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Acylated anthocyanins from the blue-violet flowers of *Anemone coronaria*

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Abstract

Five polyacylated anthocyanins were isolated from blue-violet flowers of *Anemone coronaria* 'St. Brigid'. They were identified as delphinidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(malonyl)-β-D-galactopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucuronopyranoside], and its demalonylated form, delphinidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(2-*O*-tartaryl)malonyl)-β-D-galactopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucuronopyranoside], and its cyanidin analog as well as delphinidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(2-*O*-(tartaryl)malonyl)-β-D-galactopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside].

Keywords: *Anemone coronaria*; Ranunculaceae; Blue-violet flower color; Acylated anthocyanins; Delphinidin and cyanidin 3-glucosylgalactoside-7-glucoside-3′-glucuronides; Caffeic acid; Malonic acid; Tartaric acid; Anthocyanin and tartaric acid disubstituted malonate

1. Introduction

As a part of our continuing work on flower color variation due to acylated anthocyanins in ornamental Ranunculaceae, we reported previously the isolation of 11 acylated anthocyanins in the flowers of *Consolida armeniaca* (Saito et al., 1996), *Ranunculas asiaticus* (Toki et al., 1996), and *Delphinium hybridum* (Saito et al., 1998), as well as the presence of three acylated pelargonidin glycosides in the scarlet flowers of *Anemone coronaria* 'St. Brigid Red' (Toki et al., 2001). However, no information is available on acylated anthocyanins in the bluish flowers of this plant, except regarding the presence of delphinidin and cyanidin glycosides (Lawrence et al., 1939; Harborne, 1967). As an extension of our work, we investigated the structures of the blue-violet pigments in the flowers of *Anemone coronaria* 'St. Brigid', and found five polyacylated anthocyanins, Anemone Blue Anthocyanins **1–4** and Anemone Purple Anthocyanin 1 (**5**). Among these five compounds, three (**3–5**) carried malonic acid moieties, similar to Anemone Red Anthocyanin-3 (Toki et al., 2001). In this paper, we report the isolation and structure elucidation of these five polyacylated anthocyanins.

2. Results and discussion

In a survey of the anthocyanins in the blue-violet flowers of *Anemone coronaria* 'St. Brigid' by high-performance liquid chromatography (HPLC), eight anthocyanin peaks were observed in the flowers extracted with 5% HOAc. Five pigments **1–5**, were isolated from the crude extracts and purified using Diaion HP-20 and Sephadex LH20 column chromatography, paper chromatography and HPLC, according to the procedures described previously (Toki et al., 2001). The relative concentrations of **1–5** in the extracts were 6, 15, 36, 6 and 8% respectively, as determined by HPLC analysis. The R_f values, R_t (min) and spectral properties of these five pigments **1–5** are shown in Table 1. Upon acid hydrolysis, **1–4** gave delphinidin, whereas **5** gave cyanidin as aglycones. All five pigments **1–5** contained galactose and glucose, in addition to glucuronic acid being

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detected in the products of **1–3** and **5** upon acid hydrolysis. Upon alkaline hydrolysis, all five pigments **1–5** gave caffeic acid, and **2–5** gave malonic acid as a second acid component. Additionally, tartaric acid was detected in the products of **3–5** as a third acid component.

2.1. Anemone Blue Anthocyanin *1*

The FAB mass spectrum of this pigment **1** showed a molecular ion [M^+] at *m/z* 1289 ($C_{67}H_{61}O_{34}$), composed of delphinidin with two molecules each of glucose (Glc) and caffeic acid, and one molecule each of galactose (Gal) and glucuronic acid (Glr). Based on HRMS, its elemental components were further confirmed (see Section 3.5). The ^{1}H NMR spectrum [500 MHz in DCl–DMSO-d_6 (1:99)] also supported the presence of delphinidin, three kinds of sugar molecules and two molecules of caffeic acid each with a *trans* configuration, as suggested by the large coupling constants (caffeic acid I: $J = 15.9$ Hz, and caffeic acid II: $J = 15.9$ Hz; Table 2). The four anomeric proton signals of the sugar moieties appeared at 5.59 ppm (*d*, $J = 7.0$ Hz, H-1 of 3-Gal), 5.32 ppm (*d*, $J = 7.0$ Hz, H-1 of 7-Glc A), 5.08 ppm (*d*, $J = 7.6$ Hz, H-1 of 3′-Glr), and 4.87 ppm (*d*, $J = 7.9$ Hz, H-1 of 2″-Glc B), The coupling constants (*J*) of the 3-Gal moiety were observed at 8.9 Hz (4.17 ppm, *d*, H-2″), 2.0 and 12.5 Hz (3.61 ppm, *dd*, H-3″), and broad singlet signals were also seen for H-4″ (3.54 ppm) and H-5″ (3.55 ppm), and the 3′-Glr moiety, $J = 9.8$ Hz (4.06 ppm, *d*, H-5). Therefore, the sugar structures of 3-Gal and 3′-Glr were identified to be β-D-galactose and β-D-glucuronic acid, respectively, and the other two sugars were determined to be β-D-glucose. These four sugars were confirmed to have β-D-pyranose forms based on their coupling constants. Using negative difference NOE (NOEDIF) experiments (Kondo et al., 1987), the linkages and/or positions of attachments of the sugar and caffeic acid units in **1** were determined as follows (Fig. 1). The hydroxyl group at the 3-position of delphinidin is glycosylated with galactose (3-Gal), 7-OH is glycosylated with glucose (7-Glc A), and 3′-OH is glycosylated with glucuronic acid (3′-Glr), since three pairs of strong NOEs were observed between H-4 of delphinidin and H-1 of 3-Gal, H-6 and H-8 of delphinidin and H-1 of 7-Glc A, and also H-2′ of delphinidin and H-1 of 3′-Glr by irradiation at each anomeric proton of three sugars and measurement of NOEDIF spectra. Based on its ^{1}H–^{1}H COSY spectrum, a methine proton signal at 4.17 ppm (*t*, $J = 8.9$ Hz) for H-2″ of 3-Gal was found to be shifted to a lower magnetic field, indicating that Glc B is attached to 2″-OH of 3-Gal through a glycosyl bond. This evidence was confirmed by strong NOEs between H-1 of 2″-Glc B and H-2″ of 3-Gal upon irradiation at H-1 of 2″-Glc B (see Fig. 1).

