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Oxidative biosynthesis of phenylbenzoisochromenones from phenylphenalenones

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Dedicated to Professor Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

^{13}C NMR analysis demonstrated incorporation of two ^{13}C labelled phenylalanine units into phenylphenalenones and phenylbenzoisochromenones co-occurring in *Wachendorfia thyrsiflora*. These results suggest oxidative formation of phenylbenzoisochromenones following a late branching from a common phenylphenalenone biosynthetic pathway. A dioxygenase-type mechanism, followed by decarboxylation, is suggested for the key steps of this conversion.

Keywords: *Wachendorfia thyrsiflora*; Haemodoraceae; Biosynthesis; ^{13}C NMR; Oxidative rearrangement; Phenylbenzoisochromenones; Phenylphenalenones

1. Introduction

Phenylphenalenones, which are produced by Haemodoraceae (Cooke and Edwards, 1981; Hölscher and Schneider, 1997) and Musaceae (Luis et al., 1995; Kamo et al., 1998), are accompanied in some of these plants by a number of related natural products, such as oxabenzochrysenones (naphthoxanthenones) (Cooke and Dagley, 1979; Opitz et al., 2002a), naphthalic anhydrides (Cooke and Thomas, 1975; Bazan and Edwards, 1976), phenylbenzoisochromenones (phenylnaphthalides) (Cooke, 1970; Opitz et al., 2002b), and phenylbenzoisoquinolinones (lachnanthopyridones) (Edwards and Weiss, 1972). The American species *Lachnanthes tinctoria* and *Xiphidium caeruleum* are especially rich in aza- and oxa analogues of phenylphenalenones (Bazan and Edwards, 1976; Opitz et al., 2002b). In the first part of this work we report on the co-occurrence of phenylphenalenones and phenylbenzoisochromenones in *Wachendorfia thyrsiflora*, a South African member of the Haemodoraceae.

The biosynthetic origin of the carbon skeleton of phenylphenalenones from two phenylpropanoid units and C-2 of acetate has been established previously (Harmon et al., 1977; Hölscher and Schneider, 1995a) and the involvement of open-chain diarylheptanoid intermediates was demonstrated (Hölscher and Schneider, 1995b). Oxa analogues of phenylphenalenones have been considered oxidation products derived from structurally related phenylphenalenones (Edwards and Weiss, 1974). This hypothesis has been confirmed experimentally for 2-(4′-hydroxyphenyl)-1,8-naphthalic anhydride, carrying the anhydride moiety in ring A, in *Musa acuminata* (Kamo et al., 2000). A biosynthetic route of oxidative formation of the oxalactone structure in ring B has been proposed (Edwards and Weiss, 1974; Opitz et al., 2002b). However, from the data hitherto available, a hypothetical alternative biosynthesis of phenylbenzoisochromenones through a Diels-Alder type cycloaddition of an ester intermediate (1,7-diaryl-2-oxa-heptanoid) cannot be ruled out. ^{13}C NMR analysis of phenylphenalenones and phenylbenzoisochromenones obtained from phenylalanine feeding experiments described in this work were used to investigate whether the oxidative pathway from phenylphenalenones or the hypothetical route from an ester intermediate is operating in *W. thyrsiflora*.

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2. Results and discussion

2.1. Phenylphenalenones and phenylbenzoisochromenones from W. thyrsiflora

Phenylphenalenones have been previously isolated from *W. thyrsiflora* (Edwards, 1974) and 5-methoxy-6-oxa-benzo[*def*]chrysen-1-one, a natural product related to phenylphenalenones, recently has been isolated from root cultures of this species (Opitz et al., 2002a). We now report the identification of two phenylphenalenones, anigorufone (**1**), 6-*O*-[(6″-*O*-allophanyl)-β-D-glucopyranosyl]-2,5-dihydroxy-7-phenylphenalen-1-one (**2**), and two phenylbenzoisochromenones, 5-hydroxy-3,6-dimethoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**3**), and 6-*O*-[(6″-*O*-allophanyl)-β-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**4**) from the same source (Fig. 1). Anigorufone (**1**) represents the most simple phenylphenalenone and was first isolated from *Anigozanthos rufus* (Cooke and Thomas, 1975). The allophanylglucosides **2** and **4** have been reported from *Xiphidium caeruleum* (Opitz et al., 2002b). Analytical data of compounds **1**, **2**, and **4** exactly matched those of authentical references. Although the *O*-methyl group at C-3 of the phenylbenzoisochromenone **3** is an artifact of the extraction procedure (Opitz et al., 2002b), it has no effect on the biosynthetic investigations presented here. NMR chemical shifts of **3** (see Experimental) were assigned following the strategy described for other phenylbenzoisochromenones (Opitz et al., 2002b).

