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Acylated cyanidin 3-sambubioside-5-glucosides in three garden plants of the Cruciferae

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Abstract

Seven acylated cyanidin 3-sambubioside-5-glucosides were isolated from the flowers of three garden plants in the Cruciferae. Specifically, four pigments were isolated from *Lobularia maritima* (L.) Desv., together with a known pigment, as well as, three pigments from *Lunaria annua* L., and two known pigments from *Cheiranthus cheiri* L. These pigments were determined to be cyanidin 3-*O*-[2-*O*-((acyl-II)-(β-D-xylopyranosyl))-6-*O*-(acyl-I)-β-D-glucopyranoside]-5-*O*-[6-*O*-(acyl-III)-β-D-glucopyranoside], in which the acyl-I group is represented by glucosyl-*p*-coumaric acid, *p*-coumaric acid and ferulic acid, acyl-II by caffeic acid and ferulic acid, and acyl-III by malonic acid, respectively. The distribution and biosynthesis of acylated cyanidin 3-sambubioside-5-glucosides are discussed according to the variations of acylation and glucosylation at their 3-sambubiose residues.

Keywords: *Cheiranthus cheiri* L.; *Lobularia maritima* (L.) Desv.; *Lunaria annua* L.; Cruciferae; Flower color; Acylated anthocyanins; Cyanidin 3-sambubioside-5-glucoside

1. Introduction

The Cruciferae is a large family of natural plants, involving a number of ornamental garden plants such as stock, wallflower, honesty, sweet alyssum and so on. The anthocyanins that have been isolated from this family have unusually complex structures, and occur associated with one or more cinnamic acids (Harborne, 1967; Harborne and Baxter, 1999; Honda et al., 2005). There are two different glycosyl patterns at the 3-position of the anthocyanins; such as anthocyanidin 3-sophoroside-5-glucosides and 3-sambubioside-5-glucosides. The distribution of anthocyanidin 3-sophoroside-5-glucoside is restricted to the plants of genus *Brassica* and *Raphanus* in the Cruciferae (Harborne, 1967; Honda et al., 2005). On the other hand, the distribution of anthocyanidin 3-sambubioside-5-glucoside is rather widely spread in plants of the genus *Arabidopsis*, *Matthiola*, *Orychophragonus* and *Sinapis* in this family. On the acylation of anthocyanidin 3-sambubioside-5-glucosides, anthocyanins are characteristically acylated at two positions of the sambubiose group; i.e. such as the 6-OH of the glucose moiety and the 2-OH of the xylose moiety, except for the 6-OH of the glucose at the 5-position of anthocyanidin.

In our previous study on flower color variation due to acylated anthocyanins in the Cruciferae, 12 acylated anthocyanidin 3-sambubioside-5-glucosides were identified in the flowers of *Matthiola incana* (Saito et al., 1995, 1996) and *Orycophragonus violaceus* (Honda et al., 2005) of particular interest. The aromatic acid moieties of acylated anthocyanins are responsible for production of the bluing effect in their flower colors.

In this study we wish to report upon the structure elucidation of seven new and two known acylated cyanidin 3-sambubioside-5-glucosides isolated from the flowers of the Cruciferae, and to discuss the variation of acylation in these plants.

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2. Results and discussion

2.1. Pigments 1 and 2 isolated from the flowers of Cheiranthus cheiri

Two pigments (**1** and **2**) were isolated from the rose-red flowers of *C. cheiri* 'Vega', and their proportions in the MAW (MeOH–HOAc–Water, 4:1:5, v/v/v) extract were determined to be 83% (pigment **1**) and 6% (pigment **2**) based on percentage of total absorbance of the detected anthocyanins at 530 nm by HPLC analysis. The chromatographic and spectral properties of both pigments are summarized in Table 1.