Since the three characteristic signals of two methylene protons of 7-Glc A (4.29 and 4.50 ppm, H6-a and H6-b) and also one methine proton of 2″-Glc B (4.58 ppm, H-2) were shifted to a lower magnetic field in the ^{1}H NMR spectrum, 6-OH of 7-Glc A and 2-OH of 2″-Glc B were thought to be acylated with caffeic acids I and II, respectively. This evidence was also confirmed by measurements of NOEDIF spectra between H-1 of 7-Glc A and five protons of caffeic acid I, and also between H-1 of 2″-Glc B and five protons of caffeic acid II. Therefore, the structure of **1** was determined to be delphinidin

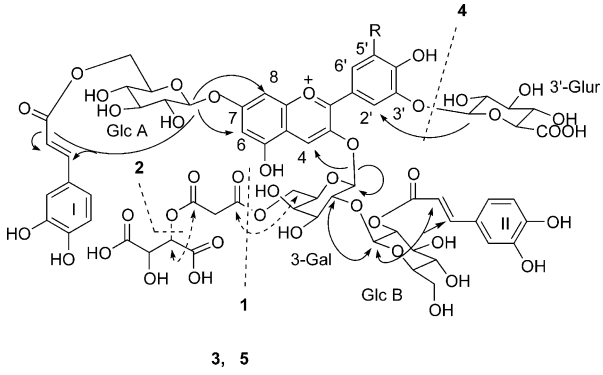

Fig. 1. Anemone Blue Anthocyanins and Anemone Purple Anthocyanin. Observed NOEs are indicated by arrows. Observed HMBCs are indicated by dotted arrows. **1**, R=OH: ABA-1; **2**, R=OH: ABA-2; **3**, R=OH: ABA-3; **4**, R=OH: ABA-4; **5**, R=H: APA-1.

Table 1
Chromatographic and spectral data for Anemone Blue Anthocyanins (ABA-1–4) and Anemone Purple Anthocyanin (APA-1)

Anthocyanins[a]	R_f values (×100)				R_t (min)	Spectral data on 0.1% HCl–MeOH			FAB–MS [M^+]
	BAW	BuH	1%HCl	HOAc-HCl		λ_{max} (nm)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)	
1	19	17	30	53	12.8	288, 332, 536	142	20	1289
2	20	19	29	56	15.6	288, 332, 539	131	25	1375
3	17	20	34	59	16.3	288, 332, 537	133	26	1507
4	24	25	35	63	16.8	288, 332, 524	117	28	1491
5	23	34	32	59	18.4	285, 329, 543	184	24	1331

[a] **1**. Delphinidin 3-[2-(2-(caffeoyl)glucosyl)galactoside]-7-[6-(caffeoyl)glucoside]-3′-[glucuronide], (ABA-1); **2**. delphinidin 3-[2-(2-(caffeoyl)glucosyl)-6-(malonyl)galactoside]-7-[6-(caffeoyl)glucoside]-3′-[glucuronide], (ABA-2); **3**. delphinidin 3-[2-(2-(caffeoyl)glucosyl)-6-(2-(tartaryl)malonyl)galactoside]-7-[6-(caffeoyl)glucoside]-3′-[glucuronide], (ABA-3); **4**. delphinidin 3-[2-(2-(caffeoyl)glucosyl)-6-(2-(tartaryl)malonyl)galactoside]-7-[6-(caffeoyl)-glucoside], (ABA-4); **5**. cyanidin 3-[2-(2-(caffeoyl)glucosyl)-6-(2-(tartaryl)malonyl)galactoside]-7-[6-(caffeoyl)glucoside]-3′-[glucuronide], (APA- 1).

3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-β-D-galactopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucuronopyranoside], which has not been found previously in plants (Harborne and Baxter, 1999).

2.2. Anemone Blue Anthocyanin 2

The FAB mass spectrum of **2** showed a molecular ion [M^+] at *m*/*z* 1375 ($C_{60}H_{63}O_{37}$). The elemental components of this pigment were confirmed by HRMS. The results of the mass analysis indicated that **2** is composed of delphinidin with two molecules each of glucose and caffeic acid, and one molecule each of galactose and glucuronic acid. In addition, **2** has one molecule of malonic acid as an aliphatic acid component. To elucidate the structure of this pigment **2**, its demalonyl derivative was prepared by treating **2** with 1% HCl–MeOH according to the procedures reported previously (Saito and Harborne, 1992; Saito et al., 1994). This revealed that demalonylated **2** was identical to **1** based on HPLC analysis. Furthermore, the ^{1}H NMR spectrum (500 MHz) of **2** was superimposable to that of **1**, except for the proton signals due to malonic acid and galactose moieties (Tables 2 and 3 and Fig. 1). The detailed structure of **2** was elucidated based on its ^{1}H–^{1}H COSY spectrum and the NOEDIF spectral techniques described for **1**. The chemical shifts of 11 aromatic protons and their coupling constants in the delphinidin and caffeic acid moieties were assigned as shown in Tables 2 and 3. Four olefinic protons of caffeic acids I and II were also assigned (Table 2), and their large coupling

