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ACYLATED ANTHOCYANINS IN *VERBENA* FLOWERS

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Key Word Index—*Verbena hybrida*; Verbenaceae; flower colour; acylated anthocyanins; cyanidin and pelargonidin 3,5-di-(6-acetylglucoside); pelargonidin 3-(6-malonylglucoside)-5-(6-acetylglucoside).

Abstract—Three new acylated anthocyanins were isolated from the deep red–purple flowers of verbena, strain UR-RP-3, and identified as the 3,5-di-*O*-(6-*O*-acetyl-β-D-glucopyranosides) of cyanidin and pelargonidin, and the 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside)-5-*O*-(6-acetyl-β-D-glucopyranoside) of pelargonidin.

INTRODUCTION

In the course of investigation of the flower colour variation in *Verbena hybrida*, we found seven acylated anthocyanins with malonic and acetic acids in the blue–purple, red–purple, maroon, magenta and scarlet flowers [1–3]. In further studies on a deep red–purple strain of this species, three new diacyl anthocyanins were isolated and identified.

RESULTS AND DISCUSSION

We isolatated three anthocynanins, one red (**1**) and two orange red pigments (**2** and **3**) from the deep red–purple flowers of *Verbena* (strain UR-RP-3) by extraction with 3% formic acid and purification with Diaion HP-20 and Sephadex LH-20 CC, PC and HPLC. Compound **2** was present as a main anthocyanin (48%) and the other two (**1**: 21%, **3**: 5%) as minor ones.

The chromatographic and spectral data of these pigments are summarized in Table 1. Acid hydrolysis of **1**–**3** gave cyanidin, pelargonidin and pelargonidin, respectively, as the aglycones, and glucose as the sole sugar component. On alkaline hydrolysis of **1** and **2**, cyanidin 3, 5-diglucoside and pelargonidin 3,5-diglucoside were obtained, but no acyl group was detected on TLC. By contrast, pigment **3** gave pelargonidin 3,5-diglucoisde and malonic acid on the same treatment.

Pigments **1** and **2**: the FAB mass spectra of **1** and **2** showed the $[M]^+$ 695 and 679, respectively, which correspond to cyanidin or pelargonidin 3,5-diglucoside with two acetic acids. The detailed structures were elucidated by ^1H NMR measurements (Table 2). The proton signals

Table 1. Chromatographic and spectral data of *Verbena* anthocyanins

Anthocyanins*	R_f values (×100)†				R_t† (min)	In 0.1% HCl–MeOH		FABMS
	BAW	BuH	1%HCl	HAc–HCl		λ_{max}(nm)	E_{440}/E_{VIS}	$[M]^+$
Verbena pigments								
Cy3aG5aG (**1**)	45	34	21	56	27.1	279, 527	12	695
Pg3aG5aG (**2**)	61	42	36	69	29.4	269, 508	18	679
Pg3mG5aG (**3**)	47	38	37	67	23.5	268, 508	19	723
Related pigments								
Cy3G5G	25	6	10	27	8.2	279, 527	12	
Pg3G5G	39	13	20	43	10.5	269, 508	19	

*Cy3aG5aG, cyanidin 3,5-diacetylglucoside; Pg3aG5aG, pelargonidin 3,5-dicacetyl-glucoside; Pg3mG5aG, pelargonidin 3-malonylglucoside-5-acetylglucoside.

†BAW, *n*-BuOH–AcOH–H_2O (4:1:5); BuOH, *n*-BuOH–2 N HCl (1:1); HAc–HCl, AcOH–HCl–H_2O (15:3:82), R_t, retention time, see experimental.

