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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 ^{1}H 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)

	Rf values(×100)				Rt	Spectral data in 0.1% HCl-MeOH			FAB-MS
Anthocyanins*	BAW	BuH	1%HCl	HAc-HCl	(min)	λ_{max}(nm)	E_{acyl}/E_{vis}(%)	E_{440}/E_{vis}(%)	$[M]^+$
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 ^{1}H NMR spectra [500 MHz in CF_3CO_2D-DMSO-d_6 (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 ($C_{60}H_{63}O_{36}$), 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 ^{1}H 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).

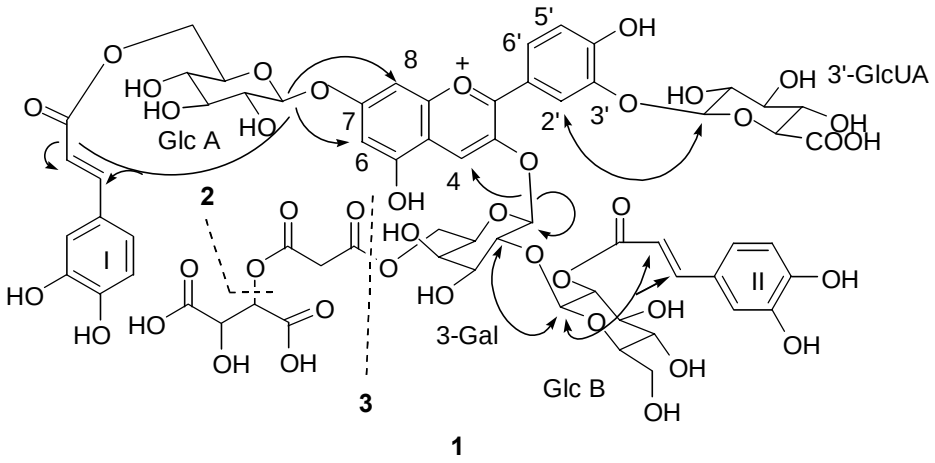

Figure. 1 Anemone Purple Anthocyanins (APAs).
Observed NOEs are indicated by arrows.
1 APA-1; **2** APA-2; **3** APA-3

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 ^{1}H-^{1}H 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 galactoside[3] 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 ^{1}H 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. ^{1}H NMR spectral data for Anemone Purple Anthocyanins (**2**) and (**3**) (500 MHz, DMSO-d_6-CF_3CO_2D, TMS as an internal standard).

	APA-1 (δ) [2]	APA-2 (δ)	APA-3 (δ)
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) 3.54 d (16.2)	3.46 d (16.7) 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 ^{1}H 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 ^{1}H-^{1}H 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-CF_3CO_2D (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% H_3PO_4, 20% AcOH, 25% MeCN) in solvent A (1.5% H_3PO_4). Preparative HPLC was carried out with AcOH-H_2O as solvent by an isocratic elution. Thin layer chromatography (TLC) was performed on Merck pre-coated cellulose plates using BAW (*n*-BuOH-AcOH-H_2O, 4:1:5), BuH (*n*-BuOH-2M HCl, 1:1), 1% HCl and HAc-HCl (AcOH-HCl-H_2O, 15:3:82) for amthocycanins, BAW, *i*-PrOH-*n*-BuOH-H_2O (7:1:2), PhOH-H_2O (4:1), and *i*-PrOH-H_2O (4:1) for sugars, and BAW, EtOAc-AcOH-H_2O (3:1:1) and EtOH-H_2O-NH_4OH (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 H_2O and eluted with AcOH-MeOH-H_2O (5:75:20). The eluate was concentrated and fractionated on Sephadex LH20 column chromatography using AcOH-MeOH-H_2O (1:6:12). The purple-red fractions were further purified by TLC [BAW, *n*-BuOH-AcOH-H_2O (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% H_3PO_4 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**): ^{1}H 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 $C_{60}H_{63}O_{36}$. Pigment (**3**): ^{1}H 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 $C_{57}H_{61}O_{33}$.

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