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Acylated anthocyanins from petunia flowers

Rune Slimestad, Atle Aaberg, Øyvind M. Andersen*

Department of Chemistry, University of Bergen, Allégt. 41, 5007 Bergen, Norway

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Abstract

Two acylated anthocyanins have been isolated from the petals of petunia (*Petunia hybrida* 'Surfinia blue'). Their complete structures were elucidated by means of homo- and heteronuclear two-dimensional NMR techniques to be malvidin 3-*O*-[6-*O*-(4-*O*-*E*-caffeoyl-α-rhamnopyranosyl)-*β*-glucopyranoside]-5-*O*-*β*-glucopyranoside and the new pigment malvidin 3-*O*-[6-*O*-(4-*O*-*Z*-*p*-coumaroyl-α-rhamnopyranosyl)-*β*-glucopyranoside]-5-*O*-*β*-glucopyranoside.

Keywords: *Petunia hybrida* 'Surfinia blue'; Solanaceae; Flowers; Anthocyanins; *Z*-*p*-Coumaroyl; Malvidin 3-*O*-[6-*O*-(4-*O*-*Z*-*p*-coumaroyl-α-rhamnopyranosyl)-*β*-glucopyranoside]-5-*O*-*β*-glucopyranoside; Malvidin 3-*O*-[6-*O*-(4-*O*-*E*-caffeoyl-α-rhamnopyranosyl)-*β*-glucopyranoside]-5-*O*-*β*-glucopyranoside; 2D-NMR

1. Introduction

Petunia (*Petunia hybrida*) has together with maize (*Zea mays*) and snapdragon (*Antirrhinum majus*) been particularly important for elucidating the anthocyanin biosynthetic pathway. Petunia has in recent years become the organism of choice for isolating flavonoid biosynthetic genes and studying their interactions and regulation (Dooner, Robbins, & Jorgensen, 1991; Gerats & Martin, 1992). The inheritance of anthocyanin pigmentation is found to be controlled by multiple independent genes that follow simple Mendelian genetics (Harborne & Swain, 1979; Griesbach, 1996). At least 35 genes are known to affect flower colour in petunia (Wiering & de Vlaming, 1984). The following anthocyanins from this plant have been characterised: the 3-glucosides of the six common anthocyanidins; the 3-rutinosides of delphinidin, cyanidin and pelargonidin; the 3-sophoroside, 3-gentiobioside and 3,7-diglucoside of cyanidin; peonidin 3-sophoroside; the 3-*p*-coumaroylrutinoside-5-glucosides of malvidin, petunidin, cyanidin and peonidin; and the 3-caffeoylrutinoside-5-glucosides of malvidin, petunidin and peonidin (Harborne, 1960; Muszynski, 1964; Harborne & Swain, 1979; Griesbach, Asen, & Leonnarat, 1991). For these acylated anthocyanins neither the binding site of the acyl moieties nor the configuration of the acyl groups have been determined properly. Recently, two diacylated malvidin derivatives have been fully identified (Tatsuzawa et al., 1997; Fukui et al., 1998). In this paper we report the isolation and complete structure elucidation of two monoacylated malvidin derivatives from this plant.

2. Results and discussion

Two compounds, **1** and **2**, were isolated from a methanolic extract of *Petunia hybrida* 'Surfinia blue' petals. They were purified by partition between water and ethyl acetate followed by adsorption chromatography on a Amberlite XAD-7 resin. The pigments were isolated by chromatography on Sephadex LH-20 and preparative HPLC.

From the HPLC chromatogram of the crude petal extract, **2** was found to account for approximately 75% of the total anthocyanin content. The UV–Vis spectrum of **2** measured during HPLC-elution, showed visible maximum at 535 nm with A_{440}/A_{535} of 8% and an absorption band around 300 nm (Table 1). This indicated the presence of an anthocyanin 3,5-diglycoside acylated with an aromatic acid (Andersen, Opheim, Aksnes, & Frøystein, 1991). The downfield part of the ^{1}H NMR spectrum of **2** showed a 1H singlet at δ 9.09 (H-4), a 2H singlet at δ 8.12 (H-2′/6′) and a 2H singlet at δ 7.12 (H-6/8). In addition, a 3H AMX spin system ($J_{AM}=8.2$ Hz, $J_{MX}=1.9$ Hz) together with a 2H AX spin system ($J_{AX}=15.9$ Hz) typical for *E*-caffeic or *E*-ferulic acid, occurred in this part of the spectrum (Table 2) (Strack & Wray, 1989). The chemical shifts of the carbons of the

* Corresponding author. Tel.: +47-55-583-460; fax: +47-55-589-490; e-mail: oyvind.andersen@kj.uib.no.