Table 2
^{1}H NMR data for Anemone Blue Anthocyanins-1–4 and Anemone Purple Anthocyanin-1 (500 MHz, DMSO-d_6–CF_3COOD, TMS as an internal standard)

	ABA-1	ABA-2	ABA-3	ABA-4	APA-1[a]
Aglycone					
4	8.74 *s*	8.88 *s*	8.91 *s*	8.73 *s*	8.92 *s**1
6	7.08 *d* (1.8)	7.07 *brs*	7.07 *brs*	7.04 *brs*	6.95 *brs**2
8	7.25 *d* (1.8)	7.25 *brs*	7.25 *brs*	7.14 *brs*	7.29 *brs**3
2′	7.78 *d* (1.8)	7.79 *d* (1.5)	7.79 *brs*	7.76 *s*	8.07 *s**4
5′	—	—	—	—	7.24 *d* (8.5)*5
6′	8.12 *d* (1.8)	8.13 *d* (1.5)	8.13 *brs*	7.76 *s*	8.68 *d* (8.5)*6
*Caffeic acid-I***					
2	6.97 *d* (1.8)	6.97 *brs*	6.98 *brs*	6.92 *brs*	7.05 *brs**15
5	6.69 *d* (7.9)	6.69 *d* (8.2)	6.69 *d* (7.9)	6.69 *d* (8.2)	6.79 *d* (7.9)*16
6	6.85 *dd* (1.8, 7.9)	6.85 *brd* (8.2)	6.86 *brd* (7.9)	6.87 *brd* (8.2)	6.95 *brd* (7.9)*17
α	6.33 *d* (15.9)	6.22 *d* (15.9)	6.22 *d* (15.9)	6.13 *d* (15.9)	6.24 *d* (15.9)*14
β	7.42 *d* (15.9)	7.41 *d* (15.9)	7.43 *d* (15.9)	7.41 *d* (15.9)	7.42 *d* (15.9)*18
*Caffeic acid-II***					
2	7.01 *d* (1.8)	6.99 *brs*	6.98 *brs*	7.00 *brs*	6.93 *brs**20
5	6.79 *d* (8.2)	6.80 *d* (8.2)	6.80 *d* (7.9)	6.77 *d* (8.2)	6.65 *d* (8.2)*21
6	6.89 *dd* (1.8, 8.2)	6.94 *brd* (8.2)	6.94 *brd* (7.9)	6.87 *brd* (8.2)	6.83 *brd* (8.2)*22
α	6.25 *d* (15.9)	6.26 *d* (15.9)	6.22 *d* (15.9)	6.13 *d* (15.9)	6.24 *d* (15.9)*19
β	7.42 *d* (15.9)	7.43 *d* (15.9)	7.41 *d* (15.9)	7.41 *d* (15.9)	7.42 *d* (15.9)*23
*3-Galactose***					
1	5.59 *d* (7.0)	5.63 *d* (6.7)	5.62 *d* (6.7)	5.58 *d* (7.0)	5.49 *d* (7.6)
2	4.17 *t* (8.9)	4.21 *t* (8.2)	4.22 *m*	4.21 *m*	4.24 *m**10
3	3.61 *dd* (2.0, 12.5)	3.66 *dd* (2.0, 8.9)	3.65 *dd* (2.0, 11.3)	3.63 *d* (11.0)	3.69 *brd* (11.3)
4	3.54 *brs*	3.75 *brs*	3.76 *brs*	3.75 *brs*	3.74 *brs*
5	3.55 *brs*	4.14 *m*	4.16 *m*	4.14 *m*	4.14 *m*
6a	3.47 *m*	4.17 *m*	4.18 *m*	4.14 *m*	4.23 *m*
6b	3.54 *m*	4.20 *m*	4.20 *m*	4.21 *m*	4.26 *m*

Coupling constants (*J* in Hz) in parentheses. **Assigned by ^{1}H–^{1}H COSY and NOEDIF.

[a] Observed major HMBC correlations (δ values of ^{13}C are in Table 4): *1 (H-4): C2, C9, C5, C3; *2 (H-6): C7, C9, C10, C8; *3 (H-8): C7, C5, C10, C6; *4 (H-2′): C2, C4′, C6′, C3; *5 (H-5′): C3′, C1′, C2′; *6 (H-6′): C2, C4′, C2′; *7 (Mal-H-α,β): Mal-CO1, Mal-CO2, Tar-CO2, 3-Gal-C6″; *8 (tartaric acid-H1): Tar-CO2, Mal-CO2; *9 (tartaric acid-H2): Tar-CO1; *10 (galactose-H2) Gal-C1; *11 (glucuronic acid-H1): C3′; *12 (glucuronic acid-H5): Glur-CO; *13 (2″-glucose-H2): 2″-Glc-C1, Caf-II-CO; *14 (caffeic acid-I-Hα): Caf-I-C1, Caf-I-CO; *15 (caffeic acid-I-H2): Caf-I-C6, Caf-I-C3, Caf-I-C4; *16 (caffeic acid-I-H5): Caf-I-C1, Caf-I-C3, Caf-I-C2, Caf-I-C4; *17 (caffeic acid-I-H6): Caf-I-C2, Caf-I-Cβ, Caf-I-C4; *18 (caffeic acid-I-Hβ): Caf-I-C2, Caf-I-C6, Caf-I-CO; *19 (caffeic acid-II-Hα): Caf-II-C1, Caf-II-CO; *20 (caffeic acid-II-H2): Caf-II-C6, Caf-II-Cβ, Caf-II-C4; *21 (caffeic acid-II-H5): Caf-II-C1, Caf-II-C3, Caf-II-C2, Caf-II-C4, Caf-II-Cβ; *22 (caffeic acid-II-H6): Caf-II-C2, Caf-II-C4, Caf-II-Cβ; *23 (caffeic acid-II-Hβ): Caf-II-C2, Caf-II-C6, Caf-II-CO.

