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Triacylated cyanidin 3-(3^X-glucosylsambubioside)-5-glucosides from the flowers of *Malcolmia maritima*

Fumi Tatsuzawa [a,*], Norio Saito [b], Kenjiro Toki [a], Koichi Shinoda [c], Atsushi Shigihara [b], Toshio Honda [b]

[a] *Faculty of Horticulture, Minami-Kyushu University, Takanabe, Miyazaki 884-0003, Japan*
[b] *Faculty of Pharmaceutical Sciences, Hoshi University, Shinagawa, Tokyo 142-8501, Japan*
[c] *National Agricultural Research Center for Hokkaido Region, Sapporo, Hokkaido 062-8555, Japan*

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Abstract

Three acylated cyanidin 3-(3^X-glucosylsambubioside)-5-glucosides (**1**–**3**) and one non-acylated cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside (**4**) were isolated from the purple-violet or violet flowers and purple stems of *Malcolmia maritima* (L.) R. Br (the Cruciferae), and their structures were determined by chemical and spectroscopic methods. In the flowers of this plant, pigment **1** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-(β-D-glucopyranoside) as a major pigment, and a minor pigment **2** was determined to be the *cis*-*p*-coumaroyl isomer of pigment **1**. In the stems, pigment **3** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) as a major anthocyanin, and also a non-acylated anthocyanin, cyanidin 3-*O*-[2-*O*-(3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) was determined to be a minor pigment (pigment **4**). In this study, it was established that the acylation-enzymes of malonic acid has important roles for the acylation of 5-glucose residues of these anthocyanins in the flower-tissues of *M. maritima*; however, the similar enzymatic reactions seemed to be inhibited or lacking in the stem-tissues.

Keywords: *Malcolmia maritima* (L.) R. Br; Cruciferae; Acylated anthocyanins; Cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside; *p*-Coumaric acid; Sinapic acid; Malonic acid; Flower color

1. Introduction

Malcolmia maritima (L.) R. Br. (Virginia stock in English) is a plant species native mainly to the Mediterranean region, and cultivated as a popular annual garden plant with white, pink, purple-violet, and violet flowers.

In the continuing work on flower color variation due to acylated anthocyanins of the ornamental plants in the Cruciferae, we have already reported the distribution of structurally complicated acylated anthocyanins in the flowers of *Matthiola incana* (Saito et al., 1995, 1996), *Orychophragonus violaceus* (Honda et al., 2005) and *Cheiranthus cheiri*, *Lobularia maritima*, and *Lunaria annua* (Tatsuzawa et al., 2006, 2007). As part of our continuing work, we are interested in the structures of the floral anthocyanins of *M. maritima*, since anthocyanins of this plant have not been thoroughly studied.

In this paper, we report the structure elucidation of cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside, a novel cyanidin glycoside pattern, and also its three acylated anthocyanin derivatives in *M. maritima* of the Cruciferae.

2. Results and discussion

Four anthocyanin pigments (**1** –**4**) (Figs. 1 and 2) were found in the methanol–acetic acid–water (MAW: 4:1:5,

* Corresponding author. Tel./fax: +81 983 22 6835.
E-mail address: fumi@nankyudai.ac.jp (F. Tatsuzawa).

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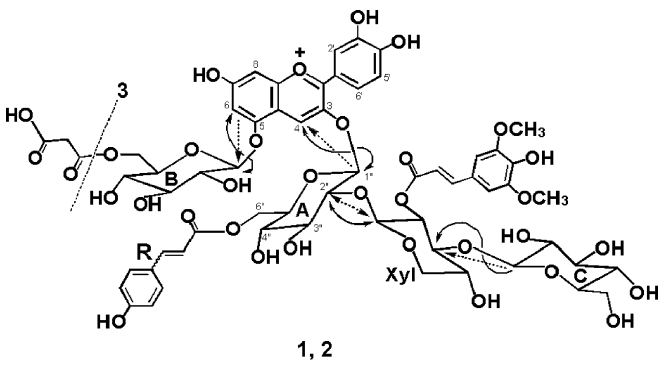
v/v/v) extracts from purple-violet to violet flowers, and also purple stems of *M. maritima* by HPLC analysis (Fig. 2). Their frequencies as estimated by HPLC were pigment **1** (64.4%), pigment **2** (8.6%) and pigment **3** (2.3%) in the MAW extract from their flowers. On the other hand, in the MAW extract from its stems pigment **1** (1.5%), pigment **3** (42.3%) and pigment **4** (3.8%) were obtained together with three other small pigment peaks.

These four pigments (**1**–**4**) were extracted from the mixture of flowers and stems with 5% HOAc, and purified using Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column chromatography (CC), preparative HPLC and TLC, according to the procedures described previously (Tatsuzawa et al., 2006, 2007). The chromatographical data and spectral properties of these pigments are shown in Table 1.

