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Differentiation-dependent levels of benzofuran-type resveratrol dimers in root cultures of *Anigozanthos preissii*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

The level of secondary compounds formed by sterile root cultures of *Anigozanthos preissii* depends on the differentiation state. Cultures showing shoot formation and accelerated growth are depleted in stilbenes, stilbene glucosides, and phenylphenalenones. Three glucosides of anigopreissin A, a benzofuran-type resveratrol dimer, were isolated from slow-growing cultures and their structures elucidated by spectrometric methods.

Keywords: *Anigozanthos preissii*; Haemodoraceae; Benzofurans; Dimers; Glucosides; Root cultures; Stilbenes

1. Introduction

Root cultures of *Anigozanthos preissii* (Haemodoraceae) are a rich source of phenylphenalenones (Hölscher and Schneider, 1997) and have been used in biosynthetic studies (Hölscher and Schneider, 1995a,b; Schmitt and Schneider, 1999; Schmitt et al., 2000). In addition to phenylphenalenones, anigopreissin A, a resveratrol dimer (Hölscher and Schneider, 1996), and simple phenylpropanoids and phenolics (Schmitt and Schneider, 2001) have been found to be produced by this culture. An obstacle in these studies has been the slow growth rate of the cultures leading to their limited availability. However, during the cultivation period, some root cultures spontaneously started differentiation. These cultures developed shoots and simultaneously showed accelerated growth rate. In order to examine the suitability of the faster growing differentiated cultures for biosynthetic studies, a comparative phytochemical study of cultures showing normal and accelerated growth was undertaken with special emphasis on stilbenoids.

2. Results and discussion

A root culture line of *Anigozanthos preissii*, propagated from one parent culture, was used in this study. The differentially developing cultures of type S (slow growth) and A (accelerated growth, shoot development) were analyzed by reversed-phase HPLC. The crude MeOH extracts and the *n*-hexane, CH_2Cl_2 and ethyl acetate fractions of cultures of both types S and A were compared. While the crude MeOH extract of type S cultures, which was analyzed using gradient I (see Experimental), exhibited the previously observed pattern of phenylphenalenones (Hölscher and Schneider, 1997) and a dominant peak of anigopreissin A (**1**) (Hölscher and Schneider, 1996), the peak of the latter compound largely dominated the extract of type A cultures. Based on the peak height, the ratio of anigopreissin A (**1**) in cultures of the type S:type A was 6:1. HPLC analyses of the CH_2Cl_2 (gradient II, see Experimental) and ethyl acetate fractions (gradient I) of cultures of type A resulted in a similar pattern with anigopreissin A (**1**) being the dominant peak with only trace amounts of other compounds detected in these fractions. The *n*-hexane extract did not show substantial absorption at the detection wavelength of 254 nm (gradient III, see Experimental). This finding indicated that the level of secondary compounds formed by sterile root

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cultures of *Anigozanthos preissii* depends on the differentiation status. Cultures showing shoot formation and accelerated growth are depleted in phenylphenalenones and significantly reduced in stilbenes. This change in biosynthetic activity of *Anigozanthos* root cultures is assumed to be due to reduced metabolic flux from primary into secondary metabolism during shoot development.

HPLC analyses of the *n*-hexane (gradient III), CH_2Cl_2 (gradient II) and ethyl acetate fractions (gradient I) of type S cultures resulted in the detection of known phenylphenalenones (anigorufone, hydroxyanigorufone, dihydroxyanigorufone) and the resveratrol dimer, anigopreissin A (**1**). Inspection of the major peaks of the ethyl acetate fraction revealed characteristic UV spectra closely resembling that of anigopreissin A (**1**), suggesting similar benzofuran-type stilbene dimers or conjugates. Isolation of these peaks from a larger quantity of cultured roots and structure elucidation by means of NMR and MS resulted in identification of compounds **2–4** (Fig. 1). The low-field region of the ^1H NMR spectra resembled that of anigopreissin A (**1**) with the exception of the missing exchangeable ^1H resonances of 4a-OH of compounds **2** and **4**, and 4b-OH of **3** and **4**. In addition, signals of one (compounds **2** and **3**) or two (compound **4**) glucose units indicated glucosidic structures. The doublets of the anomeric H-1 around δ 5.0 ($J = 7.8$ Hz) confirmed β-configuration of the glucose units. HMBC correlations between the respective glucosidic H-1 and C-4a of **2** (δ 157.2), C-4b of **3** (δ 157.0), and both C-4a (δ 157.2) and C-4b (δ 157.5) of **4** proved attachment of the glucosyl moieties to the 4-hydroxy positions of the phenyl rings. Complete sets of ^1H–^1H COSY, HMQC and HMBC correlations, corresponding to those described previously for anigopreissin A (Hölscher and Schneider, 1996), and, in addition, electrospray and HR–MS data confirmed the suggested structures.

