

ACYLATED CYANIDIN GLYCOSIDES FROM THE PURPLE-RED FLOWERS OF *ANEMONE CORONARIA*

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Abstract – Two novel polyacylated cyanidin glycosides were isolated from the purple-red flowers of *Anemone coronaria* ‘St. Brigid’ together with a known polyacylated cyanidin glycoside (Anemone Purple Anthocyanin 1). These novel pigments were identified as cyanidin 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 demalonyl derivative.

INTRODUCTION

In the course of our anthocyanin investigation on the flower pigments of *Anemone coronaria*, we have already found eight acylated anthocyanins such as three pelargonidin glycosides in the scarlet flowers,¹ and also four delphinidin glycosides and one cyanidin glycoside in the blue-violet flowers.² As the continuation of our work, we further investigated the purple-red flower pigments of *Anemone coronaria* ‘St. Brigid’, and found two novel polyacylated anthocyanins together with a known polyacylated cyanidin glycoside named as Anemone Purple Anthocyanin 1.² In this paper, we report the isolation and structure elucidation of these polyacylated anthocyanins.

RESULTS AND DISCUSSION

Three acylated anthocyanins, pigment (1), pigment (2) and pigment (3), were obtained from purple-red flowers of *Anemone coronaria* ‘St. Brigid’. By the analyses of TLC and HPLC, pigment (1) was

assumed to be Anemone Purple Anthocyanin 1 (APA-1)² as shown in Figure 1. Its structure was unambiguously confirmed by comparison of its ¹H NMR spectrum (Table 2) including 2D COSY and negative difference NOE (DIFNOE) with those of APA-1 directly.

Table 1. Chromatographic and spectral data for Anemone Purple anthocyanins (APA-1-3)

Anthocyanins*	Rf values(× 100)				Rt (min)	Spectral data in 0.1% HCl-MeOH			FAB-MS [M] ⁺
	BAW	BuH	1%HCl	HAc-HCl		λ _{max} (nm)	E _{acyl} /E _{vis} (%)	E ₄₄₀ /E _{vis} (%)	
1	32	22	36	63	16.8	288, 332, 524	117	28	1491
2	39	22	32	60	16.3	288, 332, 523	117	28	1359
3	38	21	32	59	13.6	287, 331, 522	125	28	1273

***1.** cyanidin 3-[2-(2-caffeoylglucosyl)-6-(2-tartarylmalonyl)galactoside]-7-[6-caffeoylglucoside]-3'-glucuronide (APA-1), **2.** cyanidin 3-[2-(2-caffeoylglucosyl)-6-malonylgalactoside]-7-[6-caffeoylglucoside]-3'-glucuronide (APA-2), **3.** cyanidin 3-[2-(2-caffeoylglucosyl)galactoside]-7-[6-caffeoylglucoside]-3'-glucuronide (APA-3)

The structures of novel polyacylated anthocyanins [pigments (**2**) and (**3**)] were determined as follows.

For the sake of convenience, these novel anthocyanins were named as Anemone Purple Anthocyanins 2 and 3 (APA-2 and APA-3), respectively.

Pigment-2 (APA-2): Upon acid hydrolysis, pigments (**2**) and (**3**) gave cyanidin as their aglycone, and galactose, glucose and glucuronic acid as their sugars. By alkaline hydrolysis, pigment (**3**) gave caffeic acid as its acid component. On the other hand, the same treatment of pigment (**2**) gave malonic acid in addition to caffeic acid as its acid components.

The structures of pigments (**2**) and (**3**) were elucidated based on the measurements of their ¹H NMR spectra [500 MHz in CF₃CO₂D-DMSO-d₆ (1:9)], including 2D COSY and negative difference NOE spectral techniques described previously.² The FABMS spectrum of pigment (**2**) showed its molecular ion [M⁺] at *m/z* 1359 (C₆₀H₆₃O₃₆), composed of cyanidin with two molecules each of glucose (Glc) and caffeic acid, and one molecule each of galactose (Gal), glucuronic acid (GlcUA) and malonic acid. The ¹H NMR spectrum of **2** also supported the presence of cyanidin, three kinds of sugar molecules, one molecule of malonic acid and two molecules of caffeic acid with *trans* configurations, as suggested by the large coupling constants (caffeic acid I: *J* = 15.9 Hz, and caffeic acid II: *J* = 15.9 Hz). The four anomeric proton signals of the sugar moieties appeared at 5.51 ppm (d, *J* = 7.9 Hz, H-1 of 3-Gal), 5.36 ppm (d, *J* = 6.7 Hz, H-1 of 7-Glc A), 5.18 ppm (d, *J* = 7.6 Hz, H-1 of 3'-GlcUA) and 5.24 ppm (d, *J* = 8.4 Hz, H-1 of 2''-Glc B). The coupling constants (*J*) of 3-Gal moiety were observed by 7.9 Hz (4.27 ppm, t, H-2''), and the broad singlet signal was also assigned to H-4'' (3.76 ppm).