Acid hydrolysis of all four pigments (**1**–**4**) resulted in cyanidin, glucose, and xylose. Moreover, *p*-coumaric acid and sinapic acid were detected in the hydrolysates of pigments **1**, **2** and **3**, and also malonic acid was detected in the hydrolysates of pigments **1** and **2** by TLC (Harborne, 1984). Alkaline hydrolysis of pigments **1**–**3** resulted in only one deacylated anthocyanin, whose structure was identified to be pigment **4** by the analyses of TLC and HPLC (Table 1). The deacylanthocyanin was hydrolyzed with 2 N HCl by heating in a water bath of about 80–100 °C for 1, 5, and 10 min. By the analysis of TLC and HPLC, the four intermediary glucosides (3-glucoside, 5-glucoside, 3,5-diglucoside, 3-sambubioside-5-glucoside of cyanidin) as well as deacylanthocyanin were detected in three partial hydrolysates. From these results, the structure of the deacylanthocyanin (pigment **4**) was presumed to be a glucosyl cyanidin 3-sambubioside-5-glucoside. The structures of the four anthocyanins (pigments **1**–**4**) were further elucidated based on the analyses of their ^{1}H NMR spectra (500 MHz) and ^{13}C NMR (125.78 MHz) in DMSO-d_6–CFCOOD (9:1), including 2D COSY, NOESY, ^{1}H–^{13}C HMQC, ^{1}H–^{13}C HMBC and negative difference NOE (DIFNOE) spectra.



1, 2

Fig. 1. Pigment **1**, **2** and **3** in the flowers and stems of *Malcolmia mantima*. Pigment **1** and **3** R = *trans*, Pigment **2**, R = *cis*. Observed main NOE's are indicated by arrows. Observed HMBCs are indicated by dotted arrows.

2.1. Pigment 4 and deacyl pigments 1, 2 and 3

The molecular ions $[M]^{+}$ of pigment **4** and deacyl pigments **1**, **2** and **3** were observed at *m*/*z* 905 ($C_{38}H_{49}O_{25}$), indicating that these pigments are composed of cyanidin with three molecules of glucose and one molecule of xylose. The elemental components of pigment **4** were confirmed by measuring its high-resolution FABMS (HRMS), and the mass data obtained are summarized in Section 4.4. As mentioned before deacyl pigments **1**, **2** and **3** are identical with pigment **4**. Therefore, only the structure elucidation of pigment **4** was carried out as follows.

From the analysis of the ^{1}H NMR spectra of pigment **4**, the chemical shifts of six aromatic protons of cyanidin were assigned as shown in Table 2. Also four signals of anomeric protons were observed at δ 5.59 (*d*, $J = 7.6$ Hz, glu A), δ 5.09 (*d*, $J = 7.6$ Hz, glu B), δ 4.80 (*d*, $J = 8.0$ Hz, xylose) and δ 4.34 (*d*, $J = 7.7$ Hz, glu C). Based on the observed coupling constants (Table 2), the four sugars were assumed to have a β-pyranose form.

The linkages and/or positions of the attachment of the sugar groups in this pigment whose structure was presumed to be glucosyl cyanidin 3-sambubioside-5-glucoside by the analysis of its partial hydrolysate were mainly determined by using 2D COSY, NOEDIF and HMBC experiments. By NOEDIF experiments, strong NOEs were observed between H-4 of cyanidin and H-1 of glu A, and H-6 of cyanidin and H-1 of glu B indicating that cyanidin was glycosylated with glu A at OH-3 of cyanidin and also glycosylated with glu B at OH-5 of cyanidin. A proton signal (δ 3.94, *t*, $J = 8.2$ Hz) shifted to a lower magnetic field was assigned to H-2 of glu A by the analysis of 2D COSY spectrum of pigment **4**. This resonance was correlated to the ^{13}C-1 (δ 103.9) of xylose and also to the resonance of H-1 (xylose, δ 4.80) was correlated to the ^{13}C-2 (δ 80.5) of glu A in the HMBC spectrum, supporting that xylose was linked to OH-2 of glu A forming sambubiose. Furthermore, a strong NOE was observed at H-3 of xylose by the irradiation of NOEDIF experiment at H-1 of glu C indicating that glu C was linked to the OH-3 of xylose. This result was confirmed by the analysis of HMBC spectrum (Fig. 1).

Therefore, pigment **4** and deacyl pigments **1**–**3** were determined to be cyanidin 3-*O*-[2-*O*-(3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside, which is a new cyanidin glycoside in plants (Harborne and Baxter, 1999; Andersen and Jordheim, 2006).

2.2. Pigment 1

The molecular ion $[M]^{+}$ of pigment **1** was observed at *m*/*z* 1343 ($C_{61}H_{67}O_{34}$) indicating that pigment **1** is composed of cyanidin with three molecules of glucose, one molecule each of xylose, as well as sinapic, *p*-coumaric and malonic acids. The elemental components were confirmed by measuring its HRMS, and the mass data obtained were