Table 2. ^1H and ^{13}C NMR spectral data of *Verbena* anthocyanins

	Cy3aG5aG (**1**)	Pg3aG5aG (**2**)	Pg3mG5aG (**3**)	
H or C	^1H	^1H	^1H	^{13}C
Anthocyanidin				
2				162.7
3				144.6
4	8.74 *s*	8.77 *s*	8.83 *s*	132.7
5				155.0
6	6.96 *br s*	6.94 *d* (1.9)	6.97 *d* (1.4)	104.1
7				167.8
8	7.13 *br s*	7.15 *d* (1.2)	7.21 *d* (1.4)	96.4
8a				155.6
4a				101.9
1′				119.4
2′	8.05 *d* (2)	}	}	
6′	8.27 *dd* (2, 8.8)	8.58 *d* (9.3)	8.62 *d* (8.8)	135.2
3′		}	}	
5′	7.09 *d* (8.8)	7.07 *d* (9)	7.12 *d* (8.8)	117.2
4′				165.4
3-Glucose (A)				
1	5.56 *d* (7.8)	5.52 *d* (7.6)	5.57 *d* (7.3)	100.4
2	3.63 *t* (8.8)	3.54 *t* (8.4)	3.59 *t* (8.3–8.8)	72.7
3	3.53 *t* (8.8)	3.45 *t* (8.9)	3.50 *t* (8.8)	76.5
4	3.29 *t* (9.8)	3.25 *t* (9.4)	3.31 *t* (9.3–9.8)	70.0
5	3.93 *m*	3.77 *m*	3.97 *ddd* (1.5, 7.8, 9.3)	74.1
6a	4.05 *dd* (7.8, 11.2)	4.12 *dd* (6.4, 12.2)	4.18 *dd* (7.8, 12.2)	64.6
6b	4.41 *d* (11.2)	4.31 *d* (10.5)	4.41 *d* (11.7)	
5-Glucose (B)				
1	5.23 *d* (7.9)	5.19 *d* (7.6)	5.22 *d* (7.8)	101.3
2	3.49 *t* (8.3)	3.50 *t* (7.7)	3.54 *t* (7.8–9.3)	73.1
3	3.44 *t* (8.8)	3.40 *t* (8.9)	3.44 *t* (8.8–9.3)	75.8
4	3.27 *t* (9.8)	3.23 *t* (9.3)	3.31 *t* (9.3–9.8)	70.1
5	3.81 *dd* (6.3, 9.8)	3.90 *ddd* (1.5, 9, 10)	3.82 *ddd* (2, 7.3, 9.8)	74.4
6a	4.15 *dd* (6.3, 11.7)	4.01 *dd* (7.8, 12.0)	4.11 *dd* (7.3, 12.2)	63.6
6b	4.34 *d* (11.7)	4.37 *d* (10.5)	4.41 *d* (11.7)	
Acetic acid (Ac)				
I Me-	2.04 *s*	1.99 *s*		
CO-				
II Me-	1.96 *s*	1.91 *s*	2.05 *s*	20.7
CO-				170.5
Malonic acid				
-CO- (1)				167.3
$-CH_2-$ (2)			3.45	41.6
-CO- (3)				167.9

Coupling constants (*J*, Hz) in parentheses.

of **1** and **2** were mainly assigned by ^1H–^1H COSY, and also linkages of sugars were confirmed by DIFNOE [4, 5]. The characteristic signals in the low field region were assigned to cyanidin (**1**) or pelargonidin (**2**) protons. In the sugar region 5.56–3.23 ppm, as anomeric proton signals were shifted to lower magnetic field (**1**: δ5.56 and 5.23, **2**: δ5.52 and 5.19) with large coupling constant (J = 7.6–7.8 Hz), the glycosylated sugars were shown to be the β-anomers. The large coupling constant of the sugar ring protons (**1**: J = 6.3–11.7 Hz, **2**: J = 6.4–12.2 Hz) showed the glucosyl residues are in the pyranose form. The low field shifts of both C-6 methylene protons. (**1**: δ4.41, 4.05 and δ4.34, 4.15, **2**: δ4.31, 4.11 and δ4.37, 4.01) of glucoses indicated that both primary CH_2OH were acylated. The methyl protons of both acetic acids in **1** and **2** were assigned at δ2.04 and 1.94 (**1**) or δ1.99 and 1.91 (**2**). Furthermore, both glucose moieties in **1** and **2** were confirmed to be attached to 3-OH and 5-OH of the anthocyanidins by DIFNOE experiments. Therefore, the new pigments **1** and **2** are the 3-*O*-(6-*O*-(acetyl)-β-D-glucopyranoside)-5-*O*-(6-*O*-(acetyl)-β-D-glucopyranosides) of cyanidin and perlargonidin, respectively.

Pigment **3**: the FAB mass spectrum gave its molecular ion at 723 m/z $[M]^+$ in good agreement with the mass

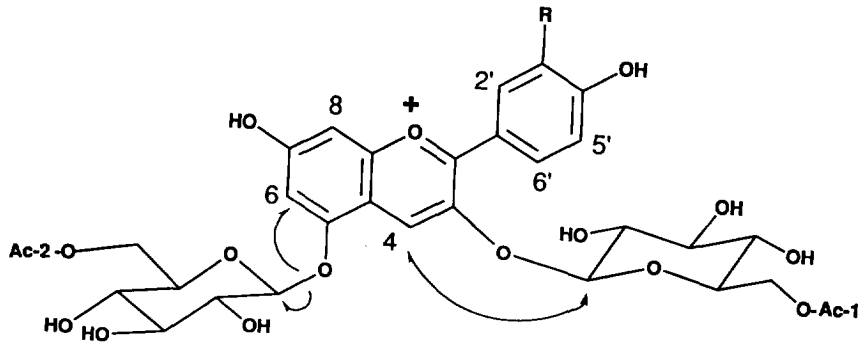

Fig. 1. Diacylated *Verbena* anthocyanins: **1** R = OH, Ac-1 = Ac-2 = acetyl; **2** R = H, Ac-1 = Ac-2 = acetyl; **3**R = H, Ac-1 = Malonyl, Ac-2 = acetyl. Observed NOEs are indicated by arrows.