The coupling constants of GlcUA moiety were 9.6 Hz (4.10 ppm, d, H-5) and 9.6 Hz (3.59 ppm, t, H-4). Therefore, the structures of 3-Gal and 3'-GlcUA were supposed to be β-D-galactose and β-D-glucuronic acid respectively, although the absolute configurations of those sugars could not be determined

unambiguously, due to their small amount available. The other two sugars were determined to be β -D-glucose. These sugars were confirmed to have β -pyranose forms based on their coupling constants. Using DIFNOE experiments, the linkages and/or positions of attachments of the sugar and acid units in pigment (2) were determined (Figure 1).

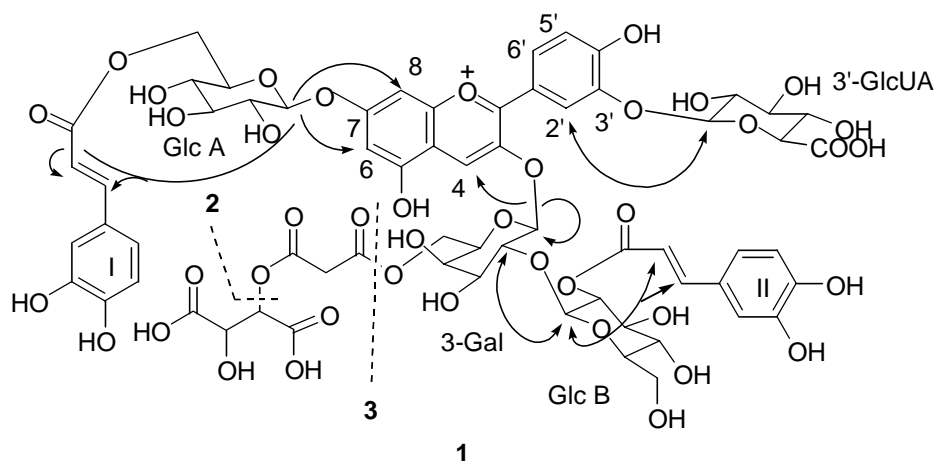


Figure. 1 Anemone Purple Anthocyanins (APAs).
Observed NOEs are indicated by arrows.

1 APA-1; 2 APA-2; 3 APA-3

The 3-OH of cyanidin was glycosylated with 3-Gal, 7-OH was glycosylated with 7-Glc A, and 3'-OH was glycosylated with GlcUA, since the three pairs of strong NOEs were observed between H-4 of cyanidin and H-1 of 3-Gal, H-6 and -8 of cyanidin and H-1 of 7-Glc A, and H-2' of cyanidin and H-1 of GlcUA by irradiation at each anomeric protons of three sugars or by irradiation at the above mentioned aromatic protons, respectively, in their DIFNOE spectra. Based on the ^1H - ^1H COSY spectrum of pigment (2), a methine proton signal at 4.27 ppm (t, $J = 7.9$ Hz) for H-2'' of 3-Gal was found to be shifted to a lower magnetic field than that (3.56 ppm) of the free 2-OH galactoside³ indicating that 2''-Glc B is attached to 2''-OH of 3-Gal through a glycosyl bond. This bonding was also confirmed by the measurement of DIFNOE spectra (Figure 1). The three characteristic signals of two methylene protons of 7-Glc A (4.25-4.28 and 4.55 ppm, H-6a and 6b) and also one methine proton of 2''-Glc B (4.64 ppm, H-2) were shifted to a lower magnetic field than those of non-acylated glucoside in the ^1H NMR spectrum.¹ This evidence was also confirmed by measurements of DIFNOE spectra between H-1 of 7-Glc A and α - and β -protons of caffeic acid II. Therefore, 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. Furthermore, two lower-shifted proton signals were assigned to the methylene protons (4.28 – 4.25 and 4.22 ppm, 6''-Ha and 6''-Hb) of 3-Gal. Therefore, we concluded that one carboxyl group of malonic acid was attached to 6''-OH of 3-Gal in this

pigment. Thus, the structure of pigment (2) (APA-2), was elucidated to be cyanidin 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, which has not been found previously in plants.^{2,4}

Table 2. ¹H NMR spectral data for Anemone Purple Anthocyanins (2) and (3) (500 MHz, DMSO-d₆-CF₃CO₂D, TMS as an internal standard).