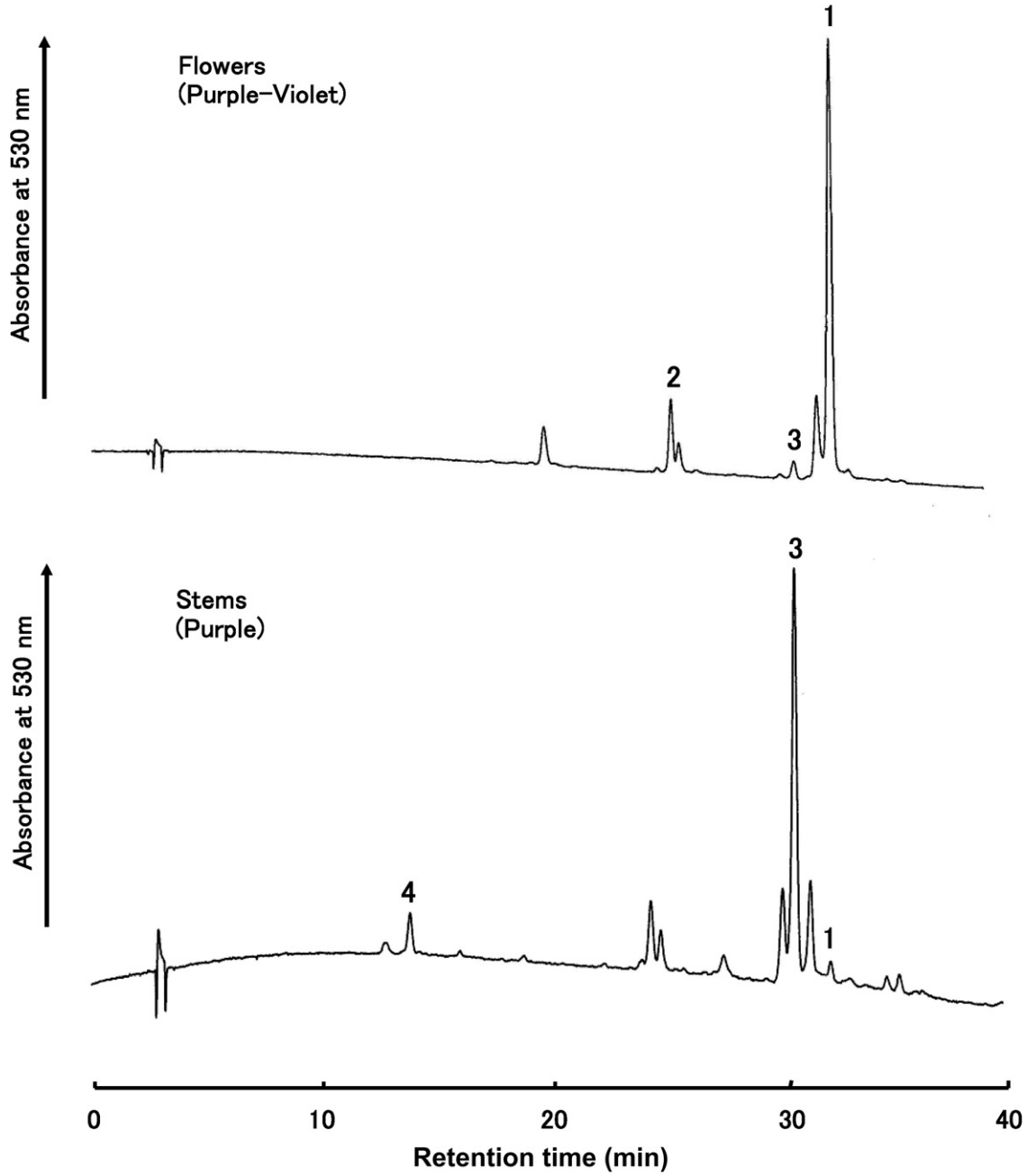


Fig. 2. Comparative HPLC profiles of anthocyanin extracts of *Malcolmia maritima*. **1**: cyanidin 3-[2-(2-(*trans*-sinapoyl)-3-(glucosyl)-xylosyl)-6-(*trans*-*p*-coumaroyl)- glucoside]-5-[6-(malonyl)-glucoside] **2**: cyanidin 3-[2-(2-(*trans*-sinapoyl)-3-(glucosyl)-xylosyl)-6-(*cis*-*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] **3**: demalonyl pigment **1**: cyanidin 3-[2-(2-(*trans*-sinapoyl)-3-(glucosyl)-xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-glucoside **4**: deacylanthocyanin: cyanidin 3-[2-(3-(glucosyl)-xylosyl)-glucoside]-5-glucoside.

summarized in Section 4.4. The detailed structure was elucidated based on the analysis of the NMR spectra.

The chemical shifts of 12 aromatic protons of cyanidin, sinapic acid and *p*-coumaric acid moieties with their coupling constants were assigned as shown in Table 2. Six protons were assigned to two methyl groups of sinapic acid. Four olefinic proton signals of sinapic and *p*-coumaric acids, with their large coupling constants ($J = 15.9$ and 15.9 Hz), showed that those acids were present in the *trans* configuration (Table 2). The chemical shifts of the sugar moieties were observed in the region of δ 5.71–3.06, where the four anomeric protons exhibited at δ 5.71 (*d*, $J = 8.0$ Hz, glu A), δ 5.20 (*d*, $J = 8.2$ Hz, xylose), δ 5.19 (*d*, $J = 8.0$ Hz, glu B), and δ 4.34 (*d*, $J = 8.0$ Hz, glu C), respectively. Based on the observed coupling constants (Table 2), the four sugars were assumed to be in the β-pyranose form. The linkages and/or positions of the attachments of the sugar and acyl groups were determined based on 2D COSY, NOESY, NOEDIF and HMBC experiments.