constants ($J = 15.9$ and 15.9 Hz) indicated that both caffeic acids have a *trans* configuration. The chemical shifts of sugar protons were observed in the region of 5.63–2.69 ppm (observed coupling constants 6.4–10 Hz). The chemical shifts of the four anomeric protons of the sugar components were determined to be at 5.63 ppm (*d*, $J = 6.7$ Hz, H-1 of 3-Gal), 5.32 ppm (*d*, $J = 6.4$ Hz, H-1 of 7-Glc A), 5.08 ppm (*d*, $J = 7.6$ Hz, H-1 of 3′-Glr), and 4.87 ppm (*d*, $J = 7.9$ Hz, H-1 of 2″-Glc B), indicating that these four sugar molecules were in the β-D-pyranose form. An analysis of the ^1H–^1H COSY and NOEDIF spectra revealed that the H-2″ proton signal of 3-Gal was shifted to a lower magnetic field at 4.21 ppm (*t*, $J = 8.2$ Hz). Therefore, Glc B was bonded to 2″-OH of 3-Gal, as in the case of **1**. The three characteristic proton signals shifted to a lower magnetic field were assigned to two methylene protons of 7-Glc A (4.29 and 4.47 ppm, H-6a and H-6b) and a methine proton of 2″-Glc B (4.82 ppm, H-2), indicating that 6-OH of 7-Glc A and 2-OH of 2″-Glc B were acylated with caffeic acid, respectively, as in the case of **1**. These linkages were confirmed by an analysis of NOEDIF spectra upon irradiations of the anomeric protons of 7-Glc and 2″-Glc B. Additionally, two new lower-shifted proton signals were assigned to the methylene protons of 3-Gal (4.17 and 4.20 ppm, 6″-Ha and 6″-Hb). Therefore, we concluded that one carboxyl group of malonic acid was attached to 6″-OH of 3-Gal in pigment. Thus, the structure of **2** was determined to be delphinidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(mal-

Table 3
^1H NMR data for Anemone Blue Anthocyanins-1–4 and Anemone Purple Anthocyanin-1 (500 MHz, DMSO-d_6–CF_3COOD, TMS as an internal standard)

	ABA-1	ABA-2	ABA-3	ABA-4	ApA-1[a]
*7-Glucose (A)***					
1	5.32 *d* (7.0)	5.32 *d* (6.4)	5.33 *d* (6.1)	5.24 *d* (7.0)	5.35 *d* (7.0)
2	3.40 *m*	3.39 *m*	3.40 *m*	3.35 *m*	3.42 *m*
3	3.44 *m*	3.38–3.41 *m*	3.43 *m*	3.41 *m*	3.36–3.42 *m*
4	3.39 *m*		3.38 *m*	3.37 *m*	
5	3.88 *m*	3.88 *m*	3.89 *m*	3.82 *m*	3.90 *m*
6a	4.29 *m*	4.29 *m*	4.29 *m*	4.30 *m*	4.30 *m*
6b	4.50 *m*	4.47 *d* (10.6)	4.48 *d* (10.7)	4.39 *d* (11.0)	4.50 *m*
*3′-Glucuronic acid***					
1	5.08 *d* (7.6)	5.08 *d* (7.6)	5.08 *d* (7.6)		5.21 *d* (7.5)*11
2	3.44 *m*	3.44 *m*	3.40 *m*		3.40 *m*
3	3.39 *m*	3.42 *m*	3.45 *m*		3.45 *m*
4	3.54 *t* (8.9)	3.54 *t* (9.2)	3.54 *t* (9.5)		3.53 *m*
5	4.06 *d* (9.8)	4.06 *d* (9.5)	4.06 *d* (9.8)		4.09 *d* (9.8)*12
*3–2″-Glucose (B)***					
1	4.87 *d* (7.9)	4.87 *d* (7.9)	4.86 *d* (8.2)	4.81 *m*	5.20 *d* (7.9)
2	4.58 *m*	4.82 *t* (8.2)	4.58 *t* (8.2)	4.56 *m*	4.60 *t* (8.6)*13
3	3.26 *t* (9.2)	3.25 *t* (9.2)	3.25 *t* (9.2)	3.15 *t* (9.0)	3.40 *m*
4	3.03 *t* (9.2)	3.05 *t* (9.2)	3.03 *t* (9.2)	2.95 *t* (9.0)	3.39 *m*
5	2.73 *m*	2.69 *m*	2.69 *m*	2.52 *m*	3.19 *m*
6a	3.09 *dd* (5.2, 11.3)	3.13 *m*	3.12 *m*	3.03 *m*	3.33–3.40 *m*
6b	3.16 *d* (10.1)	3.16 *m*	3.16 *m*	3.09 *d* (10.0)	
*Malonic acid***					
CH_2		3.33 *d* (15.9)	3.47 *d* (16.2)	3.47 *d* (15.9)	3.49 *d* (16.2)*7
		3.28 *d* (15.9)	3.52 *d* (16.2)	3.52 *d* (15.9)	3.54 *d* (16.2)*7
*Tartaric acid***					
1			5.22 *d* (2.4)	5.20 *d* (2.4)	5.23 *d* (2.7)*8
2			4.54 *d* (2.4)	4.52 *d* (2.4)	4.54 *d* (2.7)*9

Coupling constants (*J* in Hz) in parentheses. **Assigned by ^1H–^1H COSY and NOEDIF.

