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The biosynthesis of 8-phenylphenalenones from *Eichhornia crassipes* involves a putative aryl migration step

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Abstract

The first compounds of the novel 8-phenylphenalenone type were isolated from roots and leaves of *Eichhornia crassipes* (Pontederiaceae). The structures were elucidated by spectrometric methods including 1D and 2D NMR analysis. The incorporation of two molecules of [1-^{13}C]phenylalanine provides experimental evidence for the biosynthesis of the 8-phenylphenalenones. A biosynthetic pathway via diarylheptanoid and 9-phenylphenalenone intermediates, including a 1,2-aryl migration, is proposed.

Keywords: *Eichhornia crassipes*; Pontederiaceae; Phenylphenalenones; Biosynthesis; Aryl-migration

1. Introduction

Eichhornia crassipes (water hyacinth) is one of the world's most invasive aquatic weeds belonging to the Pontederiaceae plant family, pantropical freshwater aquatics (Heywood, 1993). The occurrence of putative phenylphenalenone-derived phenylphenalenes (Greca et al., 1992) and phenylnaphthalenedicarboxylic acids (Greca et al., 1993) in *E. crassipes* prompted us to reinvestigate this plant with respect to the typical phenylphenalenones, which are polycyclic natural products of the Haemodoraceae (Cooke and Edwards, 1980), Musaceae (Luis et al., 1993; Hirai et al., 1994), and Strelitziaceae (Hölscher and Schneider, 2000). Phenylphenalenones are implicated in the complex defense mechanism of plants and their role as phytoalexins and phytoanticipins in *Musa* is documented (Luis et al., 1995; Binks et al., 1997; Kamo et al., 2000; Otálvaro et al., 2002). As known from biosynthetic studies of phenylphenalenones in Haemodoraceae and Musaceae, the polyphenolic skeleton is formed by condensing two phenylpropanoic acid units (Thomas, 1971; Harmon et al., 1977; Hölscher and Schneider, 1995a; Kamo et al., 2000) and C-2 of acetate or malonate (Edwards et al., 1972; Hölscher and Schneider, 1995b). The involvement of an open-chain type diarylheptanoid (Bazan and Edwards, 1977; Hölscher and Schneider, 1995a) in the biosynthesis of phenylphenalenones and the mode of intramolecular 4 + 2-cycloaddition determines the position of the lateral phenyl ring next to a fusion atom of the phenalenone nucleus. The biosynthetic origin of peripheral oxygens from phenylpropanoid precursor units or oxidative introduction after condensation or cyclization is not yet completely clear. However, their position, particularly of the keto functionality, together with the position of the phenyl ring classifies phenylphenalenones in the major subgroups of 9- and 4-phenylphenalenones and minor subgroups of 7- and 6-phenylphenalenones. The structure

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of emenolone (Luis et al., 1993), originally supposed to be a 5-phenylphenalenone, has been revised to a 9-phenylphenalenone (Luis et al., 1995).

In this paper, the isolation and structure elucidation of two members of a novel type of phenylphenalenones, bearing the lateral phenyl ring in the unusual 8-position, are described. Biosynthetic ^{13}C NMR studies using labelled phenylpropanoid precursors indicated the involvement of an aryl migration in the rearrangement of the phenylphenalenone skeleton.