Table 1
Chromatographic and spectral properties of anthocyanins in flowers *of Malcolmia maritima*

Anthocyanins[a]	R_f value (×100)				Spectral data in 0.1% HCl–MeOH				HPLC R_t(min)	FAB mass $[M]^+$
	BAW	BuHCl	1% HCl	AHW	λ_{max} (nm)	E_{acyl}/E_{max}	E_{440}/E_{max}	$AlCl_3$		
Pigment **1**	18	21	41	81	532,320,298,282	112	14	+	31.9	1343
Pigment **2**	13	18	57	86	535,316,(298),279	121	18	+	25.0	1343
Pigment **3**	19	15	36	78	529,319,299,281	128	13	+	30.4	1257
Pigment **4**	3	4	32	64	526,278	–	14	+	13.2	905
Demalonyl pigment **1**	19	15	36	78	529,319,299,281	128	13	+	30.4	1257
Deacylanthocyanin	3	4	32	64	526,278	–	14	+	13.2	905
Cy 3-sambubioside-5-glucoside	5	6	23	58	528,273	–	17	+	12.5	–
Cy 3-sophoroside-5-glucoside	4	4	38	66	526,279	–	13	+	10.9	–

[a] Pigment **1**: cyanidin 3-[2-(2-(*trans*-sinapoyl)-3-(glucosyl)-xylosyl)-6-(*trans-p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside].Pigment **2**: cyanidin 3-[2-(2-(*trans*-sinapoyl)-3-(glucosyl)-xylosyl)-6-(*cis-p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside].Pigment **3**: demalonyl pigment 1: cyanidin 3-[2-(2-(*trans*-sinapoyl)-3-(glucosyl)-xylosyl)-6-(*trans-p*-coumaroyl)-glucoside]-5-glucoside.Pigment **4**: deacylanthocyanin: cyanidin 3-[2-(3-(glucosyl)-xylosyl)-glucoside]-5-glucoside.

A proton signal (δ 4.05 *dd*, $J = 8.0$ and 8.9 Hz) detected at a lower magnetic field, was assigned to H-2 of glu A by the analysis of its 2D COSY spectrum. This result supported that the xylose group was linked to OH-2 of glu A by forming sambubiose group at OH-3 of cyanidin. Five characteristic proton signals shifted to a lower magnetic field were also assigned to the methylene protons of glu A (δ 4.26 and 4.37, H-6a and b), glu B (δ 4.03 and 4.39, H-6a and b), and to a methine proton (δ 4.83, *dd*, $J = 8.2$ and 8.8 Hz, H-2) of xylose. Thus, three hydroxyl groups of the sugar moieties, OH-6s of glu A and B and OH-2 of xylose, were assumed to be acylated with three molecules of acids. By irradiation at H-1 of glu A, a strong NOE was observed at H-4 of cyanidin together with rather weak NOEs at H-2, −6 and -α of *p*-coumaric acid supporting that glu A was attached to OH-3 of cyanidin through a glycosidic bond. Moreover, glu A was presumed to be acylated with *p*-coumaric acid at OH-6.

In NOESY spectrum, the correlations between H-6 of cyanidin and H-1 of glu B, H-2 of glu A and H-1 of xylose, and H-3 of xylose and H-1 of glu C were observed, respectively, revealing the glycosylation patterns; such as OH-5 of cyanidin was glycosylated with glu B, OH-2 of glu A with xylose, and OH-3 of xylose with glu C. These results were confirmed by NOEDIF experiments. Irradiation of H-2 of xylose gave NOEs at H-α, -β, -2 and -6 of sinapic acid as well as H-1 and H-3 of xylose. Therefore, OH-2 of xylose was acylated with sinapic acid. These results were also confirmed by the analysis of HMBC spectrum (Fig. 1). Unfortunately, the linkage between glu B and malonic acid could not be confirmed by NOEDIF, NOESY and HMBC experiments. To confirm the acyl position of malonic acid, demalonyl pigment **1** was prepared according to the procedure described previously (Saito et al., 2007). The structure determination of demalonyl pigment **1** is performed as described in the following Section 2.3 by the same procedure as for pigment **3**, and the structure of demalonyl pigment **1** was confirmed to be identical with that of pigment **3** by the analysis of TLC, HPLC and spectroscopic data (Table 1). Since demalonyl pigment **1** was confirmed to be free from malonic acid at OH-6 of glu B, the structure of pigment **1** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-3-*O*-β-D-glucopyranosyl)-β-D-xylopyranosyl)-6-*O*- (*trans-p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside], which is a new anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Honda and Saito, 2002).

2.3. Pigment 3

The FAB mass spectrum of pigment **3** exhibited a molecular ion at 1257 *m*/*z* in agreement with the mass $C_{58}H_{65}O_{31}$ (1257.350). The elemental components were also confirmed by measuring its high-resolution FABMS: calc. for $C_{58}H_{65}O_{31}$: 1257.3501. Found: 1257.3523. As shown in Table 1, pigment **3** was identical with demalonyl pigment **1**, therefore, structure determination of pigment **3** was carried out as follows.