FAB mass spectra of pigments **1** and **2** had the same molecular ion peak $[M-1]^+$ at 888 *m/z*, corresponding to the molecular formula, $C_{41}H_{45}O_{22}$. This included the feature that both pigments are composed of cyanidin with two molecules of glucose and one molecule each of xylose and *p*-coumaric acid. In the acidic and alkaline hydrolysates, these components were detected by TLC and HPLC analyses (Harborne, 1984). The elemental components were confirmed by measuring its high-resolution FABMS (HRMS), and the mass data obtained are summarized in Section 4.5. The full assignments of proton and carbon signals of both pigments **1** and **2** are carried out with the measurements of ^1H–^1H COSY, 2D NOESY, negative difference NOE spectral experiments (NOEDIF), ^1H–^{13}C HMQC and ^1H–^{13}C HMBC spectra, respectively (Table 2 and Section 4.6.).

2.1.1. Pigment 1

The chemical shifts of 10 aromatic protons of cyanidin and *p*-coumaric acid moieties were assigned as shown in Table 2. The two chemical shifts at δ 6.23 and 7.32 were assigned to the olefinic protons of *trans*-*p*-coumaric acid with the large coupling constants ($J = 15.9$ and 15.9 Hz). The characteristic signals of the three anomeric protons appeared at δ 5.66 (*d*, $J = 7.7$ Hz, Glu A), δ 5.30 (*d*, $J = 7.7$ Hz, Glu B), and δ 4.68 (*d*, $J = 8.0$ Hz, Xylose). Based on the observed coupling constants (Table 2), the three sugars were assumed to have β-pyranose forms. The linkages and/or positions of the attachments of the sugar and acyl groups in this pigment were mainly determined by using 2D COSY, ROESY, NOEDIF,and HMBC experiments. A proton signal (δ 3.96, *t*, $J = 8.3$ Hz) shifting to a lower magnetic field was assigned to H-2 of Glu A by the analysis of the 2D COSY spectrum of pigment **1**. Furthermore, this resonance was correlated to the ^{13}C-1 signal (δ 104.8) of xylose in the HMBC spectrum, indicating that xylose was linked to the OH-2 of Glu A due to forming sambubiose at the 3-OH group of cyanidin.

This bonding was confirmed by a NOEDIF experiment as described previously (Honda et al., 2005). The two characteristic proton signals (δ 4.28 and 4.36) that was shifted to a lower magnetic field were also assigned to the methylene protons of Glu A i.e. thereby supporting the acylation with *p*-coumaric acid at the 6-OH group of Glu A. There-

Table 1
Chromatographic and spectroscopic properties of acylated anthocyanins in the flowers of the Cruciferae

Anthocyanins[a]	R_f values (×100)				Spectral data in 0.1% HCl–MeOH				R_t[b] (min)	Based on FAB-MS[c]						
	BAW	BuHCl	1% HCl	AHW	λ_{max} (nm)	E_{acyl}/E_{max}	E_{440}/E_{max}	$AlCl_3$		$[M^+]$	Cy	Glu	Xyl	*p*-C	Caf	Fer	Mal
1	21	16	22	56	530, 315, 295, 281	61	12	+	30.6	889	1	2	1	1			
2	17	13	40	67	533, (315), 295, 281	60	13	+	25.2	889	1	2	1	1			
3	10	17	36	67	530, 315, 296, 282	64	12	+	32.1	975	1	2	1	1			1
4	12	20	42	66	533, (316), (295), 281	66	14	+	26.1	975	1	2	1	1			1
5	11	12	27	63	529, 326, 295, 281	56	12	+	33.0	1005	1	2	1			1	1
6	4	4	36	72	531, (307), (297), 279	77	13	+	23.6	1051	1	3	1	1			
7	9	10	38	66	532, (315), (297), 281	91	12	+	25.0	1213	1	3	1	1	1		
8	31	32	23	55	531, 321, (297), 281	120	12	+	31.6	1051	1	2	1	1	1		
9	41	40	25	66	531, 320, 298, 281	123	12	+	32.7	1065	1	2	1	1		1	
10	6	6	35	62	527, 278	–	13	+	13.1	743	1	2	1				