[a] Observed major HMBC correlations (δ values of ^{13}C are in Table 4): *1 (H-4): C2, C9, C5, C3; *2 (H-6): C7, C9, C10, C8; *3 (H-8): C7, C5, C10, C6; *4 (H-2′): C2, C4′, C6′, C3; *5 (H-5′): C3′, C1′, C2′; *6 (H-6′): C2, C4′, C2′; *7 (Mal-H-α,β): Mal-CO1, Mal-CO2, Tar-CO2, 3-Gal-C6″; *8 (tartaric acid-H1): Tar-CO2, Mal-CO2; *9 (tartaric acid-H2): Tar-CO1; *10 (galactose-H2) Gal-C1; *11 (glucuronic acid-H1): C3′; *12 (glucuronic acid-H5): Glur-CO; *13 (2″-glucose-H2): 2″-Glc-C1, Caf-II-CO; *14 (caffeic acid-I-Hα): Caf-I-C1, Caf-I-CO; *15 (caffeic acid-I-H2): Caf-I-C6, Caf-I-C3, Caf-I-C4; *16 (caffeic acid-I-H5): Caf-I-C1, Caf-I-C3, Caf-I-C2, Caf-I-C4; *17 (caffeic acid-I-H6): Caf-I-C2, Caf-I-Cβ, Caf-I-C4; *18 (caffeic acid-I-Hβ): Caf-I-C2, Caf-I-C6, Caf-I-CO; *19 (caffeic acid-II-Hα): Caf-II-C1, Caf-II-CO; *20 (caffeic acid-II-H2): Caf-II-C6, Caf-II-Cβ, Caf-II-C4; *21 (caffeic acid-II-H5): Caf-II-C1, Caf-II-C3, Caf-II-C2, Caf-II-C4, Caf-II-Cβ; *22 (caffeic acid-II-H6): Caf-II-C2, Caf-II-C4, Caf-II-Cβ; *23 (caffeic acid-II-Hβ): Caf-II-C2, Caf-II-C6, Caf-II-CO.

onyl)-β-D-galactopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucuronopyranoside], which is another newly discovered pigment in plants (Harborne and Baxter, 1999).

2.3. Anemone Blue Anthocyanin 3

The FAB mass spectrum of **3** showed a molecular ion [M^+] at *m*/*z* 1507 ($C_{64}H_{67}O_{42}$). The elemental components of **3** were confirmed by HRMS. The results indicated that **3** is composed of delphinidin with two molecules each of glucose and caffeic acid, and one molecule each of galactose, glucuronic acid, and malonic acid, similar to **2**. Furthermore, one molecule of tartaric acid, as another aliphatic acid, was detected in pigment **3**. The presence of tartaric acid was unambiguously confirmed based on a careful investigation of its acid and alkaline hydrolysis products. The demalonyl derivative of **3**, which was prepared by treating **3** with 1% HCl–MeOH, as was used for the demalonylation of **2**, was identical with **1** by HPLC analysis. Since both tartaric and malonic acids were absent from the demalonylation product of **3**, these acids were thought to be bonded to demalonylated **3** through ester bonds. The ^{1}H NMR spectrum of **3** was superimposable to that of **2**, except for the signals of tartaric and malonic acid moieties (Table 3 and Fig. 1). All of the aromatic protons in delphinidin and caffeic acid were assigned based on an analysis of its ^{1}H–^{1}H COSY spectrum and confirmed by measurements of NOEDIF spectra, as shown in Table 2. In both caffeic acid moieties (I and II), all four olefinic proton signals exhibited large coupling constants (J = 15.9 and 15.9 Hz), supporting the presence of two caffeic acids with *trans* configurations. The signals of its sugar moieties were observed in the region of 5.62–2.69 ppm. Four anomeric protons appeared at 5.62 ppm (*d*, J = 6.7 Hz, H-1 of 3-Gal), 5.33 ppm (*d*, J = 6.1 Hz, H-1 of 7-Glc A), 5.08 ppm (*d*, J = 7.6 Hz, H-1 of 3′-Glr), and 4.86 ppm (d, J = 8.2 Hz, H-1 of 2″-Glc B). Based on the observed proton coupling constants of the sugar moieties, these sugar molecules were of the β-D-pyranoside form. The linkages of these sugars, acids, and delphinidin were confirmed and/or determined as follows. The large chemical shift of 2″-H (4.22 ppm) of 3-Gal, assigned at 4.22 ppm (*m*) by an analysis of its ^{1}H–^{1}H COSY spectrum, clearly indicated the presence of ether linkages between 1-OH of 2″-Glc B and 2-OH of 3-Gal. Acylation patterns were suggested based on the observation of three characteristic protons at 4.29 and 4.48 ppm (H-6a and H-6b of 7-Glc A) and one methine proton of H-2 in the 2″-Glc B moiety (4.58 ppm, *t*, J = 8.2 Hz). These results supported the presence of ester bonds between 6-OH of 7-Glc A and caffeic acid I, and also between 2″-OH of Glc B and caffeic acid II, which were confirmed by the measurements of NOEDIF spectra, as in the case of **2**. Furthermore, the presence of one more ester bond was confirmed by the observation of two methylene protons shifted to a lower magnetic field at 4.18 and 4.20 ppm, which were assigned to H-6″a and -6″b of 3-Gal based on an analysis of NOEDIF spectra. Thus, we assumed that 6-OH of 3-Gal was attached to malonic acid, as in **2**. The chemical shifts of 1-H and 2-H of tartaric acid were assigned at 5.22 and 4.54 ppm based on the NOEDIF spectra. Since the former proton (T-H1, 5.22 ppm) was shifted to a lower magnetic field, as can be seen in the case of T-H1 and -H2 of Anemone Red Anthocyanin 4 (5.67 and 5.60 ppm), in which the 1-OH and 2-OH were bonded to a carboxyl group (Toki et al., 2001), we reasonably deduced that 1-OH of tartaric acid of **3** was acylated with a carboxyl group of malonic acid by making an ester bond. Since the methylene proton chemical shifts (3.47 and 3.52 ppm) of malonic acid in **3** were shifted to a lower magnetic field in comparison with those (3.28 and 3.33 ppm) of **2** (monoester structure), we considered that malonic acid of **3** was esterified to both 6″-OH of 3-Gal and 1-OH of tartaric acid (see in Fig. 1). Similar δ values were observed for the methylene protons: δ 3.65, 3.58 and 3.50, 3.45 for the diester of malonic acid in Anemone Red Anthocyanins 4 and 3 (Toki et al., 2001). The proton coupling constants of tartaric acid (J = 2.4 and 2.4 Hz) supported the notion that the hydroxyl groups of tartaric acid were in a *syn* orientation. Therefore, the structure of ABA-3 was determined to be delphinidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(2-*O*-(tartaryl)malonyl)-β-D-galactopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-gluco-pyranoside]-3′-*O*-[β-D-glucuronopyranoside], which has not been isolated previously in plants (Harborne and Baxter, 1999).