	APA-1 () ²	APA-2 ()	APA-3 ()
Aglycone			
4	8.92 s	8.91 s	9.03 s
6	6.95 br s	6.85 d (1.8)	6.84 br s
8	7.29 br s	7.29 br s	7.03 d (1.8)
2'	8.07 br s	8.07 d (2.1)	8.07 d (1.8)
5'	7.24 d (8.5)	7.24 d (8.9)	7.23 d (8.9)
6'	8.68 br d (8.5)	8.70 dd (2.1, 8.9)	8.70 dd (1.8, 8.9)
Caffeic acid I			
2	7.05 br s	7.03 d (1.8)	7.03 d (1.8)
5	6.79 d (7.9)	6.78 d (8.2)	6.79 d (8.2)
6	6.95 br d (7.9)	6.95 dd (1.8, 8.2)	6.96 br d (8.2)
α	6.24 d (15.9)	6.25 d (15.9)	6.25 d (15.9)
β	7.42 d (15.9)	7.44 d (15.9)	7.45 d (15.9)
Caffeic acid II			
2	6.93 br s	6.90 d (1.8)	6.88 d (1.4)
5	6.65 d (8.2)	6.63 d (8.2)	6.63 d (8.2)
6	6.83 br d (8.2)	6.81 br d (8.2)	6.79 m
α	6.24 d (15.9)	6.25 d (15.9)	6.25 d (15.9)
β	7.42 d (15.9)	7.42 d (15.9)	7.45 d (15.9)
3-Galactose (3-Gal)			
1	5.49 d (7.6)	5.51 d (7.9)	5.46 d (7.9)
2	4.24 m	4.27 t (7.9)	4.24 t (7.9)
3	3.69 m	3.72 m	3.68 m
4	3.74 br s	3.76 br s	3.74 br s
5	4.14 m	4.16 m	3.79 m
6a	4.23 m	4.28-4.25	3.68 m
6b	4.26 m	4.22 m	3.56 m
7-Glucose A (7-Glc A)			
1	5.35 d (7.0)	5.36 d (6.7)	5.36 d (6.7)
2	3.42 m	3.41 m	3.43 m
3	3.36-3.42	3.34-3.42	3.41 m
4	3.36-3.42	3.34-3.42	3.39 m
5	3.90 m	3.91 m	3.91 m
6a	4.30 m	4.25-4.28	4.26 m
6b	4.50 m	4.55 br d (10.7)	4.56 br d (10.7)
2''-Glucose B (2''-Glc B)			
1	5.20 d (8.2)	5.24 d (8.4)	5.21 d (8.4)
2	4.60 t (8.2)	4.64 t (8.4)	4.65 t (8.4)
3	3.40 m	3.41 m	3.43 m
4	3.39 m	3.34-3.43	3.48 m
5	3.19 m	3.20-3.24	3.35-3.49
6a	3.33-3.40	3.20-3.24	3.35-3.49
6b	3.33-3.40	3.20-3.24	3.35-3.49
3'-Glucuronic acid (3'-GlcUA)			
1	5.21 d (7.5)	5.18 d (7.6)	5.18 d (8.2)
2	3.40 m	3.43 m	3.49 t (8.2)
3	3.45 m	3.30-3.42	3.39-3.45
4	3.53 m	3.59 t (9.6)	3.59 t (9.5)
5	4.09 d (9.8)	4.10 d (9.6)	4.10 d (9.5)
Malonic acid			
-CH ₂ -	3.49 d (16.2)	3.46 d (16.7)	
	3.54 d (16.2)	3.48 d (16.7)	
Tartaric acid			
	5.23 d (2.7)		
	4.54 d (2.7)		

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

Pigment-3 (APA-3): The FABMS spectrum of pigment (3) showed its molecular ion [M^+] at m/z 1273 ($C_{57}H_{61}O_{33}$), which indicated that pigment (3) is composed of cyanidin with two molecules each of glucose and caffeic acid, and one molecule each of galactose and glucuronic acid. The 1H NMR spectrum of pigment (3) was superimposable to that of pigment (2), except for the proton signals of malonic acid and galactose moieties (Table 2 and Figure 1). The detailed structure of pigment (3) was elucidated based on its 1H - 1H COSY spectrum and the DIFNOE spectral techniques described for pigment (2). Consequently, the proton chemical shifts of pigment (3) were confirmed to be identical with those of pigment (2) except for the malonyl moiety. However, two proton chemical shifts [4.25-4.28 and 4.22 ppm, H-6a and 6b of 3-Gal, pigment (2)] were shifted in the higher-magnetic field (3.68 and 3.56 ppm), and assigned to two methylene protons of 3-Gal, indicating that the 6-OH of 3-Gal is free from malonyl group. Moreover, pigment (3) was compared directly to be the demalonyl derivative of pigment (2), prepared by treatment of pigment (2) with 1% HCl-MeOH according to the procedures reported previously,⁵ which obviously revealed that both pigments were identical in all respects. Thus, the structure of pigment (3) (APA-3) was identified to be cyanidin 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 is another newly discovered pigment in plants.^{2,4}