[a] **1**: cyanidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-glucoside; **2**: cyanidin 3-[2-(xylosyl)-6-(*cis*-*p*-coumaroyl)-glucoside]-5-glucoside; **3**: cyanidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-(6-malonylglucoside); **4**: cyanidin 3-[2-(xylosyl)-6-(*cis*-*p*-coumaroyl)-glucoside]-5-(6- malonylglucoside); **5**: cyanidin 3-[2-(xylosyl)-6-(*trans*-feruloyl)-glucoside]-5-(6-malonylglucoside); **6**: cyanidin 3-[2-(xylosyl)-6-(glucosyl-*trans*-*p*-coumaroyl)-glucoside]-5-glucoside; **7**: cyanidin 3-[2-(2-*trans*-caffeoyl)-(xylosyl)-6-(glucosyl-*trans*-*p*-coumaroyl)-glucoside]-5-glucoside; **8**: cyanidin 3-[2-(2-*trans*-caffeoyl)-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-glucoside; **9**: cyanidin 3-[2-(2-*trans*-feruloyl)-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-glucoside; **10**: cyanidin 3-sambubioside-5-glucoside.

[b] See Section 4.

[c] $[M]^+$ = molecular ion mass values. Cy = cyanidin; Glu = glucose; Xyl = xylose; *p*-C = *p*-coumaric acid; Caf = caffeic acid; Fer = ferulic acid; Mal = malonic acid with their molecular numbers in each anthocyanins.

Table 2-1
^{1}H NMR spectroscopic data (δ) of acylated anthocyanins in the flowers of the Cruciferae (500 MHz in DMSO-d_6–CF_3CO_2D, TMS as an internal standard) [coupling constants (J in Hz) in parentheses]