2.4. Anemone Blue Anthocyanin 4

The FAB mass spectrum of **4** showed a molecular ion [M^+] at *m*/*z* 1331 ($C_{58}H_{59}O_{36}$). This result indicated that **4** was composed of delphinidin with two molecules each of glucose and caffeic acid, and one molecule each of galactose, malonic acid and tartaric acid. The ^{1}H NMR spectrum of **4** was very similar to that of **3**, except for the signals for the glucuronic acid moiety. The detailed structure of **4** was elucidated based on measurements of its ^{1}H NMR spectra, including 2D COSY and NOEDIF spectral techniques, as was the case for **3**. Eleven aromatic proton signals of delphinidin and caffeic acid were assigned based on an analysis of its ^{1}H–^{1}H COSY spectrum, as shown in Table 2. Four olefinic protons of caffeic acids I and II were also assigned at 6.13 and 7.41 ppm (*d*, J = 15.9 and 15.9 Hz, caffeic acid I), and 6.13 and 7.41 ppm (*d*, J = 15.9 and 15.9 Hz, caffeic acid II). The large coupling constants of these olefinic protons indicated that both molecules of caffeic acid have *trans* configurations. The signals of three anomeric protons of

the sugars appeared at 5.58 ppm (*d*, $J=7.0$ Hz, H-1 of 3-Gal), 5.24 ppm (*d*, $J=7.0$ Hz, H-1 of 7-Glc A), and 4.81 ppm (*m*, H-1 of 2″-Glc B). Based on the observed coupling constants, these three sugars had β-D-pyranose forms. A proton signal (4.21 ppm) shifted to a lower magnetic field was assigned to H-2″ of 3-Gal, supporting the notion that 2″-glucose (2″-Glc B) was linked to 2″-OH of the 3-Gal residue. This bonding was confirmed by an analysis of 2D COSY and NOEDIF spectra. The three proton signals shifted to a lower magnetic field were assigned to two methylene protons (4.30 and 4.39 ppm, H-6a and H-6b of 7-Glc A) and a methine proton (4.56 ppm, H-2 of 2″-Glc B), indicating that 6-OH of 7-Glc A and 2-OH of 2″-Glc B were acylated with caffeic acids I and II, respectively. Both linkages were confirmed by an analysis of their NOEDIF spectra. Additionally, two proton signals were shifted to a lower magnetic field at 4.14 and 4.21 ppm, similar to those in **3**, and assigned to methylene protons of 3-Gal (H-6″a and H-6″b), suggesting that 6″-OH of 3-Gal was acylated with malonic acid. Furthermore, H-1 of tartaric acid was shifted to a lower magnetic field at 5.20 ppm (*d*, $J=2.4$ Hz). Therefore, 1-OH of tartaric acid appeared to be acylated with malonic acid. This result was further confirmed by the observation of the methylene protons of malonic acid at 3.47 and 3.52 ppm. These chemical shifts were closely related to those in **3** (3.47 and 3.52 ppm), but were shifted to a magnetic field lower than those for the mono-ester of malonic acid (3.28 and 3.33 ppm), as observed in **2**. Consequently, the structure of **4** was determined to be delphinidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(2-*O*-(tartaryl)malonyl)-β-D-galactopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]. This is the first time this pigment **4** has been reported in plants (Harborne and Baxter, 1999).