The ^{1}H NMR spectrum of pigment **3** showed the presence of one molecule of cyanidin, three molecules of glucose, and one molecule each of xylose, *p*-coumaric acid and sinapic acid. By the analysis of its NMR spectra, it was revealed that proton chemical shifts of pigment **3** were almost in agreement with those of pigment **1** except for the proton signals of glu B and malonic acid moieties (Tables 2 and 3). In particular, upfield shifts of methylene protons (δ 3.78–3.45, H-6a and b) of glu B in pigment **3** were observed in comparison to those (δ 4.03 and 4.39, H-6a and b) of pigment **1**. Therefore, OH-6 of glu B in pigment **3** was free from malonic acid. Other proton signals of pigment **3** were assigned by the same process as described for pigment **1** (Tables 2 and 3). Thus, pigment **3** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-6-*O*- (*trans-p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside), which is a new anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and

Table 2
^{1}H NMR spectroscopic data (δ) of anthocyanins isolated from *Malcolmia maritima* (500 MHz, DMSO-d_6–CF_3COOD, TMS as an internal standard)

	Pigment **1**	Pigment **2**	Pigment **3**	Pigment **4**
Cyanidin				
4	8.71 *s*	8.56 *s*	8.67 *s*	8.79 *s*
6	6.99 br*s*	6.86 *d*(2.2)	6.93 br*s*	6.95 *d*(1.8)
8	7.02 br*s*	7.18 *d*(2.2)	6.96 br*s*	7.08 *d*(1.8)
2′	8.01 br*s*	7.96 *d*(2.2)	7.96 *d*(2.2)	8.03 *d*(2.2)
5′	7.07 *d*(8.6)	7.06 *d*(8.3)	7.02 *d*(8.9)	7.02 *d*(8.9)
6′	8.47 br*d*(8.6)	8.41 *dd*(2.2, 8.3)	8.41 *dd*(2.2, 8.9)	8.34 *dd*(2.2, 8.9)
p-coumaric acid				
2,6	7.30 *d*(8.0)	7.29 *d*(8.3)	7.33 *d*(8.6)	
3,5	6.71 *d*(8.0)	6.50 *d*(8.3)	6.73 *d*(8.6)	
α	6.25 *d*(15.9)	5.69 *d*(12.8)	6.21 *d*(15.9)	
β	7.35 *d*(15.9)	6.46 *d*(12.8)	7.30 *d*(15.9)	
Sinapic acid				
2,6	7.02 *s*	7.01 *s*	6.96 *s*	
α	6.54 *d*(15.9)	6.51 *d*(15.9)	6.48 *d*(15.9)	
β	7.57 *d*(15.9)	7.54 *d*(15.9)	7.51 *d*(15.9)	
OMe	3.80 *s*	3.84 *s*	3.80 s	
Malonic acid				
–CH2–	3.34 *s*	3.33 *s*		
Glucose A				
1	5.71 *d*(8.0)	5.65 *d*(7.0)	5.65 *d*(7.6)	5.59 *d*(7.6)
2	4.05 *dd*(8.0, 8.9)	4.05 *dd*(7.0, 7.9)	4.00 *t**(8.3)	3.94 *t**(8.2)
3	3.62 *t**(8.5)	3.55 *m*	3.58 *t**(9.5)	3.64 *t**(8.9)
4	3.42 *m*	3.31 *m*	3.38 *m*	3.33 *m*
5	4.00 *m*	3.90 *m*	3.97 *dd*(6.1, 11.9)	3.46 *m*
6a	4.26 *m*	4.29 *m*	4.23 *m*	3.54 *m*
6b	4.37 *m*	4.47 br*d*(12.5)	4.31 *m*	3.69 br*d*(11.6)
Glucose B				
1	5.19 *d*(8.0)	5.20 *d*(6.7)	5.03 *d*(7.6)	5.09 *d*(7.6)
2	3.55 *m*	3.53 *m*	3.48 *dd*(7.6, 8.3)	3.44 *dd*(7.6, 8.9)
3	3.42 *m*	3.20–3.90	3.33 *m*	3.35 *m*
4	3.26 *m*		3.24 *m*	3.28 *m*
5	3.28 *m*		3.48	3.40–3.50
6a	4.03 *m*	4.35 *m*	3.45–3.78	3.62–3.73
6b	4.39 br*d*(10.4)	4.35 *m*		
Glucose C				
1	4.34 *d*(8.0)	4.32 *d*(7.3)	4.28 *d*(8.0)	4.34 *d*(7.7)
2	2.98 *dd*(8.0, 8.5)	2.95 *t**(8.3)	2.94 *t**(8.6)	3.04 *dd*(7.7, 8.9)
3	3.14 *t**(8.9)	3.11 *t**(8.6)	3.07 *t**(8.9)	3.18 *m*
4	3.06 *t**(9.2)	3.03 *t**(9.2)	3.01 *t**(9.2)	3.10–3.37
5	3.26 *m*	3.26 *m*	3.24–3.90	
6a	3.25–3.90	3.20–3.90		
6b				
Xylose				
1	5.20 *d*(8.2)	5.19 *d*(7.0)	5.15 *d*(7.7)	4.80 *d*(8.0)
2	4.83 *dd*(8.2, 8.8)	4.80 *t**(8.9)	4.78 *dd*(7.7, 8.9)	3.14 *m*
3	3.71 *m*	3.69 *m*	3.68 *t**(9.5)	3.31 *t**(8.5)
4	3.42 *m*	3.40 *m*	3.18 *m*	3.01 *t**(9.2)
5a	3.20–3.90	3.20–3.90	3.22–3.75	3.54 *m*
5b				3.66 *m*