	Pigment **1**	Pigment **2**	Pigment **3**	Pigment **4**	Pigment **5**
Cyanidin					
4	8.70 *s*	8.67 *s*	8.74 *s*	8.59 *s*	8.79 *s*
6	6.93 *d* (1.6)	6.99 *d* (1.9)	7.00 *br s*	6.90 *br s*	6.97 *br s*
8	7.00 *br s*	7.01 *d* (1.9)	7.04 *br s*	6.90 *br s*	7.05 *br s*
2′	7.99 *d* (2.2)	8.06 *d* (2.5)	8.05 *br s*	8.02 *d* (2.5)	8.04 *d* (2.2)
5′	7.01 *d* (8.9)	7.08 *d* (8.9)	7.07 *d* (8.9)	7.07 *d* (8.9)	7.07 *d* (8.9)
6′	8.32 *dd* (2.2, 8.9)	8.34 *dd* (2.5, 8.9)	8.38 *br d* (8.9)	8.32 *dd* (2.5, 8.9)	8.39 *dd* (2.2, 8.9)
Hydroxycinnamic acid (I)					
2	7.15 *d* (8.2)	7.54 *d* (8.9)	7.32 *d* (8.3)	7.31 *d* (8.9)	7.06 *br s*
3	6.73 *d* (8.5)	6.63 *d* (8.9)	6.73 *d* (8.3)	6.51 *d* (8.9)	
5	6.73 *d* (8.5)	6.63 *d* (8.9)	6.73 *d* (8.3)	6.51 *d* (8.9)	6.75 *d* (8.3)
6	7.15 *d* (8.2)	7.54 *d* (8.9)	7.32 *d* (8.3)	7.31 *d* (8.9)	6.96 *dd* (1.3, 8.3)
α	6.23 *d* (15.9)	5.81 *d* (12.8)	6.27 *d* (15.9)	5.72 *d* (15.9)	6.32 *d* (15.9)
β	7.32 *d* (15.9)	6.87 *d* (12.8)	7.38 *d* (15.9)	6.50 *d* (15.9)	7.41 *d* (15.9)
OMe					3.75 *s*
Malonic acid (II)					
CH_2			3.36 *s*	3.53 *s*	3.40 *s*
Glucose A					
1	5.66 *d* (7.7)	5.75 *d* (7.6)	5.72 *d* (7.3)	5.71 *d* (7.6)	5.70 *d* (7.6)
2	3.96 *t** (8.3)	4.05 *t** (8.5)	4.03 *t** (8.0)	4.04 *m*	4.02 *m*
3	3.71 *t** (8.9)	3.74 *t** (9.2)	3.77 *t** (9.2)	3.75 *m*	3.76 *m*
4	3.42 *t** (9.2)	3.42 *t** (8.9)	3.46 *t** (9.2)	3.36 *m*	3.49 *t** (9.2)
5	3.95 *m*	3.98 *m*	4.03 *m*	3.97 *m*	4.02 *m*
6a	4.28 *dd* (6.1, 10.4)	4.23 *dd* (8.6, 11.6)	4.29 *dd* (7.6, 11.6)	4.36 *m*	4.25 *dd* (6.9, 11.6)
6b	4.36 *d* (10.4)	4.45 *d* (11.6)	4.44 *d* (11.6)	4.49 *m*	4.47 *d* (11.6)
Glucose B					
1	5.30 *d* (7.7)	5.16 *d* (7.6)	5.18 *d* (7.6)	5.22 *d* (7.6)	5.17 *d* (7.6)
2	3.49 *m*	3.58 *m*	3.57 *t** (8.6)	3.55 *m*	3.55 *m*
3	3.36 *t** (8.6)	3.39 *m*	3.43 *m*	3.30–3.80	3.42 *t** (8.9)
4	3.23 *m*	3.32 *m*	3.28 *m*		3.27 *m*
5	3.51 *m*	3.54 *m*	3.80 *m*		3.79 *m*
6a	3.51 *m*	3.68 *dd* (5.5, 11.6)	4.08 *dd* (6.4, 11.6)	3.97 *m*	3.95 *dd* (7.3, 11.6)
6b	3.76 *d* (10.4)	3.86 *d* (11.6)	4.41 *d* (11.6)	4.42 *m*	4.41 *d* (11.6)
Xylose					
1	4.68 *d* (8.0)	4.74 *d* (7.6)	4.74 *d* (7.7)	4.73 *d* (7.0)	4.74 *d* (7.7)
2	2.98 *t** (8.6)	3.02 *t** (7.9)	3.04 *t** (8.3)	3.01 *m*	3.03 *t** (8.3)
3	3.12 t (8.6)	3.15 *t** (8.9)	3.17 *t** (8.9)	3.16 *m*	3.17 *t** (8.9)
4	3.23 *m*	3.24 *m*	3.28 *m*	3.26 *m*	3.27 *m*
5a	3.51 *m*	3.52 *m*	3.58 *m*	3.54 *m*	3.54 *m*
5b	2.93 t (11.3)	2.94 t (11.3)	3.00 t (11.3)	2.96 *m*	2.99 t (11.0)

s = singlet; *d* = doublet; *t** = distorted triplet; *m* = multiplet; *dd* = double doublet.

fore, pigment **1** was determined to be cyanidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside] (Fig. 1). This is the first report on the isolation of this pigment from the Cruciferae, although this pigment has been found in *Sambucus canadensis* (Johansen et al., 1991).

2.1.2. Pigment 2

The ^{1}H NMR spectrum of pigment **2** was superimposed on that of pigment **1** except for signals of a *p*-coumaric acid moiety (Table 2). Particularly, the chemical shifts of the olefinic protons were shifted to a higher magnetic field at δ 5.81 and 6.87 with smaller coupling constants ($J = 12.8$ and 12.8 Hz) in comparison with those (δ 6.23, 15.9 Hz and δ 7.32, 15.9 Hz) of pigment **1**. Since the configuration of *p*-coumaric acid was confirmed to be *cis*, pigment **2** was determined to be cyanidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(*cis*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside] (Fig. 1). This structure was further confirmed by analysis of ^{13}C NMR spectra (Section 4.6). This is also the first report on its isolation from the Cruciferae, but its occurrence in *S. canadensis* (Johansen et al., 1991) has been reported.