2.5. Anemone Purple Anthocyanin 5

The molecular ion [M^+] of **5** was observed at *m*/*z* 1491 ($C_{64}H_{67}O_{41}$), indicating that the components of **5** were identical to those of **3**, except for the aglycone part. **5** was shown to have cyanidin instead of the delphinidin unit in **5**. The elemental components of **5** were confirmed by HRMS, where its molecular ion was seen as a Na salt (see Section 3.5). The ^{1}H NMR spectrum of **5** was superimposable to that of **3**, except for the signals of its aglycone (cyanidin) moiety (Table 2). Six proton signals of cyanidin and 10 proton signals of caffeic acid were assigned as shown in Table 2. Two sets of olefinic proton signals in the caffeic acid moieties exhibited large coupling constants ($J=15.9$ Hz for caffeic acid I and 15.9 Hz for caffeic acid II), which indicated that both caffeic acids have *trans* configurations. The signals of four anomeric protons appeared at 5.49 ppm (*d*, $J=7.6$ Hz, H-1 of 3-Gal), 5.35 ppm (*d*, $J=7.0$ Hz, H-1 of 7-Glc A), 5.21 ppm (*d*, $J=7.5$ Hz, H-1 of 3′-Glr), and 5.20 ppm (*d*, $J=7.9$ Hz, H-1 of 2″-Glc B), and the observed coupling constants of these sugars were 7.0–11.3 Hz, supporting the notion that these sugars had β-D-pyranose forms. The linkage between 2″-OH of 3-Gal and 1-OH of 2″-Glc B was confirmed by the large chemical shift (4.24 ppm) of 2″-H of 3-Gal, as observed for the other pigments **1–4**. Five characteristic proton signals shifted to a lower magnetic field were also assigned to four methylene protons of 3-Gal (4.23 and 4.26 ppm, H-6″a and H-6″b) and 7-Glc A (4.30 and 4.50 ppm, H-6″a and H-6″b) and one methine proton of 2″-Glc B (4.60 ppm, *t*, $J=8.6$ Hz, H-2). Therefore, 6-OH of 7-Glc A and 2-OH of 2″-Glc B appeared to be acylated with caffeic acid, and 6″-OH of 3-Gal was acylated with malonic acid, as in the case of **3**. The chemical shift of H-1 of the tartaric acid moiety appeared at 5.23 ppm (*d*, $J=2.7$ Hz), indicating that 1-OH of tartaric acid was acylated with malonic acid. These linkages were confirmed by measurements of the NOEDIF spectra of the sugar and tartaric acid moieties using the same process as for **3**. Furthermore, this structure was confirmed by an analysis of ^{13}C NMR, ^{1}H–^{13}C COSY and HMBC spectra. Since these spectra showed overlapping ^{13}C signals in the sugar region, which were also relatively weak, we could not completely assign the ^{13}C chemical shifts due to the sugar moieties (Table 4). The ^{13}C chemical shifts of **1–4** are also shown in Table 4, and the main HMBC correlations between proton and ^{13}C signals in **5** are shown in Table 2. The linkages of 3-Gal and CO-1 of malonic acid, and those of malonic acid and tartaric acid were ascertained by the HMBC correlation between H-1 of tartaric acid and CO-2 of malonic acid (Table 3). Thus, the structure of **5** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(2-*O*-(tartaryl)malonyl)-β-D-galactopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucuronopyranoside], which is a new pigment in plants (Harborne and Baxter, 1999).

Harborne (1967), found two different kinds of anthocyanidin glycosides in *Anemone coronaria* cultivars, for example, pelargonidin 3-lathyroside in scarlet flowers and delphinidin 3-diglycosides in blue flowers. Additionally, our previous study (Toki et al., 2001), we found that the scarlet flower of this plant contained three acylated pelargonidin 3-lathyrosides, Anemone Red Anthocyanins 2–4, along with pelargonidin 3-lathyroside itself (1). In contrast the blue-violet flower of *A. coronaria* 'St. Brigid' contained three acylated delphinidin 3-glucosylgalactoside-7-glucoside-3′-glucuronides **1–3** and one acylated delphinidin 3-glucosyl-galactoside-7-glucoside **4**, in which malonic and caffeic acids were found as the acid components. Notably, **3** and **4** also contained tartaric acid as an additional acid component. Moreover, a cyanidin analog was also found in this flower, and its structure was determined to be

Table 4
^{13}C NMR data for Anemone Blue Anthocyanins-1–3 and Anemone Purple Anthocyanin-1 (125.78 MHz, DMSO-d_6–CF_3COOD, δ values in ppm)

	APA-1*	ABA-1	ABA-2	ABA-3
Anthocyanidin				
2	163.1	163.0	162.4	163.1
3	145.4	145.3	145.2	145.2
4	134.0	134.5	135.6	133.8
5	155.0	154.9	154.7	154.7
6	102.6	102.0	102.4	102.3
7	165.3	165.2	165.2	165.2
8	94.1	92.0	94.1	93.5
9	156.9	158.5	158.8	158.2
10	112.6	112.6	112.4	112.6
1′	119.5	118.4	118.3	118.4
2′	116.8	115.2	115.2	115.2
3′	145.8	148.2	148.2	148.3
4′	156.0	157.0	157.6	156.8
5′	114.0	148.1	148.2	146.3
6′	131.9	133.5	132.4	132.4
Caffeic acid I				
1	125.7	125.8	125.8	125.8
2	115.1	114.8	114.8	114.8
3	145.6	145.5	145.6	145.6
4	148.3	148.3	148.3	148.4
5	115.9	115.9	115.9	115.9
6	121.3	121.3	121.4	121.4
α	114.0	113.8	113.9	113.4
β	144.7	144.6	144.8	144.7
C=O	166.5	166.6	166.6	166.8
Caffeic acid II				
1	125.4	125.4	125.4	125.4
2	114.7	114.6	114.6	114.6
3	145.4	145.4	145.4	145.4
4	148.3	148.1	148.2	148.2
5	115.8	115.9	115.9	115.9
6	121.0	121.0	121.1	121.1
α	114.0	113.8	113.8	113.8
β	144.7	144.6	144.6	144.6
C=O	165.8	166.0	166.0	166.0
Malonic acid				
C=O	165.8		167.8	165.9
CH_2	40.7		41.2	40.6
C=O	165.8		166.9	165.8
Tartaric acid				
C=O	171.7			171.6
C1	74.2			74.2
C2	69.7			69.6
C=O	167.9			167.9
*Sugars***				
3-Gala-1	101.0	101.0	100.9	100.9
3-Gala-6	63.2	59.9	63.0	63.0
7-Gluc-1	99.9	99.8	99.8	99.8
7-Gluc-6	64.9	63.0	64.1	64.5
3′-Gluc acid-1	101.0	101.1	102.2	102.1
3′-Gluc acid-5	77.5	78.5	78.4	78.2
3′-Gluc acid-CO	170.1	170.0	170.0	170.0
2″-Gluc-1	99.9	100.0	99.7	99.8
2″-Gluc-6	61.5	59.9	60.3	60.3

* Assigned by the analysis of ^1H–^{13}C COSY (HMQC) and HMBC spectra.