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Table 1
Chromatographic and spectral data on the 4‴-*O*-*Z*-*p*-coumaroyl (**1**) and 4‴-*O*-*E*-caffeoyl (**2**) of malvidin 3-*O*-(6-*O*-α-rhamnopyranosyl-β-glucopyranoside)-5-*O*-β-glucopyranoside and petanin (**3**). Relative retardation (R_{rel}) are measured using cyanidin 3-*O*-β-glucopyranoside as reference compound

	TLC; hR_F (R_{rel})		On-line HPLC		
Compound	FHW	BAW	vis. max. (nm)	A_{440}/A_{max} (%)	t_R (min)
1	90 (3.0)	56 (1.4)	535	6	12.3
2	81 (2.7)	50 (1.3)	535	8	13.2
3	83 (2.9)	49 (1.4)	536	22	14.4

aglycone and acyl moiety assigned by the HMBC (Fig. 1) and SEFT NMR experiments were in agreement with the presence of malvidin and one caffeic acid moiety. In the ^{1}H NMR spectrum three anomeric proton signals were detected, and the proton resonances belonging to the respective sugar moieties were assigned by DQF-COSY and TOCSY experiments (Table 2). On the basis of these data together with the resonances shown in the sugar-region of the SEFT spectrum, the presence of two glucopyranose and one rhamnopyranose units were identified (Tables 2–3, Andersen et al., 1991).

The connection sites of the sugars of **2** on the aglycone were derived from the HMBC experiment. Cross-peaks between the anomeric proton at δ 5.59 and the carbon at δ 146.25 (C-3), and the anomeric proton at δ 5.29 and the carbon at δ 156.75 (C-5), revealed that the aglycone 3- and 5-positions were both connected to a glucopyranosyl with β-linkages showing coupling constants of 7.7 and 7.8 Hz, respectively. A cross-peak between the anomeric proton at δ 4.82 and the carbon at δ 67.08 revealed that the rhamnopyranosyl was connected to the 6-position on the 3-glucopyranoside. The downfield shift of this carbon resonance (4.72 ppm) compared to the analogous signal of the 5-glucopyranosyl (Table 2) also confirmed this connection site. A downfield shift of H-4‴ (δ 5.02) together with a cross-peak in the HMBC spectrum between this resonance and the carbon signal at δ 168.98 revealed that the caffeic acid moiety was connected to the rham-

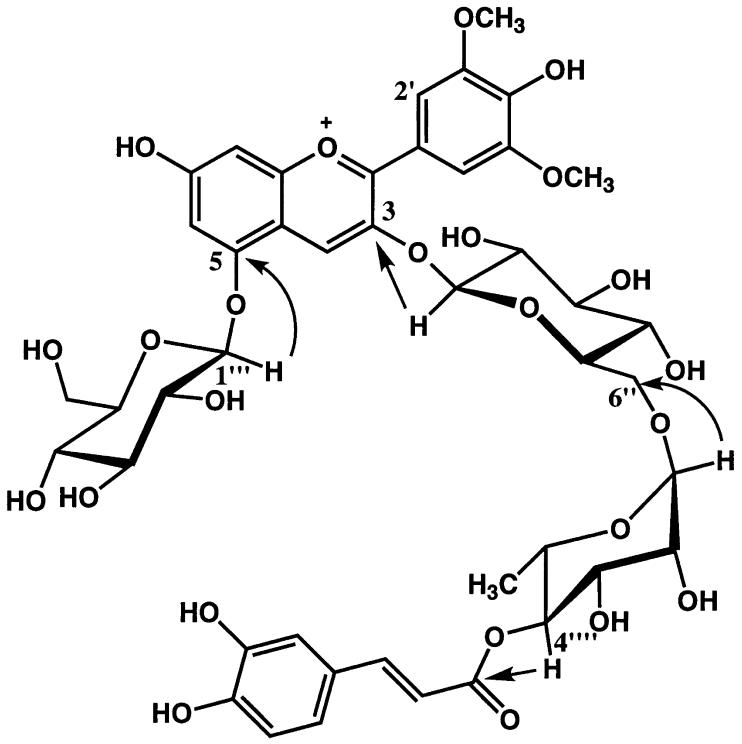

Fig. 1. Structure of **2**. The arrows are showing some important long-range coupling interactions detected in the heteronuclear multiple bond correlation (HMBC) NMR experiment.