2.2. Pigments 3, 4 and 5 isolated from the flowers of Lunaria annua

Pigments **3**–**5** were isolated from the red-purple flowers of *L. annua*, and their proportions in the MAW extract were determined to be 53.9% (pigment **3**),

Table 2-2
^{1}H NMR spectroscopic data (δ) of acylated anthocyanins in the flowers of the Cruciferae (500 MHz in DMSO-d_6-CF_3CO_2D, TMS as an internal standard) [coupling constants (J in Hz) in parentheses]

	Pigment **6**	Pigment **7**	Pigment **8**	Pigment **9**
Cyanidin				
4	8.77 *s*	8.71 *s*	8.70 *s*	8.73 *s*
6	7.01 *d* (2.0)	6.99 *br s*	6.97 *d* (1.8)	6.99 *d* (1.5)
8	7.04 *d* (2.0)	7.01 *br s*	6.99 *d* (1.8)	6.99 *d* (1.5)
2′	8.05 *d* (2.2)	8.01 *d* (2.1)	8.00 *d* (2.5)	8.02 *d* (2.4)
5′	7.07 *d* (8.9)	7.06 *d* (8.9)	7.06 *d* (8.9)	7.08 *d* (8.9)
6′	8.38 *dd* (2.2, 8.9)	8.48 *dd* (21, 8.9)	8.48 *dd* (2.5, 8.9)	8.48 *dd* (2.4, 8.9)
Hydroxycinnamic acid (I)				
2	7.53 *d* (8.9)	7.51 *d* (8.9)	7.39 *d* (8.9)	7.39 *d* (8.9)
3	7.05 *d* (8.9)	7.04 *d* (8.9)	6.78 *d* (8.9)	6.79 *d* (8.9)
5	7.05 *d* (8.9)	7.04 *d* (8.9)	6.78 *d* (8.9)	6.79 *d* (8.9)
6	7.53 *d* (8.9)	7.51 *d* (8.9)	7.39 *d* (8.9)	7.39 *d* (8.9)
α	6.39 *d* (15.9)	6.37 *d* (15.9)	6.36 *d* (15.9)	6.52 *d* (15.9)
β	7.44 *d* (15.9)	7.41 *d* (15.9)	7.51 *d* (15.9)	7.60 *d* (15.9)
Hydroxycinnamic acid (II)				
2		7.11 *br s*	7.11 *d* (1.8)	7.32 *d* (1.6)
3				
5		6.82 *d* (7.9)	6.82 *d* (8.3)	6.86 *d* (8.2)
6		7.04 *m*	7.03 *dd* (1.8, 8.3)	7.16 *dd* (1.6, 8.2)
α		6.36 *d* (15.6)	6.27 *d* (15.9)	6.27 *d* (15.9)
β		7.51 *d* (15.6)	7.35 *d* (15.9)	7.35 *d* (15.9)
OMe				3.87 *s*
Glucose A				
1	5.71 *d* (7.9)	5.69 *d* (7.3)	5.69 *d* (8.0)	5.71 *d* (7.6)
2	4.01 *m*	4.01 *t** (8.3)	4.01 *t** (8.3)	4.04 *t** (8.2)
3	3.77 *t** (8.9)	3.61 *t** (8.9)	3.60 *t** (8.6)	3.64 *t** (8.8)
4	3.41 *t** (9.5)	3.40 *m*	3.41 *m*	3.41 *t** (9.2)
5	4.02 *m*	3.96 *m*	3.94 *m*	3.96 *m*
6a	4.33 *dd* (6.7, 11.6)	4.31 *m*	4.31 *m*	4.30 *dd* (7.1, 11.0)
6b	4.42 *d* (11.6)	4.33 *m*	4.31 *m*	4.35 *d* (11.0)
Glucose B				
1	5.08 *d* (7.6)	5.07 *d* (7.6)	5.08 *d* (7.4)	5.09 *d* (7.6)
2	3.56 *m*	3.53 *m*	3.52 *m*	3.55 *m*
3	3.40 *m*	3.39 *m*	3.36–3.54	3.35–3.55
4	3.23 *m*	3.23 *m*		
5	3.54 *m*	3.50–3.80		
6a	3.75 *m*			
6b	3.82 *d* (9.8)	3.81 *m*	3.89 *m*	3.82 *m*
Glucose C				
1	4.98 *d* (7.4)	4.97 *d* (7.3)		
2	3.25 *m*	3.34 *m*		
3	3.31 *m*	3.20–3.55		
4	3.25–3.58			
5				
6a				
6b				
Xylose				
1	4.74 *d* (7.7)	5.14 *d* (7.9)	5.13 *d* (8.0)	5.15 *d* (8.3)
2	3.04 *t** (8.0)	4.65 *t** (8.9)	4.64 *t** (8.9)	4.68 *t** (8.6)
3	3.17 *t** (8.9)	3.41 *m*	3.39 *m*	3.45 *m*
4	3.27 *m*	3.35–3.45 *m*	3.26 *t** (9.2)	3.29 t (8.9)
5a	3.54 *m*	3.91 *m*	3.89 *m*	3.92 *m*
5b	2.99 *t** (10.4)	3.21 t (8.9)	3.21 t (9.8)	3.22 *m*