** The rest of the sugar ^{13}C resonances were appeared at the following δ values and these δ values of the ^{13}C resonances could not be assigned. APA-1: 76.2, 75.9, 75.5, 75.5, 74.7, 74.2, 73.9, 73.9, 73.4, 73.1, 72.8, 71.2, 69.4, 68.7. ABA-3: 76.4, 76.0, 75.9, 75.2, 74.2, 73.9, 73.8, 73.0, 72.8, 72.7, 72.1, 71.2, 69.2, 67.8. ABA-2: 76.4, 76.0, 75.9, 75.2, 74.2, 73.8, 73.1, 72.8, 71.8, 71.2, 69.6, 69.2, 67.6. ABA-1: 76.5, 75.9, 75.5, 75.0, 74.1, 73.8, 73.0, 72.4, 71.2, 69.5, 68.0, 67.5.

identical to that of **3**, except for the aglycone (cyanidin). Regarding the acylation pattern of *Anemone* anthocyanins known thus far in blue-violet cultivars, all are acylated with two molecules of caffeic acid at 6-OH of 7-Glc A and 2-OH of 2″-Glc B, and **3–5** have diester structures in the malonic acid residue of these pigments. These diester structures are formed by two carboxyl groups of malonic acid forming ester bonds between both 6″-OH of 3-Gal and 1-OH of tartaric acid.

3. Experimental

3.1. Plant material

The tubers of *Anemone coronaria* 'St. Brigid Blue' were purchased from Takii Nursery Co., Ltd, Kyoto, Japan, and grown in the experimental farm of Minami Kyusyu University. Fresh blue-violet flowers (chromaticity value $b/a=-1.6$, Violet-Blue 96B by R.H.S. color chart) were collected in spring and dried at 45 °C. These flowers were stored in a refrigerator.

3.2. Extraction and purification of Anemone anthocyanins

The dried petals (ca. 50 g) were immersed in 5% HOAc overnight at room temperature. The extract was subjected to Diaion HP-20 column chromatography (CC) and washed with H_2O. The pigments were eluted with MeOH–HOAc–H_2O (75:5:20). After concentration, the eluate was fractionated with Sephadex LH-20 CC using MeOH–HOAc–H_2O (6:1:12). The frs. were further purified by PC [BAW, *n*-BuOH–HOAc–H_2O (4:1:2) and 15% HOAc] and prep. HPLC. Prep. HPLC was performed on a Hitachi 6200 system using an Inertsil ODS-2 column (20×250 mm) with HOAc–H_2O as solvent. Anemone Blue Anthocyanin **1** (12 mg), Anemone Blue Anthocyanin **2** (27 mg), Anemone Blue Anthocyanin **3** (105 mg), Anemone Blue Anthocyanin **4** (8 mg) and Anemone Purple Anthocyanin 1 (**5**) (22 mg) were obtained from this extract.

3.3. Analysis of anthocyanins

Pigments were identified by standard procedures involving deacylation with base and acid hydrolysis (Harborne, 1984). TLC was carried out on microcrystalline cellulose (Avicel SF, Funakoshi). The solvents used for anthocyanins were BAW, AHW (HOAc–HCl–H_2O, 15:3:82), BuHCl (*n*-BuOH–2N HCl, 1:1), and 1% HCl; those used for sugars were BAW, *i*-PrOH–*n*-BuOH–HOAc-H_2O (7:1:2), and PhOH–H_2O (4:1), and those for acids were BAW, EtOH–HOAc–H_2O (3:1:1) and EtOH–H_2O–NH_4OH (16:3:1). HPLC was performed as described previously using an Inertsil ODS-2 column (4.6×250 mm) at 35 °C with a flow rate of 0.8 ml/min and monitoring at 520 nm (Toki et al., 2001). The solvent was applied as a linear gradient from 20 to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN) in solvent A (1.5% H_3PO_4) with 40-min elution. NMR spectra were measured in CF_3COOD–DMSO-d_6 (1:9) or DCl–DMSO-d_6 (1:99) and recorded at 500 MHz on a JEOL JNM GX-500 instrument for ^1H and ^{13}C NMR and at 125.78 MHz. Chemical shifts were reported in δ values relative to TMS. FAB–MS were recorded on JEOL SX-102 spectrometer in the positive mode with a magic bullet and negative mode with a glycerol matrix. Chromatographic and spectral (NMR, UV, FAB–MS) data of compounds **1–5** are shown in Tables 1–3.

3.4. Demalonylation or partial demalonylation of anthocyanins

Each acylated anthocyanin was dissolved in 1% HCl–MeOH solution and allowed to stand for 4–6 days at room temperature (Saito and Harborne, 1992; Saito et al., 1994). The demalonylated or partially demalonylated anthocyanins were then analyzed by HPLC or TLC.

3.5. High-resolution FABMS

Anemone Blue Anthocyanin **1**: HR–FABMS calc. for $C_{57}H_{61}O_{34}$: 1289.3044. Found: 1289.3109. Anemone Blue Anthocyanin **2**: HR–FABMS calc. for $C_{60}H_{63}O_{37}$: 1375.3048. Found: 1375.3130. Anemone Blue Anthocyanin **3**: HR–FABMS calc. for $C_{64}H_{67}O_{42}$: 1507.3107. Found: 1507.3120. Anemone Purple Anthocyanin 1 (**5**): HR–FABMS calc. for $C_{64}H_{66}O_{41}Na$: 1513.3025. Found: 1513.3030.

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