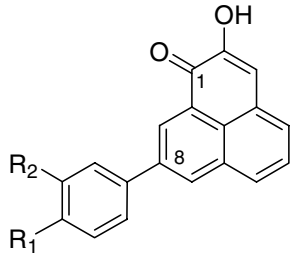
2. Results and discussion

2.1. Isolation and structure elucidation

The *n*-hexane and $CHCl_3$ soluble fractions of extracts from roots and leaves of *E. crassipes* were separated by TLC and the two major components further purified by reversed-phase HPLC. EIMS and HREIMS indicated two compounds **1** and **2** of the molecular mass *m/z* 288 ($C_{19}H_{12}O_3$) and *m/z* 304 ($C_{19}H_{12}O_4$), matching the masses and elemental composition of hydroxyanigorufone and dihydroxyanigorufone from *Anigozanthos* (Cooke and Thomas, 1975), but the characteristic intense $[M + 1]^+$ base peak ion of 9-phenylphenalenones was not visible in the spectra. The corresponding isomeric 4-phenylphenalenones, irenolone (Luis et al., 1993) and 4-(3,4-dihydroxyphenyl)-2-hydroxyphenalen-1-one (Kamo et al., 2001), which are known from *Musa*, differed in their published UV data from the measured spectra of **1** and **2**. Consequently, compounds **1** and **2** were suggested to be new natural products.

In addition to the molecular formula of $C_{19}H_{12}O_3$ of **1**, the ^1H NMR and $^1H,^1H$ COSY spectra also exhibited signals, which are consistent with the structure of an arylphenalenone. A spin system of three adjacent aromatic protons (δ 8.12, 7.82 and 7.68), a singlet at δ 7.22, and a pair of doublets of a two-spin system (δ 8.87 and 8.63), together represent the number of six protons of a substituted phenalenone tricyclus. However, the latter spin system does not show the anticipated $^3J = 8$ Hz coupling of protons, as do, for example, the doublets of adjacent H-7 and H-8 of 9-phenylphenalenones. Instead, a coupling constant of $^4J = 1.9$ Hz was found for these signals, indicating an altered position of the aryl ring at the phenalenone nucleus, presumably at C-8. The doublets at δ 7.79 and 7.06, each integrating for two protons, are typical of a 4-substituted aryl ring. The structure of compound **1** was further clarified by HMBC and HMQC spectra. The HMBC correlation of five protons (δ 8.87, 8.63, 8.12, 7.82 and 7.22) of the phenalenone core structure with the quaternary carbon at δ 123.9 not only defined this carbon as the central C-9b but, together with further HMBC and HMQC cross signals, formed the basis for assigning the position of the aryl substituent and the oxygenated carbons. Three-bond long-range heteronuclear correlations (HMBC) of the singlet of H-3 (δ 7.22) and the most downfield signal in the NMR spectrum H-9 (δ 8.87) with the carbon signal at δ 181.2 defined the position of the carbonyl group (C-1). An additional HMBC cross-peak of H-3 with the downfield carbon signal δ 151.8 indicated a hydroxyl group at C-2. Most important, attachment of the lateral aryl substituent to the phenalenone nucleus to C-8 was confirmed by HMBC cross signals of the doublets of H-9 and H-7 (δ 8.63) with the quaternary C-1′ (δ 131.6). These assignments confirm the suggested structure of this new compound 2-hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one (**1**), representing the first phenylphenalenone with an aryl substituent at position C-8.

The NMR signal assignment of 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one (**2**) (*m/z* 304) followed the same strategy as for compound **1**, except that the 4-hydroxyphenyl-group was substituted by a 3,4-dihydroxyphenyl ring.



1: $R_1 = OH$, $R_2 = H$
2: $R_1 = R_2 = OH$

2.2. Biosynthesis

The 8-phenylphenalenones **1** and **2** described here represent a novel type of phenylphenalenones. In contrast, as a consequence of the biosynthesis described above, "conventional" phenylphenalenones of plant origin bear the lateral phenyl ring without exception next to a fusion carbon at C-9, C-7, C-6 or C-4. However, the position of the lateral aryl substituent at C-8 of new compounds **1** and **2** is inconsistent with the known biosynthetic pathway of phenylphenalenones. The novelty of these natural products and the unusual position of the lateral aryl prompted us to study their biosynthesis by feeding stable labelled precursors to *E. crassipes* plants and analyzing biosynthetically formed metabolites using ^{13}C NMR spectroscopy.