Since glucosyloxy moieties regiospecifically occur in the 4-position but not at the other hydroxyl groups, one could speculate that glucosidation of the hypothetical monomeric resveratrol units takes place already prior to dimerization. Thus, resveratroloside (Nonaka et al., 1977) could be a direct biosynthetic precursor of compounds

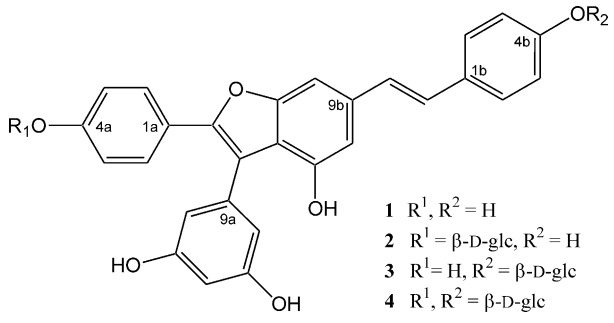

Fig. 1. Structures of anigopreissin A (**1**) and its glucosides **2–4** from root cultures of *Anigozanthos preissii*.

2–4. According to the polyphyletic evolution of stilbene biosynthesis (Tropf et al., 1994), stilbenes are distributed among unrelated plant families. Very few stilbene di- and oligomeric forms, which predominantly occur in the Gnetaceae and Vitaceae (Gorham, 1995), contain a completely unsaturated benzofuran ring system. The first compound of that type, anigopreissin A (**1**), was isolated from *Anigozanthos preissii* and *Musa cavendish* (Hölscher and Schneider, 1996) and an isomeric dimer, viniferifuran, has been reported from *Vitis vinifera* (Ito et al., 1999).

3. Experimental

3.1. Plant material and culture conditions

Root cultures of *Anigozanthos preissii* (L.) were established as previously described (Hölscher and Schneider, 1997), propagated from a parent culture, and aseptically grown on LS agar (Linsmaier and Skoog, 1965) at 23 °C in the dark. After approximately 10 weeks, the roots (approx. 5 g fresh mass) were transferred to liquid LS medium (140 ml in 300-ml conical flasks) and maintained at 23 °C on a gyratory shaker (85 rpm) under permanent diffuse light (4.4 μmol m^{-2} s^{-1}). After cultivation for further 16 weeks, the slow growing roots (culture type S) reached a fresh mass of 25–30 g, and were harvested. After 8–10 weeks in liquid medium, several subcultures spontaneously started differentiation (shoot growth) without changing external conditions. These shoot-developing cultures (accelerated growth, culture type A) were subcultured by transfer of approximately 10 g of plant material into fresh medium and were harvested with a fresh mass of 25–30 g after 4 weeks.

3.2. Extraction and purification

For analysis, roots of type A and type S of *A. preissii* were separately frozen with liquid nitrogen, ground, and exhaustively extracted with MeOH at room temperature. The MeOH extracts were evaporated and partitioned between *n*-hexane–H_2O, $CHCl_3$–H_2O, and EtOAc–H_2O. The crude MeOH extract and the *n*-hexane, $CHCl_3$, and EtOAc fractions were analyzed by reversed-phase HPLC on a LiChrospher 100 RP-18 column (5 μm; 250×4 mm); diode array detection 200–600 nm, monitoring wavelength 254 nm; 1.0 ml min^{-1}; solvent A: H_2O; solvent B: MeCN containing 0.1% trifluoroacetic acid; gradient I: 0 min 80% A/20% B, 30 min 30% A/70% B; gradient II: 0 min 70% A/30% B, 30 min 10% A/90% B; gradient III: 0 min 40% A/60% B, 30 min 100% B. In order to enable comparability, both type A and S cultures were extracted with the same amount of solvent and analyzed using identical aliquots.