EXPERIMENTAL

General. NMR spectra were recorded in DMSO- d_6 -CF₃CO₂D (9:1) with TMS as internal standard using a JEOL JNM GX-500 spectrometer. FABMS spectra were recorded on a JEOL JMS SX-102 spectrometer. UV-VIS spectra were obtained on an MPS-2000 (Shimadzu) spectrophotometer in 0.1% HCl-MeOH. HPLC was performed on an Inertsil ODS-2 column (4.6×250 mm for analysis and 20×250 mm for preparation) at 35°C, and monitoring at 530 nm for anthocyanins, and at 320 nm for UV-absorbing compounds. Solvent systems employed for analysis were as follows: a linear gradient elution for 40 min from 25-85% solvent B (1.5% H₃PO₄, 20% AcOH, 25% MeCN) in solvent A (1.5% H₃PO₄). Preparative HPLC was carried out with AcOH-H₂O as solvent by an isocratic elution. Thin layer chromatography (TLC) was performed on Merck pre-coated cellulose plates using BAW (*n*-BuOH-AcOH-H₂O, 4:1:5), BuH (*n*-BuOH-2M HCl, 1:1), 1% HCl and HAc-HCl (AcOH-HCl-H₂O, 15:3:82) for anthocyanins, BAW, *i*-PrOH-*n*-BuOH-H₂O (7:1:2), PhOH-H₂O (4:1), and *i*-PrOH-H₂O (4:1) for sugars, and BAW, EtOAc-AcOH-H₂O (3:1:1) and EtOH-H₂O-NH₄OH (16:3:1) for acids.

Plant materials. The tubers of *Anemone coronaria* 'St. Brigid' were purchased from Takii Nursery Co., Ltd, Kyoto, Japan, and grown in the experimental farm of Minami Kyusyu University. Fresh purple-red flowers were collected in spring and dried at 45°C. These flowers were stored in a refrigerator.

Extraction and isolation. Dry purple-red flowers (100 g) of *Anemone coronaria* 'St. Brigid' were immersed in 10 L of 5% AcOH at rt for overnight. The filtrate extract was absorbed on Diaion HP-20 column, washed with H₂O and eluted with AcOH-MeOH-H₂O (5:75:20). The eluate was concentrated and fractionated on Sephadex LH20 column chromatography using AcOH-MeOH-H₂O (1:6:12). The purple-red fractions were further purified by TLC [BAW, *n*-BuOH-AcOH-H₂O (4:1:2) and 15% AcOH], and preparative HPLC according to the procedures described previously.^{1,2} Pure pigment (1) (112 mg), pigment (2) (20 mg) and pigment (3) (28 mg) were obtained from the extract.

Fresh flowers (0.1 g) and dry flowers (0.01 g) of this plant were immersed with 30 mL of 20% MeOH containing 1.5% H₃PO₄ or 5% AcOH, respectively, for several hours at rt. The purple-red anthocyanin solutions were obtained and analyzed by TLC and HPLC. Three anthocyanins of pigments (1-3) were confirmed to be present in the extracts of both flower materials.

Demalonylation of 2. Pigment (2) was dissolved in 1% HCl-MeOH solution and allowed to stand for 4-6 days at ambient temperature according to the standard procedure.⁵ Acid hydrolysis (2N HCl in MeOH at 60-80 °C for 1.5 h) and alkaline hydrolysis (2N NaOH solution at rt 1 h) of pigments were carried out according to the standard procedure.^{5,6}

Spectral data. Pigment (2): ¹H NMR data was described in the Table 2, and chromatographic and UV-VIS spectral data were indicated in Table 1. FABMS (*m/z*): 1359 [M⁺] for C₆₀H₆₃O₃₆. Pigment (3): ¹H NMR data was described in the Table 2, and chromatographic and UV-VIS spectral data were indicated in Table 1. FABMS (*m/z*): 1273 [M⁺] for C₅₇H₆₁O₃₃.

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