Coupling constants (J in Hz) in parentheses.
s = singlet, brs = broad singlet, *t** = distorted triplet, *m* = multiplet, *dd* = double doublet.

Baxter, 1999; Honda and Saito, 2002). For further confirmation of the identity between the structures of pigment **3** and demalonyl pigment **1**, demalonyl pigment **1** was prepared by treatment of pigment **1** with 1 N HCl solution (Saito and Harborne, 1992; Saito et al., 2007), and compared with pigment **3** directly. The chromatographic and spectroscopic data of demalonyl pigment **1** are shown in Table 1. These data including the analyses of mass and NMR spectra of demalonyl pigment **1** and pigment **3** revealed that both pigments were quite identical with each other.

Table 3
^{13}C NMR spectroscopic data (d) of anthocyanins isolated from *Malcolmia maritima* (125.78 MHz, DMSO-d_6–CF_3COOD, δ values in ppm)

	Pigment **1**	Pigment **3**	Pigment **4**
Cyanidin			
2	162.3	162.3	162.4
3	144.4	144.4	144.6
4	131.3	131.7	132.2
5	155.0	155.2	155.2
6	105.1	105.0	104.1
7	167.4	167.7	167.5
8	96.1	96.3	96.1
9	155.1	155.3	155.3
10	111.4	111.7	111.5
1′	119.7	119.8	119.6
2′	117.6	117.7	117.8
3′	146.7	146.7	146.4
4′	155.5	155.5	155.3
5′	116.7	116.7	116.8
6′	128.7	128.7	128.2
p-Coumaric acid			
1	125.0	125.1	
2,6	130.5	130.7	
3,5	115.7	116.0	
4	160.0	160.1	
7α	113.8	113.8	
8β	145.4	145.4	
9	166.9	166.9	
Sinapic acid			
1	125.0	125.1	
2,6	106.5	106.6	
3,5	148.3	148.3	
4	138.5	138.6	
7α	114.3	115.8	
8α	145.2	145.4	
9	166.1	166.1	
OMe	56.4	56.4	
Malonic acid			
–CH2–	41.3		
COO	167.0		
COO	168.1		
Glucose A			
1	97.8	97.9	98.9
2	77.2	77.9	80.5
3	76.6	77.3	77.0
4	–	70.8	69.2
5	73.3	73.5	73.1
6	64.6	63.2	60.5
Glucose B			
1	101.9	102.5	101.5
2	73.4	77.7	77.1
3	–	76.1	75.9
4	–	73.9	69.6
5	73.3	73.5	77.6
6	64.2	61.4	61.3
Glucose C			
1	103.2	103.3	104.0
2	73.3	73.5	73.9
3	75.8	76.7	76.2
4	70.3	70.3	–
5	–	–	–
6	61.3	61.1	61.3
Xylose			
1	101.4	101.4	103.9
2	72.1	72.2	73.0
3	83.7	83.7	87.4
4	–	77.2	70.4
5	56.4	60.9	61.3

2.4. Pigment 2

The molecular ion $[M]^+$ of pigment **2** was observed at m/z 1343 ($C_{61}H_{67}O_{34}$) which was identical with that of pigment **1** supporting that pigment **2** is composed of cyanidin with three molecules of glucose, one molecule each of xylose, sinapic acid, *p*-coumaric acid and malonic acid. The elemental components were also confirmed by measuring its HRMS, and the data obtained were summarized in Section 4.4. The ^1H NMR spectrum of pigment **2** was similar to that of pigment **1** except for signals of *p*-coumaric acid moiety as shown in Table 2. Particularly, the chemical shifts of the olefinic protons were shifted to a higher magnetic field at δ 5.69 and 6.46 with smaller coupling constants ($J = 12.8$ and 12.8 Hz) in comparison with those (δ 6.25, $J = 15.9$ Hz, and δ 7.35, $J = 15.9$ Hz) of pigment **1**. Therefore, the configuration of *p*-coumaric acid was confirmed to be in the *cis*-form, and the structure of pigment **2** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-6-*O*-(*cis*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside], which is a new anthocyanin in plants. The *cis*-configuration was unambiguously confirmed by comparison of TLC and HPLC (Table 1) with an authentic sample prepared from its *trans*-derivative (pigment **1**) as shown in Section 4 (Saito and Harborne, 1992).