2.2. Biosynthesis

Known details about the biosynthesis of phenylphenalenones are summarized in Fig. 2A. Cinnamic and *p*-coumaric acid, derived from phenylalanine, condense with one molecule of acetyl- (or malonyl-)SCoA ester and after a number of further steps (Hölscher and Schneider, 1995a; Schmitt and Schneider, 1999), give a diarylheptanoid, 1-phenyl-7-(3,4-dihydroxyphenyl)-hepta-1,3-dien-5-one (**5**), capable of forming a phenylphenalenone by cyclisation (Hölscher and Schneider, 1995b). The primary cyclisation product is still unknown. However, taking into account that only a single oxygen atom of the former carboxyl groups, which have been involved in condensation, is retained in the diarylhepta-1,3-dien-5-one **5**, a cyclic compound of the same oxidation state in the part of the molecule derived from the C-7 chain, such as compound **6**, is a promising candidate. This compound **6**, trivial name lachnanthocarpone, is known from *L. tinctoria* (Edwards and Weiss, 1970) and *W. thyrsiflora* (Edwards, 1974). Reduction of the hydroxyl at C-6 of compound **6** would result in anigorufone (**1**). Hydroxylation of **6** at C-2 and tautomerization would give **7** (the aglycon of **2**), and after oxidative rearrangement including decarboxylation, compounds of the phenylbenzoisochromenone-type, which may be further modified at C-3, C-5, and C-6.

A shorter route through cyclization of an oxa-diarylheptanoid **8** (Fig. 2B) would not require phenylphenalenone intermediates such as **6** and **7** and oxidative rearrangement, but would result directly in phenyl-

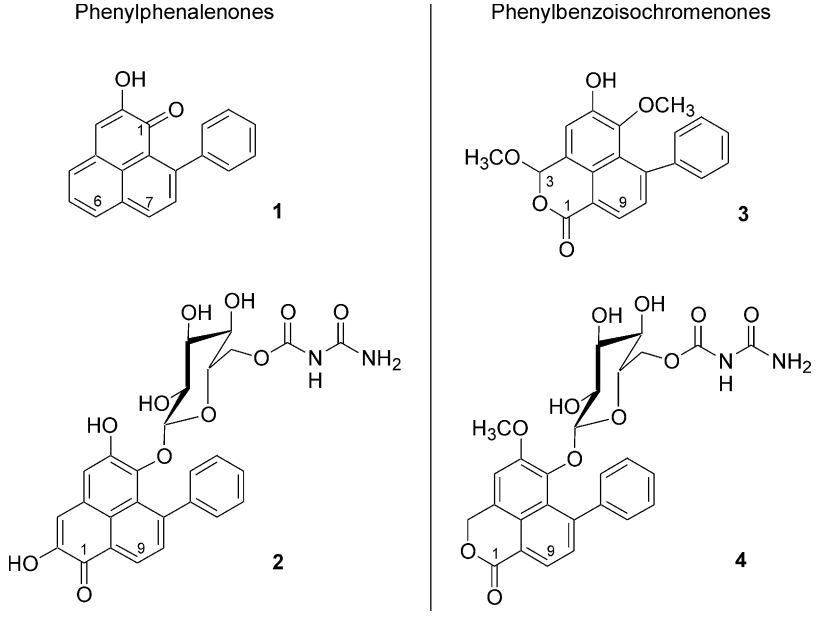

Fig. 1. Structures of compounds from root cultures of *Wachendorfia thyrsiflora* analyzed for incorporation of [1-^{13}C]phenylalanine: anigorufone (**1**), 6-*O*-[(6″-*O*-allophanyl)-β-D-glucopyranosyl]-2,5-dihydroxy-7-phenylphenalen-1-one (**2**), 5-hydroxy-3,6-dimethoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**3**), and 6-*O*-[(6″-*O*-allophanyl)-β-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**4**).

benzoisochromenones. The ester intermediate **8** could be formed from a condensation product of cinnamoyl- and malonyl-SCoA with *p*-hydroxybenzyl alcohol. The biosynthetic precursor of *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, has been found in many plants including a Haemodoraceae, *Anigozanthos preissii* (Schmitt and Schneider, 2001).