Experimental evidence for the phenylpropanoid origin of 2-hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one (**1**) was obtained from incorporating [1-^{13}C]phenylalanine (Fig. 1.). In comparison with the non-labelled reference compound, the ^{13}C NMR spectrum of **1** isolated from the labelling experiment demonstrated two en-

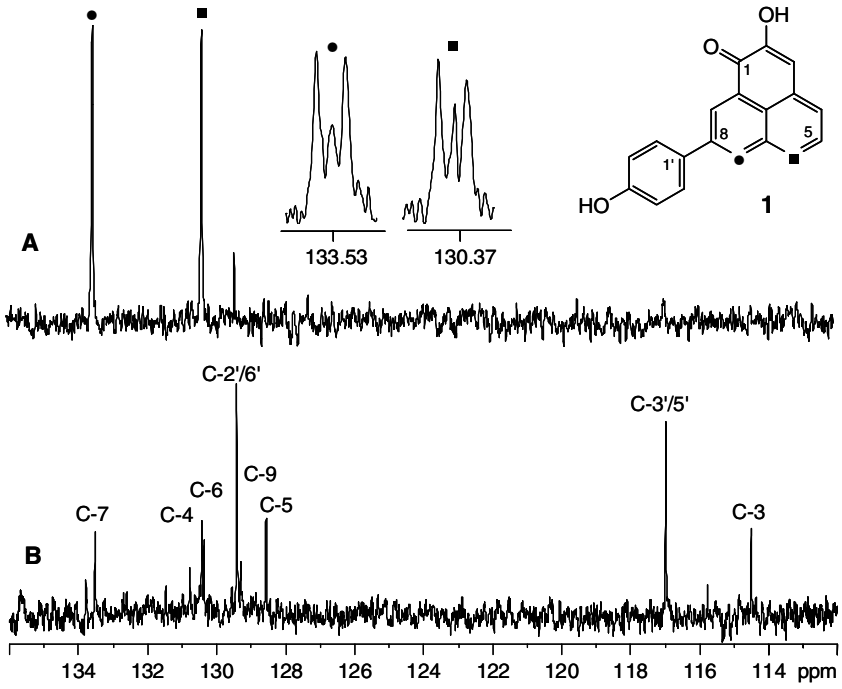


Fig. 1. Partial ^{13}C NMR spectra of the 8-phenylphenalenone **1** from *E. crassipes*. (A) Compound **1** isolated after incorporation of two molecules [1-^{13}C]phenylalanine. ●, ■ = ^{13}C. (B) unlabelled reference.

hanced signals at δ 130.37 and 133.53, corresponding to C-6 and C-7. Close inspection of both signals revealed them as pseudo triplets composed of a doublet and a central singlet (extensions in Fig. 1). The doublets showed a coupling constant of $^{2}J_{C-C}$ = 2.7 Hz, which exactly matched corresponding $^{2}J_{C-C}$ couplings in other phenylphenalenones (Schneider et al., 2003). The C–C coupling clearly confirmed ^{13}C enrichment of C-6 and C-7 of the [6,7-$^{13}C_2$]-isotopomer of **1** and unambiguously discriminates C-6 (δ 130.37) from C-4, which shows a very similar chemical shift value (δ 130.43); however, distance between C-4 and C-7 is too great for coupling to be observed. The singlets at δ 133.53 and 130.37, appearing between the lines of the doublets, indicate the occurrence of single labelled isotopomers [6-^{13}C]**1** and [7-^{13}C]**1**. No displacement of the labels to other positions was observed. Incorporation of 7.6% ^{13}C in singly labelled (C-6 and C-7) and 16.0% in doubly labelled isotopomers of **1** (both C-6 and C-7) was calculated from EI-MS data.

Enrichment of two carbon atoms, C-6 and C-7, of compound **1** from the labelled carboxyl group of phenylalanine is fully consistent with the incorporation of two entire phenylpropanoid units via the diarylheptanoid pathway, except with regard to the position of the lateral aryl substituent. Thus, 1,2-migration from C-9 to the adjacent position C-8 must be the reason for the unusual position of the cinnamate-derived aryl ring.