3. Concluding remarks

As already described (Tatsuzawa et al., 2006), there are two glycosidic patterns in the Cruciferae at OH-3 of anthocyanidins, 3-sambubioside and 3-sophoroside. In this study, it is demonstrated that an additional new 3-glycosidic pattern is found in *Malcolmia maritima*, cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside. This glycosidic pattern in *M. maritima* is comparable to the former pattern of 3-sambubioside; however, it is noteworthy that this pigment has novel glycosidic pattern, 3^X-glucosylsambubioside, in which sumbubioside is xylosyl(1–2)glucose. The observed glycosidic pattern seems to be unique based on the consideration of previous observations. This is the first report of the occurrence of this trisaccharide in conjunction with anthocyanins (Andersen and Jordheim, 2006) or flavonoids (Williams, 2006). From a chemotaxonomical point of view, this result is very interesting in the classification of *M. maritima* in the Cruciferae, and the glycosylation of the trisac-

charide at OH-3 of anthocyanin indicated a advanced characteristic in comparison with that of the sambubiose.

Regarding the acylations, the anthocyanins of *M. maritima* exhibited common patterns like the anthocyanins in the Cruciferae, in which OH-6 of glu A and OH-2 of glu C or xylose in these pigments were acylated with hydroxycinnamic acids, and also OH-6 of glu B was acylated with a malonic acid (Giusti et al., 1998; Igarashi et al., 1990; Mori et al., 2006; Otsuki et al., 2002). These acylations with hydroxycinnamic acids in anthocyanin pigments are also responsible for the bluing effect and also for stability of the flower color of *M. maritima* (Honda and Saito, 2002).

4. Experimental

4.1. General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using eight mobile phases: BAW (*n*-BuOH–HOAc–H_2O, 4:1:2), BuHCl (*n*-BuOH–2 N HCl, 1:1, upper layer), AHW (HOAc–HCl–H_2O, 15:3:82), 1% HCl for anthocyanins, and BAW, EAA (EtOAc–HOAc–H_2O, 3:1:1), ETN (EtOH–NH_4OH–H_2O, 16:1:3) and EFW (EtOAc–HCOOH–H_2O, 5:2:1) for sugars and organic acid with UV light and aniline hydrogen phthalate spray reagent (Harborne, 1984).

Analytical HPLC was performed on a LC 10A system (Shimadzu), using a Waters C18 (4.6 $\phi \times 250$ mm) column at 40°C with a flow rate of 1 mL/min and monitoring at 530 nm. The eluant was applied as a linear gradient elution for 40 min from 20% to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O).

UV–Vis spectra were recorded on UV–Vis Multi Purpose Spectrophotometer (MPS-2450, Shimadzu) in 0.1% HCl-MeOH (from 200 to 700 nm).

High resolution FAB mass (FABMS) spectra were obtained in the positive ion mode using the magic bullet (5:1 mixture of dithiothreitol and dithioerythritol) as a matrix. NMR spectra were determined at 500 MHz for ^1H spectra and at 125.78 MHz for ^{13}C spectra in CF_3COO–DMSO-d_6 (1:9). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants are in Hz.

4.2. Plant materials

The seeds and seedling of *Malcolmia maritima* (L.) R. Br. were purchased from Unwins Seeds Ltd. (Histon, Cambridge, UK) and Sakata Co. Ltd (Yokohama, Japan), and grown in greenhouses at the Experimental farm of Minamikyushu University. The flowers exhibited from violet [Violet 87B by Royal Horticultural Society color chart and chromaticity value, $b^*(-36.48)/a^*(36.42)$] to a purple-violet [Purple-Violet 81B, $b^*(-27.46)/a^*(46.07)$]. The stems exhibited a purple [Purple 77A by RHS color chart and chromaticity value, $b^*(5.70)/a^*(12.36)$]. Flowers and stems were collected from winter to spring seasons in Japan and dried overnight at 40 °C, and kept in a refrigerator at about 4 °C. The chromaticity values were recorded on a SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co., Ltd.).

4.3. Isolation of anthocyanins

Dried flowers (ca. 60 g) of *Malcolmia maritima* were immersed in 5%HOAc–H_2O (3L; HOAc–H_2O, 1:19) at room temp. for 5 h and extracted. The extract was passed through a Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column ($90\phi \times 150$ mm), on which acylated anthocyanins were adsorbed. Then, the column was thoroughly washed with H_2O (2 L) and eluted with 5% HOAc–MeOH (500 mL) to recover the anthocyanins. After concentration, the eluates were separated and purified with paper chromatography (PC) using BAW. The separated pigments were further purified by TLC (15% HOAc) and prep. HPLC. Prep. HPLC was performed on a Waters C18 ($19\phi \times 150$ mm) column at 40 °C with a flow rate of 4 mL/min and monitoring at 530 nm. The solvent used was as follows: a linear gradient elution for 16 min from 60% to 80 % solvent B in solvent A. Each fraction was transformed to a Diaion HP-20 column, on which pigments were adsorbed. Anthocyanin pigments were eluted with 5% HOAc–MeOH followed by addition of excess of Et_2O, and then dried. The purified pigments from the flowers were obtained as follows; pigment **1** (ca. 10 mg), pigment **2** (ca. 3 mg) and pigment **3** (ca. 1 mg).