Table 2
^{1}H NMR spectral data for malvidin 3-*O*-[6-*O*-(4-*O*-*Z*-*p*-coumaroyl-α-rhamnopyranosyl)-*β*-glucopyranoside]-5-*O*-*β*-glucopyranoside (**1**), malvidin 3-*O*-[6-*O*-(4-*O*-*E*-caffeoyl-α-rhamnopyranosyl)-*β*-glucopyranoside]-5-*O*-*β*-glucopyranoside (**2**) and petunidin 3-*O*-[6-*O*-(4-*O*-*E*-*p*-coumaroyl-α-rhamnopyranosyl)-*β*-glucopyranoside]-5-*O*-*β*-glucopyranoside (=petanin) (**3**)

Positions	**1** (δ (ppm), *J* (Hz))	**2** (δ (ppm), *J* (Hz))	**3** (Andersen et al., 1991) (δ (ppm), *J* (Hz))
Aglycone			
4	9.07 s	9.09 s	8.97 s
6	7.12 d 1.8	7.12 s	7.06 s
8	7.14 d 1.8	7.12 s	7.06 s
2′	8.09 s	8.12 s	7.94 d 2.1
6′	8.09 s	8.12 s	7.78 d 2.1
OMe	4.09 s	4.10 s	4.02 s
3-O-β-Glucopyranoside			
1	5.63 d 7.7	5.59 d 7.7	5.69 d 7.7
2	3.79 dd 7.7, 9.2	3.81 dd 7.7, 8.9	3.85 dd 7.7, 8.9
3	3.67 dd 9.2, 9.5	3.69 dd 8.9, 9.4	3.75 t 8.9
4	3.58 t 9.6	3.57 dd 9.0, 9.4	3.61 dd 8.9, 9.6
5	3.87 dd 9.5, 6.2	3.92 m	3.97 ddd 9.6, 1.4, 6.3
6A	4.06 d 11.5	4.12 d 11.6	4.16 dd 1.4, 11.7
6B	3.79 dd 6.2, 11.5	3.82 dd 6.6, 11.6	3.85 dd 6.3, 11.7
6″-O-α-Rhamnopyranosyl			
1	4.78 s	4.82 s	4.83 s
2	3.86 s	3.91 s	3.93 s
3	3.85 d 9.4	3.92 d 9.5	3.94 d 9.5
4	4.94 t 9.5	5.02 t 9.6	4.99 t 9.5
5	3.78 dd 9.5, 6.3	3.82 dd 9.5, 6.2	3.86 dd 9.5, 6.3
6	0.98 d 6.3	1.12 d 6.2	1.09 d 6.3
5-O-β-Glucopyranoside			
1	5.28 d 7.7	5.29 d 7.8	5.31 d 7.8
2	3.7–3.8 m	3.80 dd 7.8, 8.9	3.82 dd 7.8, 8.7
3	3.7–3.8 m	3.77 m	3.71 dd 8.7, 9.1
4	3.7–3.8 m	3.64 m	3.65 dd 9.1, 9.3
5	3.76 dd 9.3, 6.1	3.71 m	3.72 dd 9.3, 6.8
6A	4.04 d 12.3	4.02 d 12.2	4.06 d 11.7
6B	3.89 dd 6.1, 12.3	3.89 dd 5.9, 12.2	3.92 dd 6.8, 11.7
	4‴-*Z*-*p*-coumaroyl	4‴-*E*-*p*-caffeoyl	4‴-*E*-*p*-coumaroyl
2	7.69 d 8.7	7.22 d 1.9	7.46 d 8.6
3	6.80 d 8.7	-	6.88 d 8.6
5	6.80 d 8.7	6.86 d 8.2	6.88 d 8.6
6	7.69 d 8.7	7.00 dd 8.2, 1.9	7.46 d 8.6
7 (*β*)	5.69 d 12.8	6.30 d 15.8	6.27 d 15.9
8 (α)	6.88 d 12.9	7.58 d 15.8	7.61 d 15.9

nosyl 4-position. Thus, **2** was identified as malvidin 3-*O*-[6-*O*-(4-*O*-*E*-caffeoyl-α-rhamnopyranosyl)-*β*-glucopyranoside]-5-*O*-*β*-glucopyranoside.