The involvement of aryl migration in the biosynthesis of the 8-phenylphenalenones seems to resemble the formation of the isoflavonoids from flavonoids. Since isoflavonoids are of scientific and economic interest because of their benefits to human health, phytoestrogenic activity, and the role of isoflavone phytoalexins in legumes, their biosynthesis has been thoroughly investigated. Based on tracer studies (Al-Ani and Dewick, 1984) and enzymatic investigations (Kochs and Grisebach, 1986), a cytochrome P450-catalyzed formation has been proposed (Hakamatsuka and Sankawa, 1993). This oxidative mechanism involves hydrogen abstraction, aryl migration from C-2 to C-3 and 2-hydroxylation. The intermediary 2-hydroxyisoflavanone undergoes dehydration by way of a second enzyme, a dehydratase, to form the $\Delta_{2,3}$-double bond in the isoflavonoid C-ring. Recently, identification of genes encoding isoflavone synthase (presumably 2-hydroxyisoflavone synthase) confirmed P450 proteins as the key enzymes catalyzing the aryl migration of flavonoids (Steele et al., 1999; Jung et al., 2000).

In *E. crassipes*, migration of the aryl substituent to C-8 of phenylphenalenones **1** and **2** may follow a mechanism, which might be similar to isoflavonoid biosynthesis. Hydrogen abstraction at C-8 would result in radical formation followed by aryl migration. Hydroxylation at C-9 and dehydration would give 8-phenylphenalenones such as compounds **1** and **2**. In this hypothetical mechanism, the substrate for 1,2-aryl migration is uncertain.

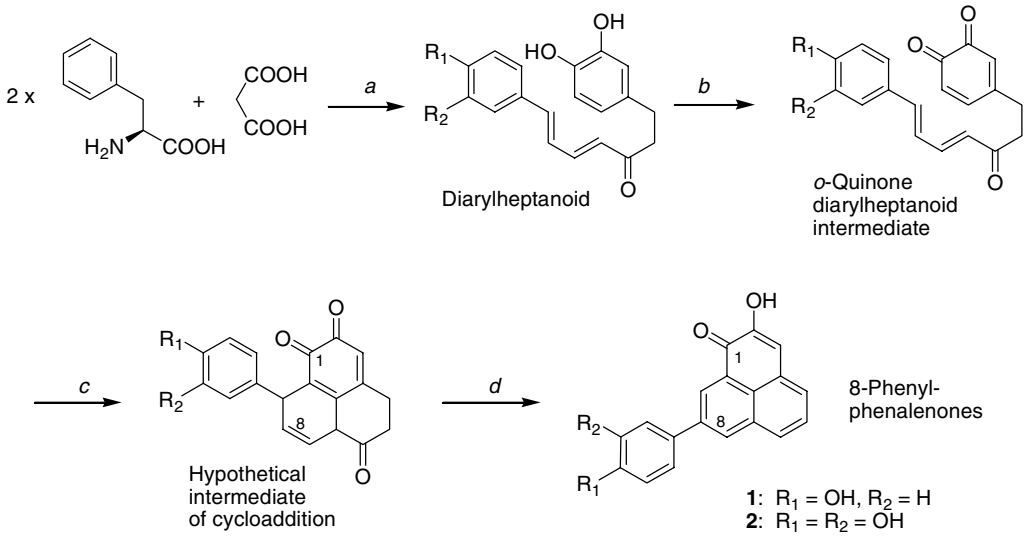


Fig. 2. Hypothetical pathway of 8-phenylphenalenone biosynthesis in *E. crassipes* (Pontederiaceae). (*a*) Diarylheptanoid biosynthesis through the phenylpropanoid pathway. (*b*) Oxidative formation of an *o*-quinone diarylheptanoid. (*c*) intramolecular 4 + 2-cycloaddition. (*d*) 1,2-aryl migration from C-9 to C-8 position (see text for details) followed by reduction and dehydrogenation.