The hypothetical pathways discussed here can be discriminated by feeding experiments as phenylalanine would be anticipated to be incorporated twice into phenylbenzoisochromenones through the diarylheptanoid pathway (Fig. 2A) but only once through the "ester pathway" (Fig. 2B). As demonstrated previously, the labelled carboxyl carbon atom of [1-^{13}C]phenylpropanoids appears at C-6 and C-7 of anigorufone (**1**) and other phenylphenalenones. In the phenylbenzoisochromenones formed through the diarylheptanoid route (Fig. 2A), the label of [1-^{13}C]phenylalanine would be expected to be incorporated into both C-1 and C-9. In contrast, incorporation of only one label (C-9) would be expected in phenylbenzoisochromenones synthesized via the hypothetical "ester pathway" (Fig. 2B) through loss of a C-2 unit including the labelled carboxyl group from one of the phenylpropanoids. The latter conversion of phenylpropanoic acids through benzaldehyde would lead to *p*-hydroxybenzyl alcohol (Abd El-Mawla and Beerhues, 2002).

The results of feeding [1-^{13}C]phenylalanine to *W. thyrsiflora* clearly indicated that the phenylbenzoisochromenones are formed through the diarylheptanoid pathway (Fig. 2A). This was demonstrated by ^{13}C signals of C-1 and C-9 in compounds **3** (C-1: δ 166.3, C-9: δ 128.7) and **4** (C-1: δ 166.7, C-9: δ 127.6), which were enhanced compared to those in spectra of unlabelled reference compounds. The spectra of biosynthetically labelled and unlabelled compound **4** are depicted in Fig. 3 as an example. Due to the long relaxation properties of the lactone carbon atom C-1 of phenylbenzoisochromenones, the corresponding signal appears less intense in comparison with that of the methine carbon