For isolation of glucosides **2–4**, the EtOAc fraction of type S cultures (320 g) was prepared as described earlier, passed through a RP-18 cartridge (elution with methanol), and subjected to semi-preparative HPLC on a Nucleosil 100-7 C_{18} column (7 μm; 250×21 mm); UV detection 290 nm; 11.5 ml min^{-1}; solvent A: H_2O; solvent B: MeCN; gradient IV: 0 min 80% A/20% B, 30 min 30% A/70% B, 35 min 30% A/70% B. Finally, the fractions containing separated glucosides **2–4** were further purified using the same method yielding pure glucosides **2** (2.1 mg; R_t 22.5 min), **3** (0.8 mg; R_t 21.2 min), and **4** (1.3 mg; R_t 14.8 min).

Table 1
^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.75 MHz) spectroscopic data for anigopreissin A glucosides **2–4** from root cultures of *Anigozanthos preissii* (DMSO-d_6–C_6H_6-d_6 5:3)

Position	**2**		**3**		**4**	
	δ ^{1}H (*J* in Hz)	δ ^{13}C	^{1}H (*J* in Hz)	δ ^{13}C	^{1}H (*J* in Hz)	δ ^{13}C
1a		124.2		121.2		124.1
2a/6a	7.95 *d* (*J* = 8.8)	127.5	7.54 *d* (*J* = 8.8)	127.7	7.56 *d* (*J* = 8.8)	127.2†
3a/5a	7.07 *d* (*J* = 8.8)	116.2	6.86 *d* (*J* = 8.8)	115.4	7.05 *d* (*J* = 8.8)	116.5
4a		157.2		157.6		157.1§
7a		148.5		149.1		148.6
8a		116.8		115.5		116.8
9a		135.0		135.0		134.9#
10a/14a	6.49 *d* (*J* = 2.2)	108.6	6.59 *d* (*J* = 2.2)	108.6	6.51 *d* (*J* = 2.2)	108.6
11a/13a		158.3		158.0		158.3
12a	6.41 *t* (*J* = 2.2)	102.1	6.54 *t* (*J* = 2.2)	101.8	6.47 *t* (*J* = 2.2)	102.1
1b		128.2		130.9		130.9
2b/6b	7.49 *d* (*J* = 8.6)	127.8	7.55 *d* (*J* = 8.6)	127.6	7.54 *d* (*J* = 8.6)	127.5‡
3b/5b	6.91 *d* (*J* = 8.6)	115.6	7.19 *d* (*J* = 8.6)	116.5	7.14 *d* (*J* = 8.6)	116.1
4b		157.5		157.0		157.2§
7b	7.17 *d* (*J* = 16.3)	128.0	7.21 *d* (*J* = 16.3)	127.2	7.32 *d* (*J* = 16.3)	127.7‡
8b	7.11 *d* (*J* = 16.3)	125.7	7.21 *d* (*J* = 16.3)	127.1	7.19 *d* (*J* = 16.3)	127.1†
9b		135.4		134.6		135.0#
10b	7.32 *s*	100.0	7.35 *s*	100.1	7.34 s	100.1
11b		155.5		155.1		155.4
12b		117.5		117.7		117.9
13b		152.2		151.8		152.2
14b	6.98 *s*	106.4	7.06 *s*	106.5	7.02 s	106.6
OH-4a			9.71 *s*			
OH-4b	9.71 *s*					
OH-11a/13a	9.38 *s*		9.40 *s*		9.39 *s*	
OH-13b	9.70 *s*		9.89 *s*		9.75 *s*	
1′	5.01 *d* (*J* = 7.8)	100.2			5.00 *d* (*J* = 7.8)	100.2
2′	3.39–3.49 m	73.3			3.41–3.48 m	73.3
3′		76.8*				76.7*
4′		69.8				69.8
5′		77.1*				77.1*
6′A	3.84 *dd* (*J* = 12.0, 5.5)	60.8			3.84 *dd* (*J* = 12.0, 5.5)	60.8
6′B	3.64 *m*				3.66 *m*	
OH-2′	5.45 *d* (*J* = 4.9)				5.45 *d* (*J* = 4.9)	
OH-3′	5.23 *d* (*J* = 4.2)				5.23 *d* (*J* = 4.2)	
OH-4′	5.16 *d* (*J* = 5.9)				5.18 *d* (*J* = 5.9)	
OH-6′	4.68 *t* (*J* = 5.9)				4.69 *t* (*J* = 5.9)	
1″			5.07 *d* (*J* = 7.8)	100.4	5.02 *d* (*J* = 7.8)	100.6
2″			3.51–3.56 m	73.3	3.41–3.48 m	73.4
3″				76.7*		76.8*
4″				69.8		69.9
5″				77.1*		77.2*
6″A			3.96 *dd* (*J* = 12.0, 5.5)	60.7	3.91 *dd* (*J* = 12.0, 5.5)	60.9
6″B			3.74 *m*		3.66 *m*	
OH-2″			5.52 *d* (*J* = 4.9)		5.47 (*J* = 4.9)	
OH-3″			5.30 *d* (*J* = 4.2)		5.26 *brs*	
OH-4″			5.24 *d* (*J* = 5.9)		5.20 *d* (*J* = 4.5)	
OH-6″			4.78 *t* (*J* = 5.9)		4.74 *t* (*J* = 5.9)	