As with the isoflavonoid biosynthesis (Hakamatsuka and Sankawa, 1993), it seems likely that rearrangement occurs immediately after cyclization rather than on the stage of the open-chain diarylheptanoids (Fig. 2). We speculate that a non-aromatic tricyclic intermediate formed by the intramolecular 4 + 2-cycloaddition is the preferred substrate of the hypothetical 8-phenylphenalenone synthase. A triketo intermediate proposed by Bazan and Edwards (1977) (Fig. 2) is considered a suitable precursor of almost all "conventional" phenylphenalenones and might be involved in the formation of the novel 8-phenylphenalenones as well. In order to confirm this hypothesis, further studies are needed to elaborate the mechanism of aryl migration in the course of formation of 8-phenylphenalenones.

3. Experimental

3.1. Plant material

Plants of *E. crassipes* (Mart.) Solms were obtained from the botanical gardens of the universities of Jena and Halle/Saale (Germany), where this species is permanently maintened. The plants were grown in water (2 l in 3 l plastic container) under greenhouse conditions (day 24–26 °C, night 18–21 °C; humidity: 60–70%; the natural photoperiod was supplemented with 16 h illumination from Phillips Sun-T Agro 400 Na lights).

3.2. Isolation and purification

Roots and leaves (870 g fr. wt) of freshly harvested plants were frozen with liquid N_2, ground and exhaustively extracted with Me_2CO at room temperature. The Me_2CO extract was evaporated (<40 °C) and partitioned between *n*-hexane–H_2O and $CHCl_3$–H_2O. The *n*-hexane and $CHCl_3$ fractions were subjected to TLC (silica gel 60 F_{254}: 20 × 20 cm, 0.5 mm layer thickness; solvent toluene–Me_2CO 3:2, v:v). The two major coloured TLC zones (compound **1**: R_f 0.62, compound **2**: R_f 0.38) were scraped off, eluted with Me_2CO, and passed through an RP-18 cartridge (elution with Me_2CO and EtOH). Further purification was achieved by means of semipreparative reversed-phase HPLC on a Nucleosil 100 RP-18 column (10 μm; 250 × 10 mm, UV 284 nm); a linear gradient MeCN–H_2O (0.1% TFA) from 15% to 95% MeCN in 60 min at a flow rate of 3.5 ml min^{-1} was used. Analytical reversed-phase HPLC runs on a LiChrospher 100 RP-18 column (5 μm, 250 × 4 mm; gradient MeCN–H_2O (0.1% TFA) from 15% to 95% MeCN in 60 min at a flow rate of 0.8 ml min^{-1}, PDA detection 200–600 nm; compound **1**: R_t 26.2 min; **2**: R_t 32.5 min) were used for purity checks. Amounts of compounds isolated from *E. crassipes* were as follows **1**: 5.2 mg, **2**: 2.0 mg.

3.3. Spectroscopic methods

NMR spectra were recorded on a Bruker DRX 500 spectrometer at 500.13 MHz for ^1H and 125.75 MHz for ^{13}C using standard Bruker pulse sequences. TMS was used as internal standard. ^1H NMR, ^1H–^1H COSY, 2D NOESY, HMBC, and HMQC experiments were recorded in a 2.5 mm inverse detection microprobe head; broadband decoupled ^{13}C spectra were run using a 2.5 mm broadband microprobe head. Mass spectra were recorded on a MasSpec sector field mass spectrometer (Micromass Ltd., Manchester, UK) with a direct insertion probe. UV spectra were obtained

from a Perkin Elmer UV/VIS Spectrometer Lambda-16 and the IR data were recorded on a Magna-IR Spectrometer 550.