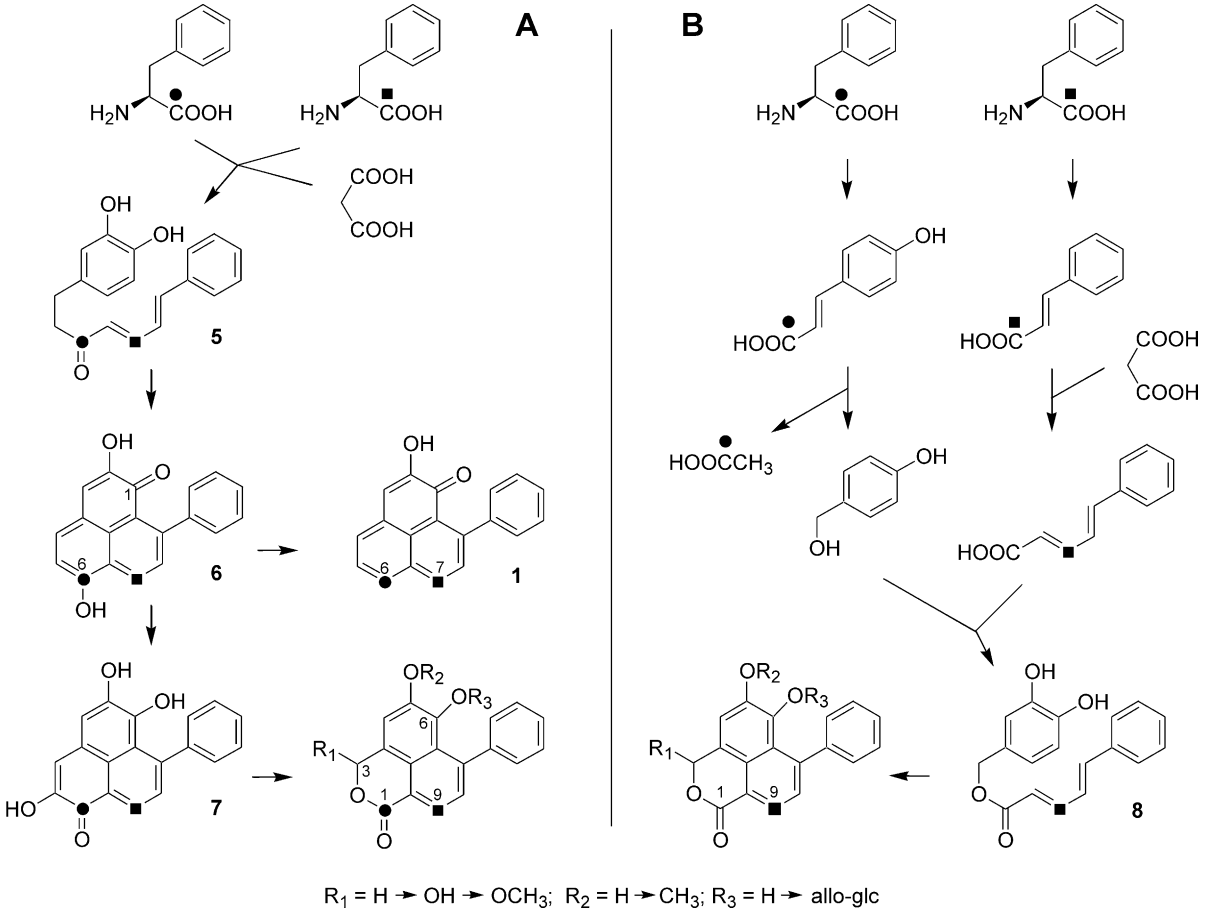

Fig. 2. Two hypothetical pathways of phenylbenzoisochromenone biosynthesis: (A) From phenylalanine via intramolecular cycloaddition of diarylheptanoid **5** and subsequent oxidative rearrangement. This route represents a branch of the phenylphenalenone pathway. (B) From phenylalanine via intramolecular cycloaddition of an oxa-analogue diarylheptanoid ("ester intermediate") **8**. ●, ■ = ^{13}C.

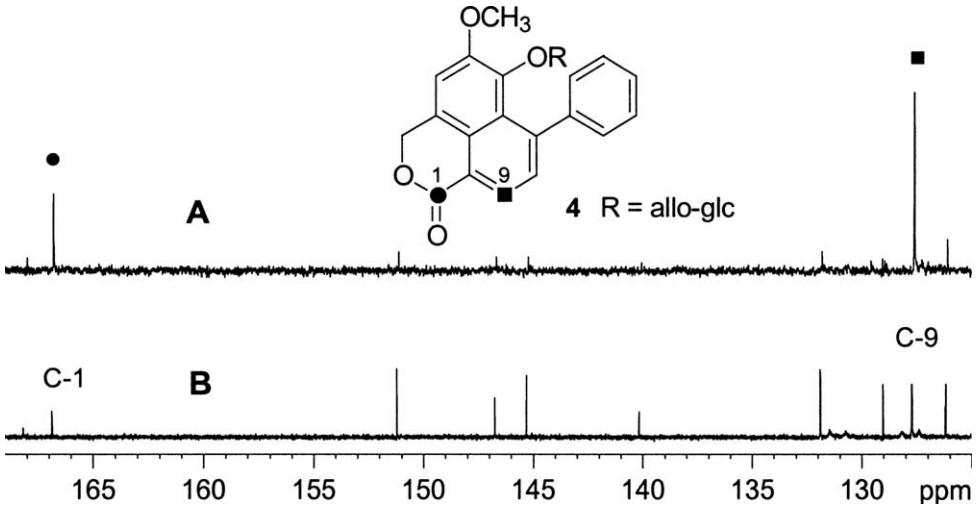

Fig. 3. Partial ^{13}C NMR spectra (MeOH-d_4) of the phenylbenzoisochromenone glucoside **4** from *Wachendorfia thyrsiflora* isolated (A) after incorporation of [1-^{13}C]phenylalanine and (B) unlabelled reference. ●, ■ = ^{13}C.