Compound **1** accounted for 10% of the total anthocyanin content. Its UV–Vis spectrum was similar to that of **2** with one exception: the relative absorbance A_{300}/A_{535} was 0.6 for **1** compared to 1.0 for **2**, indicating different acyl moieties in these pigments. The ^{1}H and ^{13}C resonances of **1** were assigned using the same homo- and heteronuclear NMR experiments as for **2**. In addition, a HSC spectrum of **1** showed direct proton–carbon correlations which supported the assignments. A ROESY experiment of **1** was used to confirm the connection sites between the glucose units and the aglycone moiety, and to assign the chemical shifts of H-6 and H-8 (Fig. 2). The low-field part of the ^{1}H NMR spectrum of **1** revealed a 4H AA′XX′ spin system (J=8.7 Hz) similar to what is found in *p*-coumaric acid (Table 2, (Andersen et al., 1991)). The 2H AX spin system at δ 6.88 (H-α) and δ 5.69 (H-*β*) with a relative small coupling constant

Table 3
^{13}C data of **1** and **2** together with petanin (**3**)

Positions	**1** δ (ppm)	**2** δ (ppm)	**3** (Andersen et al., 1991) δ (ppm)
Aglycone			
2	164.28	164.56	163.79
3	146.07	146.25	146.09
4	134.61	134.99	134.08
5	156.74	156.75	156.89
6	105.66	105.61	105.54
7	169.87	169.90	169.73
8	97.65	97.64	97.55
9	nd	153.33	156.61
10	113.23	113.31	113.03
1′	119.57	119.50	119.58
2′	110.88	110.97	109.52
3′	149.89	149.89	149.76
4′	147.33	146.75	147.55
5′	149.89	149.89	146.16
6′	110.88	110.97	114.03
OMe	57.26	57.26	57.22
3-O-β-Glucopyranoside			
1	102.47	102.79	102.54
2	74.81	74.78	74.73
3	78.19	78.25	78.23
4	71.13	71.32	71.31
5	77.54	77.66	77.69
6	66.92	67.08	67.33
6″-O-α-Rhamnopyranosyl			
1	101.78	102.13	102.15
2	72.07	72.09	72.09
3	70.32	70.34	70.39
4	74.91	75.28	75.40
5	67.67	67.85	67.88
6	17.77	17.84	17.85
5-O-β-Glucopyranoside			
1	102.80	103.07	102.69
2	74.81	74.78	74.84
3	77.87	77.90	77.88
4	70.98	70.97	70.95
5	78.74	78.73	78.64
6	62.20	62.16	62.13
	4‴-*Z*-*p*-coumaroyl	4‴-*E*-*p*-caffeoyl	4‴-*E*-*p*-coumaroyl
1	127.47	127.73	127.16
2	133.83	115.38	131.31
3	115.78	146.75	116.92
4	160.10	149.59	161.26
5	115.78	116.50	116.92
6	133.83	122.99	131.31
7 (β)	145.54	147.36	147.09
8 (α)	116.83	114.99	114.98
9	167.66	168.98	169.09

nb: not detected.

between these signals ($J=12.9$ Hz), was in accordance with *Z*-*p*-coumaric acid (Nakatani et al., 1995). Thus, **1** was assigned to be malvidin 3-*O*-[6-*O*-(4-*O*-*Z*-*p*-coumaroyl-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside.

In the genus *Solanum* the presence of a pleiotropic gene (Ac) has been suggested to control three aspects of anthocyanin synthesis; methylation of the aglycone, addition of glucose to the 5-hydroxyl and acylation of rutinose with *p*-coumaric acid (Harborne & Swain, 1979). When the complete structure of these acylated pigments from *Solanum* sp. have been elucidated, the *p*-coumaroyl moiety has exposed the *E*-configuration (Andersen et al., 1991; Opheim & Andersen, 1992; Tatsuzawa et al., 1997; Fukui et al., 1998). In this context it is interesting to note that the acyl moiety of **1**, isolated from *Petunia hybrida* 'Surfinia blue', displayed the rare *Z*-configuration.

3. Experimental

3.1. Extraction

300 g (fr. wt) of *Petunia hybrida* 'Surfinia blue' flowers was collected at Toppe garden market (Bergen, Norway) on August 12, 1997. A voucher specimen has been deposited in the herbarium of the Chemistry Department, University of Bergen. The material was stored at $-20^{\circ}C$ in a plastic bag. Extraction was accomplished using 1.5 l MeOH (containing 0.1% TFA, v/v) for 24 h (3 times). After removal of the methanol under reduced pressure, the water enriched extract was partitioned against ethyl acetate (5×0.2 l EtOAc). A glass column (6.7×45 cm, Pyrex) filled with a slurry of 0.5 kg Amberlite XAD-7 was used to clean the anthocyanin extract using distilled water as eluent. The anthocyanins were thereafter eluted using 3.5 l MeOH (containing 0.1% TFA). After evaporation of the solvent 4.6 g purified extract was obtained.