Dried stems (ca. 100 g) of *M. maritima* was immersed in 5% HOAc–H_2O (5 L) at room temp. for 5 h. The extract was purified by the same process as for the dried flowers as described above. The pigments were obtained as follows; pigment **1** (ca. 1 mg), pigment **3** (ca. 20 mg) and pigment **4** (ca. 5 mg).

4.4. Analyses of anthocyanins

The identification of anthocyanins was carried out by standard procedures involving deacylation with acid, and both alkaline and acid hydrolyses (Harborne, 1984). The data of TLC (R_f value), HPLC ($R_{t\text{-min}}$), UV–VIS (λ_{max}), and FABMS spectra are shown in Table 1 and also described in Sections 4.4.1.– 4.4.4.

4.4.1. Pigment *1*

Dark purple-red powder; for UV–Vis and TLC, see Table 1; for ^1H and ^{13} C NMR spectra, see Tables 2 and 3; HR-FABMS calc. for $C_{61}H_{67}O_{34}$: 1343.3514. Found: 1343.3481.

4.4.2. Pigment *2*

Dark purple-red powder; for UV–Vis and TLC, see Table 1; for ^1H and ^{13} C NMR spectra, see Tables 2 and 3; HR-FABMS calc. for $C_{61}H_{67}O_{34}$: 1343.3514. Found: 1343.3494.

4.4.3. Pigment **3** (demalonyl pigment **1**)

Dark purple-red powder; for UV–Vis and TLC, see Table 1; for ^{1}H and ^{13}C NMR spectra, see Tables 2 and 3; HR-FABMS calc. for $C_{58}H_{65}O_{31}$: 1257.3510. Found: 1257.3523.

4.4.4. Pigment **4** and deacyl pigments **1**, **2** and **3**

Dark red powder; for UV–Vis and TLC, see Table 1; for ^{1}H and ^{13}C NMR spectra, see Tables 2 and 3; HR-FABMS calc. for $C_{38}H_{49}O_{25}$: 905.2563. Found: 905.2550.

4.4.5. Deacylanthocyanin

Pigments **1** and **3** (ca.10 mg) were dissolved in 2 N NaOH (1 mL) under a degassed syringe and allowed to stand for 15 min. Then the solution was sufficiently acidified with 2 N HCl and evaporated in vacuo to dryness. The residue was dissolved in 1% HCl–MeOH and applied on TLC (BAW) to yield a deacylanthocyanin (ca. 5 mg). The characteristic properties of deacylanthocyanin are shown in Table 1.

4.4.6. Demalonyl pigment **1**

Pigment **1** (ca. 5 mg) was dissolved in 1 N HCl solution (2 ml) and allowed to stand at room temperature for 2 weeks. At this point, demalonylated pigment **1** was formed in its solution (Saito and Harborne, 1992; Saito et al., 2007). Demalonylated pigment **1** was then absorbed on the resin column of Diaion HP-20 and eluted with 5% HOAc–MeOH from the column. After evaporation of the solvent in vacuo, the concentrated residue was dissolved in a small volume of 5% HOAc–MeOH followed by addition of excess Et_2O, from which solids were then dried in vacuo to give a demalonyl pigment **1** powder (ca. 3 mg). The characteristic properties of demalonyl pigment **1** are shown in Table 1.

4.4.7. Isomerization of pigment **1** by sunlight

Pigment **1** (ca. 1 mg) was dissolved in 5% HOAc–MeOH (2 ml), and exposed for sunlight for half an hours to transform it into the *cis*-isomer by about 50% (Saito and Harborne, 1992). The isomer was isolated and purified by TLC and HPLC. After this process, the purified isomer of pigment **1** (ca. 0.5 mg) was obtained: UV–Vis in 0.1% HCl–MeOH 535, 316, (298), 279 nm; TLC ($R_f \times 100$) BAW 13, BuHCl 18, 1% HCl 57, AHW 86; HPLC (R_t (min)) 25.0; FAB mass $[M]^+$ 1343.

4.4.8. Partial hydrolysates of deacylanthocyanin

Three kinds of acid hydrolysis for deacylanthocyanin were performed as following. Three 1 N HCl solutions of deacylanthocyanin (containing ca. 1 mg in 5 mL 1 N HCl each) were hydrolyzed by heating on a water bath (80–100 °C) for 1, 5, and 10 min, respectively. These three partial hydrolysates were quickly analyzed by TLC and HPLC with authentic cyanidin glycosides. Four intermediary glucosides were found in these partial hydrolysates, and identified to be 3-glucoside (R_t; 15.9 min by HPLC, TLC; AHW 0.10, BAW 0.15), 5-glucoside (R_t; 16.8, TLC; AHW 0.12, BAW 0.25), 3,5-diglucoside (R_t;13.3, TLC; AHW 0.22, BAW 0.07), and 3-sambubioside-5-glucoside (Rt;12.6, TLC; AHW 0.58, BAW 0.05) of cyanidin as well as deacylanthocyanin (R_t; 13.2, TLC; AHW 0.64, BAW 0.03).

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