C-9. In the spectrum obtained after [1-^{13}C]phenylalanine feeding, both resonances of C-1 and C-9 showed approximately equal enhancement (compounds **3**: 8%/10% and **4**: 13%/12%) when compared with the spectrum of the unlabelled compound. The same was found for phenylphenalenones (compound **1**: C-6, δ 130.2, 18%/C-7, δ 136.2, 18% and **2**: C-1, δ 182.2, 11%/C-9, δ 128.6, 11%), confirming previous feeding experiments with phenylpropanoic acids (Hölscher and Schneider, 1995a,b; Schmitt and Schneider, 1999). From the equal incorporation rates into both positions, reincorporation of [1-^{13}C]acetate, derived from side chain degradation in the hypothetical ester pathway, can be ruled out. Jasmonic acid, which was added to the culture medium, stimulated the formation of compounds under study, especially anigorufone (**1**) (data not shown) but did not influence significantly the level or alter the ratio of ^{13}C of both labelled positions.

A hypothetical dioxygenase-type mechanism (Jefford and Cadby, 1981) from lachnanthocarpone (**6**) to the phenylbenzoisochromenones is shown in Fig. 4. Initial binding of O_2 at C-2, followed by a four-membered cyclic transition state (**9**) would result in the ring-opened intermediate (**10**). At this stage, the oxalactone (**11**) could be formed, containing the former endocyclic C-2 as exocyclic carboxyl functionality. A natural product of that type, a 7-allophanylglucoside of 3-carboxy-5,6-dihydroxy-7-phenylbenzoisochromenone (**11**), recently was isolated from *X. caerulem* (Opitz et al., 2002b). Decarboxylation at C-3 would give phenylbenzoisochromenones such as compound **12**, representing the 5-*O*-demethyl aglycone of **4**, which may undergo hydroxylation at the formerly carboxylated position C-3 to result in 3,5,6-trihydroxylated 7-phenylbenzoisochromenones.

3. Experimental

3.1. Plant material and administration of [1-^{13}C]phenylalanine

Root cultures of *W. thyrsiflora* were established from seeds as described for other in vitro cultures of a Haemodoraceae (Hölscher and Schneider, 1997). The sterile cultures were grown in liquid M3 (Murashige and Skoog, 1962) medium (100 ml) in conical flasks (volume 300 ml) on a gyratory shaker (85 rpm) at 23 °C under

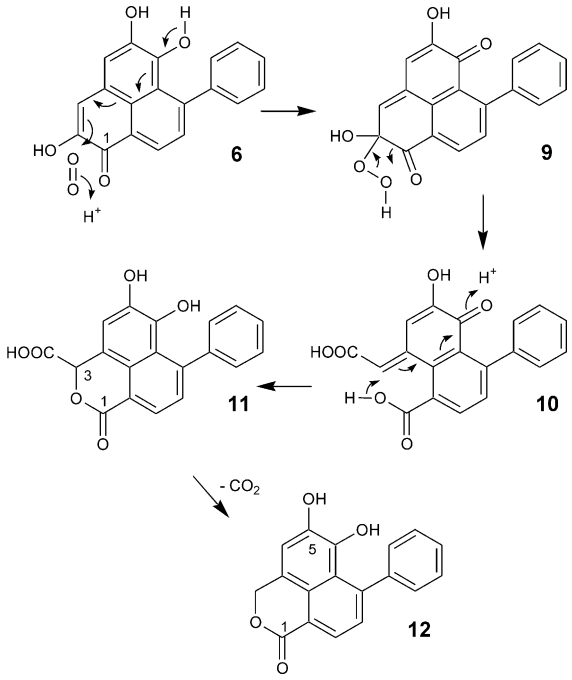

Fig. 4. Hypothetical dioxygenase-catalyzed oxidative rearrangement of phenylphenalenones to phenylbenzoisochromenones with decarboxylation as the final step.

permanent diffuse light (4.4 μmol m^{-2} s^{-1}). Root cultures with and without application of jasmonic acid (JA) (50 μM final concentration) were used for identification of compounds **1–4**. For feeding experiments, the roots (approximately 10 g fresh weight) were transferred to fresh medium two days prior to administration of the labelled compound. [1-^{13}C]Phenylalanine (5 mg, 99% ^{13}C, Cambridge Isotope Laboratories) was dissolved in H_2O (1 ml) and added to the root culture through a membrane filter to a final concentration of 290 μM and incubated for 5 days. JA (50 μM final concentration) was added to the medium simultaneously.

