that of intermediate **7** (Fig. 4) implies another biogenetic relation between chalcone and phenylphenalenone biosynthesis. In both types of substrates, carbonyl groups in analogous positions are supposed to be reduced by CHR (which was proposed to be referred to more generally as polyketide reductase, PKR (Heller & Forkmann, 1994) followed by removal of the resulting hydroxyl function. The carbonyls to be reduced are, in the chalcone intermediate, that originating from the first acetate condensed to the starter phenylpropanoid and, in phenylphenalenone biosynthesis, one of the carbonyls of the symmetrical intermediate **7** (Fig. 4).

Although not demonstrated experimentally, the oxygen at C-6′ of chalcones is presumably removed during biosynthesis by the way of water elimination. The corresponding reaction of the phenylphenalenone biosynthesis, forming intermediate **9** from **8**, is supposed to operate in an analogous manner. The loss of oxygen at C-3 of intermediate **8** is thought to be the reason that 7-hydroxy-9-phenylphenalenones never have been found in plants.

The reduction of the Δ^6 double bond of intermediate **9** finds its parallel in the formation of dihydrochalcones from the corresponding chalcones.

Compounds of the diarylheptanoid type (e.g. **12**) are committed intermediates of the phenylphenalenone biosynthesis in root cultures of *A. preissii*. 1-Phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-diene-5-one was efficiently incorporated into anigorufone (Hölscher & Schneider, 1995). During this conversion, the catechol moiety of **12** is expected to become readily oxidized to form an orthoquinone type compound like 1-(4-hy-droxyphenyl)-7-(3,4-dioxophenyl)-hepta-1,3-dien-5-one(**14**) (Fig. 4). Conversion of intermediate **10** to the catechol **12** and further to the orthoquinone **14** might be catalysed by a polyphenol oxidase (PPO) having both monophenolase and diphenolase activity. Double enzyme activity of a PPO very recently was also demonstrated in a Rosaceae species (*Pyrus communis*) (Espín, Morales, Varón, Tudela, & Garcia-Cánovas, 1997). Interestingly, phloretic acid (**6**) served as an efficient substrate in the monophenolase assay (Espín, Morales, Varón, Tudela, & Garcia-Cánovas, 1995).

Biomimetically, the orthoquinone formation has been previously demonstrated by periodate oxidation (Bazan, Edwards, & Weiss, 1978). Compound **14** containing both an 1,3-diene and a dienophile, the latter being activated by the adjacent orthoquinone structure, was suggested to be the substrate of an intramolecular Diels–Alder reaction yielding hydroxyanigorufone (**1**). The C-5 to C-7 structural part of the orthoquinone intermediate (probably existing as keto and enol form **14** and **15**, Fig. 4), especially the sp^3-hybridized C-7, readily allows the diene and the dienophile to become arranged in *exo*-orientation and, consequently, to undergo a (4 + 2)-cycloaddition.

In summary, feeding experiments using multiply-labelled precursors of the dihydrophenylpropanoic acid type together with considerations on related biosynthetic reactions led us to a detailed proposal for the early steps of phenylphenalenone biosynthesis. NMR spectroscopic techniques including coupled HPLC–NMR enabled isotopomer analysis of biosynthetic intermediates in the microgram range.

4. Experimental

4.1. Plant material and administration of labelled compounds

Root cultures of *A. 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 (12–15 g) were transferred to fresh medium. Labelled precursors (5 mg in each experiment) were dissolved in EtOH: H_2O 7:3 (1 ml) and administered to intact root cultures through a membrane filter. The incubation times are stated in the Results section.

4.2. Labelled compounds

[1-^{13}C]-3, [2-^{13}C]-3 and [2-^{13}C]-**5** were synthesized by an Erlenmeyer type reaction following standard procedures. [2-^{13}C]-**4** and [2-^{13}C]-**6** were prepared from the corresponding unsaturated compounds by hydrogenation at room temperature using hydrogen gas and Pd–C (10%) as a catalyst in MeOH. For the synthesis of [2-^{13}C,2,2,3–$^{2}H_3$]-**4**, [2-^{13}C,2-$^{2}H_1$]-**3** was first prepared from [2-^{13}C,U–^{2}H]malonic acid by the Erlenmeyer method. Subsequent hydrogenation with deuterium gas using the conditions described above afforded [2-^{13}C,2,2,3–$^{2}H_3$]-4 labelled with >98% ^{2}H in positions 2a and 3, and 70% in position 2b. The ^{13}C atom excess was 99% in each case.

4.3. Extraction of roots and isolation of phenylphenalenones

Roots were frozen with liquid N_2, ground, and extracted with MeOH at room temperature. The resi-

due obtained by evaporation (<40°) of the crude MeOH extract was fractionated by partitioning between *n*-hexane–H_2O and CH_2Cl_2–H_2O. The CH_2Cl_2 fraction was first measured by ^{13}C NMR and then further used either for HPLC–NMR analysis (see below) or subjected to TLC (toluene–Me_2CO 3:2) for isolation of phenylphenalenones (**1**, R_t 0.63 min; bis-hydroxyanigorufone, R_t 0.68 min). Final purification was carried out by reversed-phase HPLC (RP-18, 250 × 4 mm, 5 μm) using a linear gradient MeCN–H_2O (0.1% TFA) from 2:3 to 17:3 in 30 min (**1**, R_t 16 min; bis-hydroxyanigorufone, R_t 23 min). From one root culture (12–15 g fw) about 0.5 mg of pure **1** and about 0.1 mg bis-hydroxyanigorufone was obtained. The analytical data (R_t, ^1H and ^{13}C NMR, MS) exactly matched those of authentic references (Hölscher & Schneider, 1997).

4.4. ^{13}C NMR of isolated compounds and extracts

Bruker DRX 500 NMR spectrometer: 500.13 MHz (^1H), 125.75 MHz (^{13}C), Me_2CO-d_6, TMS as int. standard. A 2.5 mm microprobe was used for all measurements.

4.5. LC-NMR coupling

The CH_2Cl_2 fractions obtained as described above were dissolved in H_2O–MeCN 4:1 and passed through a RP18 cartridge. The efflux was evaporated to dryness and samples of 200 μg dry wt in 100 μl MeCN–D_2O 1:1 were used for stopped-flow HPLC–^1H NMR analysis. HPLC mode: LiChrospher 100 RP18 (250 × 4 mm), 5 μm; MeCN–D_2O (0.1% TFA) from 3:17 to 7:13 in 30 min; UV 280 nm; 0.8 ml min^{-1}. A Merck–Hitachi LiChrograph L-6200A gradient pump was connected to a Bruker DRX 500 NMR spectrometer (4 mm inverse detection LC probe, detection volume 120 μl). ^1H Spectra (500.13 MHz) were measured with a spectral width of 12,000 Hz, and data were acquired into 32 K data points. An acquisition time of 1.36 s and a relaxation delay of 1.80 s were used. Double solvent suppression of MeCN and the residual water in the MeCN–D_2O gradients were performed by presaturation, applying standard Bruker pulse sequences. For calibration, the suppressed signal of MeCN was set to δ 2.0.

4.6. Mass spectrometry

GC–MS measurements of the TMS derivatives were carried out using a Fisons MD-800 instrument. Ionization: EI 70 eV; injector temp.: 250°C; column: DB-5 MS (0.32 × 15m, 0.25 μm film thickness); column temp.: 60°C for 1 min, then elevated to 110°C at 25° min^{-1} and to 290°C at 10° min^{-1}; carrier gas: He, flow rate 1.3 ml min^{-1}. Mass spectra were recorded on a Micromass MasSpec (70 eV).

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