3.2. Isolation

0.1 g of the extract was dissolved in 3 ml of 30% MeOH. The sample was subjected to a 200 g bed of Sephadex LH-20, which was slurry packed with 30% MeOH (0.1% TFA) in a C 26/100 column (Pharmacia). 840 ml of 30% MeOH and 780 ml of 60% MeOH, both containing 0.1% TFA, were used to elute the pigments in 11 fractions (145 ml each). Fraction 6 contained compound **1**, whereas fractions 8–10 contained compound **2**. The pigments were further purified on an Econosil C_{18} column (25×2.2 cm, 10 μm, Alltech) connected to a Gilson 305/306 preparative HPLC system equipped with a 1040A diode array detector (Hewlett Packard). Two solvent systems were used: A (H_2O–HCO_2H, 9:1) and B (MeOH–HCO_2H–H_2O, 5:1:4). The elution profile consisted of a linear gradient from 5 to 45% B during 5 min, an increase to 70% B during the next 7 min, followed by 100% B during the next 2 min and finally an isocratic

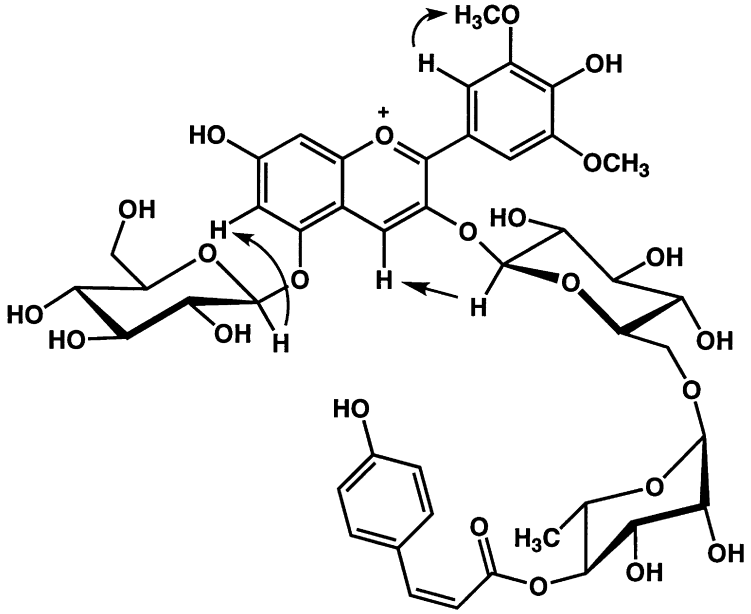

Fig. 2. Structure of **1**. The arrows are showing some important nuclear Overhauser effects obtained by the rotating frame Overhauser enhancement spectroscopy (ROESY) NMR experiment.

elution of 100% B during 2 min. The flow rate was 12 ml min^{-1}, which gave a backpressure on the column prior to injection of 77 bar. Aliquots of 100–200 μl were injected through a Rheodyne injector with a 500 μl sample loop.

3.3. Analytical HPLC

HPLC was carried out on an HP-1050 module system (Hewlett Packard) using an ODS-Hypersil column (20 × 0.5 cm, 5 mm, Hewlett Packard). Solvents were the same as for prep. HPLC and the elution profile consisted of a linear gradient from 10 to 100% B during 17 min, followed by an isocratic step for the next 4 min. The flow rate was 1.2 ml min^{-1} and aliquots of 10 μl were injected. UV spectra were recorded on-line and spectral measurements were made over the wavelength range 210–600 nm in steps of 2 nm.

3.4. NMR spectroscopy

The DQF-COSY (double quantum filtered ^{1}H–^{1}H shift correlation spectroscopy), TOCSY (total correlation spectroscopy), ROESY (rotating frame Overhauser enhancement spectroscopy), SEFT (heteronuclear coupling modulated spin echo Fourier transformation) and HSC (one-bond heteronuclear shift correlation) experiments were performed on a Bruker DMX 400 spectrometer at 400.13 and 100.62 MHz for ^{1}H and ^{13}C, respectively. The data were obtained using a 5 mm ^{1}H–^{13}C dual probe at 298 K. The one-bonding (HSQC) and long-range (HMBC) heteronuclear shift correlations on **1** and **2**, respectively, were obtained on a Bruker DRX 600 spectrometer equipped with a 5 mm TBI probe at 298 K. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (CF_3CO_2D–CD_3OD; 1:9) were used as secondary references (δ 49.0 and δ 3.4 from TMS, respectively). The coupling constants were determined from the ^{1}H and DQF-COSY spectra.

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