*, §, #, †, ‡May be reversed in one column.

3.3. Spectroscopic methods

^1H and ^{13}C NMR, ^1H–^1H COSY, HMBC, and HMQC spectra were recorded on a Bruker Avance DRX 500 NMR spectrometer. An inverse detection microprobe head (2.5 mm) was used at 500.13 MHz for acquisition of ^1H NMR, ^1H–^1H COSY, and heteronuclear 2D correlation spectra, and a broadband microprobe head (2.5 mm) for measuring ^{13}C NMR spectra at 125.75 MHz. Me_2CO–d_6–C_6H_6-d_6 5:3 was used as a solvent and TMS as internal standard. ESI-MS and ESI-HRMS were measured in the positive ion mode on a Micromass Quattro II tandem quadrupole mass spectrometer. UV spectra were obtained from a diode array detector in H_2O–MeCN during the HPLC runs.

3.3.1. Anigopreissin A (*1*)

Systematic name: 4-Hydroxy-3-(3,5-dihydroxyphenyl)-2-(4-hydroxyphenyl)-6-[2-(4-hydroxyphenyl)-vinyl]-benzo[*b*]furan. UV (MeCN–D_2O) λ_{max} nm: 292, 359; gradient I: R_t 19.9 min, II: R_t 12.7 min; for further analytical data, see Hölscher and Schneider (1996).

3.3.2. Anigopreissin A-4a-O-β-D-glucopyranoside (*2*)

Systematic name: 4-Hydroxy-3-(3,5-dihydroxyphenyl)-2-(4-*O*-β-D-glucopyranosyl-phenyl)-6-[2-(4-hydroxyphenyl)-vinyl]-benzo[*b*]furan. HPLC gradient I: R_t 13.7 min, II: R_t 8.3 min; UV (MeCN–D_2O) λ_{max} nm: 292, 359; ESI-MS (70 eV): *m*/*z* 615 (rel. int. 100) $[M+H]^+$, 453 (86) $[M+H-glc]^+$; HR-MS: *m*/*z* 615.18700 (calc. 615.18664 for $C_{34}H_{31}O_{11}$); for NMR data, see Table 1.

3.3.3. Anigopreissin A-4b-O-β-D-glucopyranoside (*3*)

Systematic name: 4-Hydroxy-3-(3,5-dihydroxyphenyl)-2-(4-phenyl)-6-[2-(4-*O*-β-D-glucopyranosyl-phenyl)-vinyl]-benzo[*b*]furan. HPLC gradient I: R_t 13.0 min; UV (MeCN–D_2O) λ_{max} nm: 292, 359; ESI-MS (70 eV): *m*/*z* 637 (rel. int. 100) $[M+Na]^+$, 614 (11) $[M]^+$; HR-MS: *m*/*z* 637.16820 (calc. 637.16858 for $C_{34}H_{30}O_{11}Na$); for NMR data, see Table 1.

3.3.4. Anigopreissin A-4a,4b-di-O-β-D-glucopyranoside (*4*)

Systematic name: 4-Hydroxy-3-(3,5-dihydroxyphenyl)-2-(4-*O*-β-D-glucopyranosyl-phenyl)-6-[2-(4-*O*-β-D-glucopyranosyl-phenyl)-vinyl]-benzo[*b*]furan. HPLC gradient I: R_t 8.2 min; UV (MeCN–D_2O) λ_{max} nm: 292, 359; ESI-MS (70 eV): *m*/*z* 777 (rel. int. 100) $[M+H]^+$, 615 (80) $[M+H-glc]^+$, 453 (13) $[M+H-2glc]^+$; HR-MS: *m*/*z* 777.23946 (calc. 777.23680 for $C_{40}H_{41}O_{16}$); for NMR data, see Table 1.

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