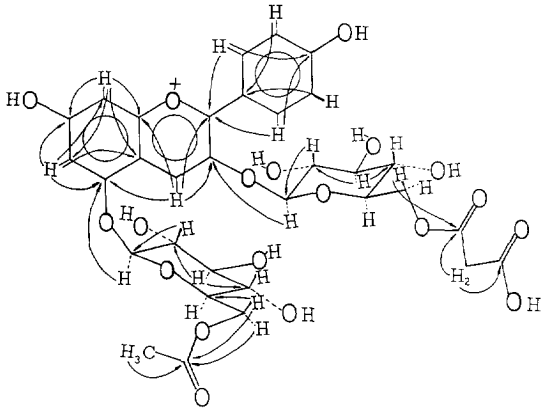

Fig. 2. HMBC correlations in pigment **3**.

calculated for $C_{32}H_{35}O_{19}$. Analysis of the ^1H and ^{13}C NMR, DQF-COSY and HOHAHA spectra [8–10] indicated the presence of one molecule of pelargonidin, two of glucose, one each of acetic acid and malonic acid. Because all observed vicinal J values of ring protons of both glucose units were *ca* 6–10 Hz, these must be β-D-glucopyranose forms (Table 2). The four methylene protons of both glucose units assigned by DQF-COSY and difference HOHAHA experiments were shifted to a low magnetic field (δ4.41, 4.18 and δ4.41, 4.11), indicating that these two hydroxyl groups (OH-6) of 3-Glc and 5-Glc are acylated.

In order to determine the attachment positions of the glucose and acyl units in the pigment molecule, DIFNOE, HMBC [11], HMQC [12] and DEPT [13] spectra were measured. By irradiation using the DIFNOE method of the H-1 at δ5.57, an NOE effect was observed at H-4 of pelargonidin. Also by irradiation of the H-1 at δ5.22, an NOE effect was observed at H-6 of pelargonidin. Therefore, the 3-Glc and 5-Glc were attached to OH-3 and OH-5 of pelargonidin, respectively, through a glycosidic bond (Fig. 1). The attachments of acetic and malonic acids were determined to be acylated with both 6-OH of 3- and 5-Glcs by HMQC and HMBC spectral analysis. The carbonyl C-1 of malonyl unit was correlated with H-6b of 3-Glc, and carbonyl C of acetyl unit was correlated with H-6a and b of 5-Glc as shown in Fig. 2. Therefore, **3** is the new anthocyanin, pelargonidin 3-*O*-(6-*O*-(malonyl)-β-D-glucopyranoside)-5-*O*-(6-*O*-(acetyl)-β-D-glucopyranoside). This is the first report of a diacylated anthocyanin with two different aliphatic acids [6, 7].

EXPERIMENTAL

Materials. Deep red–purple flower petals of *Verbena hybride*, strain UR-RP-3 were collected in the garden of Minami-Kyushu University.

Extraction and isolation. The fr. petals (*ca* 220 g) were extracted with 3% HCO_2H, and filtered. In the crude extract 15 peaks of anthocyanin were detected by HPLC. The filtrate was adsorbed on HP-20 resin column, and successively washed with 0.5% HCO_2H. The pigments were eluted with HCO_2H–MeOH–H_2O (1:10:9), concd, and further purified with LH-20 CC, PC (BAW and 15% HOAc) and HPLC (HCO_2H solvent system). Pigment **1** (25 mg), **2** (150 mg) and **3** (16 mg) were isolated.

Analysis. Characterization of these 3 anthocyanins was carried out by standard procedures involving deacylation with alkaline and acid hydrolysis [1–3, 14]. FAB mass spectra were measured in JMX GX-400 (JEOL), ^1H (400 MHz) and ^{13}C (100.53 MHz) NMR on JNM GX-400 (JEOL) in TFA-*d*1–DMSO-d_6 (9:1) with int. standard TMS. Alalyt. HPLC was performed on a Inertsil ODS-2 column (4.6 × 250 mm) at 35° with a flow rate of 0.8 ml min^{-1} monitoring at 520 nm. Solvent systems used were as follows; a linear gradient elution for 40 min from 25 to 85% B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in A (1.5% H_3PO_4).

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