3.4. 2-Hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one (*1*)

Orange solid, UV (MeOH) λ_{max} nm (log ε): 197 (3.7), 276 (1.9), 418 (0.6); IR (KBr) ν_{max} cm^{-1}: 1623, 1208, 838; ^{1}H NMR (acetone-d_6, 500 MHz): δ 8.87 (1H, *d*, *J* = 1.9 Hz, H-9), 8.63 (1H, *d*, *J* = 1.9 Hz, H-7), 8.12 (1H, *d*, *J* = 8.3 Hz, H-6), 7.82 (1H, *d*, *J* = 7.4 Hz, H-4), 7.79 (2H, *d*, *J* = 8.6 Hz, H-2′/6′), 7.68 (1H, *dd*, *J* = 8.3, 7.4 Hz, H-5), 7.22 (1H, *s*, H-3), 7.06 (2H, *d*, *J* = 8.6 Hz, H-3′/5′); ^{13}C NMR (acetone-d_6, 125 MHz): δ 181.2 (C-1), 158.9 (C-4′), 151.8 (C-2), 140.7 (C-8), 133.9 (C-6a), 133.53 (C-7), 131.6 (C-1′), 130.43 (C-4), 130.37 (C-6), 129.6 (C-3a), 129.5 (C-2′/6′), 129.4 (C-9), 128.6 (C-5), 123.9 (C-9b), 117.0 (C-3′/5′), 114.5 (C-3), C-9a not detected; EIMS: *m/z* 289 $[M + 1]^+$ (rel. int. 20), 288 $[M]^+$ (100), 287 (18), 260 (33); HREIMS: *m/z* 288.077776 (Calc. for $C_{19}H_{12}O_3$, 288.078644).

3.5. 2-Hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one (*2*)

Orange solid, UV (MeOH) λ_{max} nm (log ε): 196 (3.8), 275 (2.4), 427 (0.6); IR (KBr) ν_{max} cm^{-1}: 1635, 1208, 836; ^{1}H NMR (acetone-d_6, 500 MHz): δ 8.84 (1H, *d*, *J* = 2.0 Hz, H-9), 8.60 (1H, *d*, *J* = 2.0 Hz, H-7), 8.12 (1H, *d*, *J* = 8.2 Hz, H-6), 7.81 (1H, *d*, *J* = 7.1 Hz, H-4), 7.68 (1H, *dd*, *J* = 8.2, 7.1 Hz, H-5), 7.39 (1H, *d*, *J* = 2.2 Hz, H-2′), 7.28 (1H, *dd*, *J* = 8.3, 2.2 Hz, H-6′), 7.21 (1H, *s*, H-3), 7.03 (1H, *d*, *J* = 8.3 Hz, H-5′); ^{13}C NMR (acetone-d_6, 125 MHz): δ 181.2 (C-1), 151.5 (C-2), 146.9 (C-3′), 146.7 (C-4′), 140.7 (C-8), 134.0 (C-6a), 133.6 (C-7), 132.3 (C-1′), 130.4 (C-4), 130.4 (C-6), 129.8 (C-3a), 129.4 (C-9), 128.5 (C-5), 124.0 (C-9b), 121.4 (C-6′), 117.0 (C-5′), 115.4 (C-2′), 114.7 (C-3), C-9a not detected; EIMS: *m/z* 304 $[M]^+$ (rel. int. 100), 276 (36); HREIMS: *m/z* 304.075283 (Calc. for $C_{19}H_{12}O_4$, 304.073559).

3.6. Application of labelled precursor

For feeding experiments, small plants of *E. crassipes* (about 80 g) were selected and transferred to fresh water (100 ml) in a 200 ml-Erlenmeyer flask one day before administering the precursor. [1-^{13}C]Phenylalanine (340 μM) (5.6 mg; 99% ^{13}C; Deutero GmbH Kastellaun) was dissolved in water (1 ml), and added to the flask containing the plant. During the experiment the flask was moved on a gyratory shaker (200 rpm). 2-Hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one (**1**) was isolated 4 d after administering [1-^{13}C]phenylalanine as described above and subjected to ^{13}C NMR analysis (Fig. 1) and EI-MS: *m/z* 289 $[M + 1]^+$ (30), 288 $[M]^+$ (100).

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