3.2. Isolation of metabolites

Cultured roots were separated from the medium, frozen in liquid N_2, ground, and extracted with MeOH at room temperature. After evaporation (<40 °C) the extracts were partitioned between *n*-hexane–H_2O, CH_2Cl_2–H_2O and EtOAc–H_2O. Compounds **1–4** were isolated by means of reversed-phase HPLC (flow rate 3.5 ml min^{-1}; DAD 200–600 nm) on a LiChrospher 100 RP18 column (10 μm; 250×10 mm). MeCN–H_2O gradient (a) (3:7→13:7 over 30 min, then 9:1 over another 5 min) was used for the CH_2Cl_2 fraction and (b) (1:19→1:1 over 40 min) for the EtOAc fraction. Anigorufone (**1**) (R_t 31.8 min, gradient (a) and 5-hydroxy-3,6-dimethoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**3**) (R_t 26.0 min, gradient a) were isolated from the CH_2Cl_2 fraction, 6-*O*-[(6″-*O*-allophanyl)-β-D-glucopyranosyl]-2,5-dihydroxy-7-phenylphenalen-1-one (**2**) (R_t 25.9 min, gradient b) and 6-*O*-[(6″-*O*-allophanyl)-β-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**4**) (R_t 26.6 min, gradient (b) from the EtOAc fraction.

3.3. Spectroscopic methods

NMR spectra were measured on a Bruker DRX 500 NMR spectrometer, operating at 500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C. Chemical shifts are given in δ values referring to TMS as internal standard. ^{1}H NMR, ^{1}H–^{1}H COSY, HMQC, and HMBC 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. Electron impact mass spectra (EI–MS) and high-resolution spectra (HR-EI–MS) were recorded on a MasSpec sector field mass spectrometer (Micromass Ltd., Manchester, UK) with a direct insertion probe. UV spectra were obtained from an Agilent G1315B diode array detector during analytical HPLC in MeCN–H_2O.

3.4. Analytical data

5-Hydroxy-3,6-dimethoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**3**). UV (MeCN–H_2O) λ_{max} 266, 340, 386 nm; ^{1}H NMR (MeOH-d_4, 500 MHz) δ 8.22 (1H, *d*, J=7.4 Hz, H-9), 7.58 (1H, *s*, H-4), 7.32 (1H, *d*, J=7.4 Hz, H-8), 7.34 (5H, *m*, phenyl protons), 6.47 (1H, *s*, H-3), 3.96 (3H, *s*, 6-OCH_3), 3.67 (3H, *s*, 3-OCH_3); ^{13}C NMR (MeOH-d_4, 125 MHz) δ 166.3 (C-1), 147.2 (C-7), 145.2 (C-6), 145.0 (C-5), 144.7 (C-1′), 130.5 (C-2′/6′), 129.9 (C-8), 128.7 (C-9), 128.0 (C-3′/5′), 127.8 (C-4′), 125.6 (C-9b), 122.0 (C-9a), 119.3 and 119.4 (C-3a and C-6a), 115.4 (C-4), 103.9 (C-3), 57.8 (6-OCH_3), 56.4 (3-OCH_3); EIMS *m*/*z* 336 $[M]^+$ (50), 305 (100), 289 (30); HREIMS *m*/*z* 336.0998 (calc. for $C_{20}H_{16}O_5$, 336.0998). Analytical data (^{1}H NMR, MS, UV) of anigorufone (**1**) and glucosides **2** and **4** exactly matched those of reference compounds previously isolated from *A. preissii* (Hölscher and Schneider, 1997) and *X. caeruleum* (Opitz et al., 2002b).

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