10.8% (pigment **4**) and 19.5% (pigment **5**), respectively, by the same procedure as for *C. cheiri*. The chromatographic data and spectroscopic properties of these pigments are shown in Table 1.

The FAB mass spectra of pigments **3**–**5** gave their molecular ions at 975, 975 and 1005 m/z, respectively, in good agreement with the masses calculated for $C_{44}H_{47}O_{25}$, $C_{44}H_{47}O_{25}$ and $C_{45}H_{49}O_{26}$. These values indi-

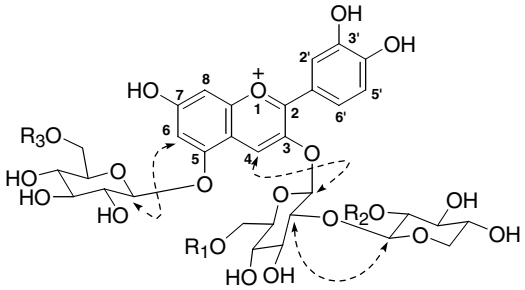

Fig. 1. Acylated cyanidin 3-sambubioside-5-glucosides in three garden plants of the Cruciferae. Observed NOEs are indicated by arrows.

Pigment	R_1	R_2	R_3	Source
1	*trans*-*p*-coumaroyl			*C. cheiri* and *L. maritima*
2	*cis*-*p*-coumaroyl			*C. cheiri*
3	*trans*-*p*-coumaroyl		malonyl	*L. annua*
4	*cis*-*p*-coumaroyl		malonyl	*L. annua*
5	*trans*-feruloyl		malonyl	*L. annua*
6	glucosyl-*trans*-*p*-coumaroyl			*L. maritima*
7	glucosyl-*trans*-*p*-coumaroyl	*trans*-caffeoyl		*L. maritima*
8	*trans*-*p*-coumaroyl	*trans*-caffeoyl		*L. maritima*
9	*trans*-*p*-coumaroyl	*trans*-feruloyl		*L. maritima*

cated that pigments **3** and **4** were composed of cyanidin with one molecule each of *p*-coumaric acid, malonic acid, and xylose, and two molecules of glucose. Pigment **5** was also composed of cyanidin with one molecule each of ferulic acid, malonic acid, and xylose, and two molecules of glucose. These components were detected in the acid hydrolysates by TLC and HPLC analyses. On alkaline hydrolysis, cyanidin 3-sambubioside-5-glucoside (**10**) was identified as the deacyl anthocyanin by analysis of TLC, HPLC and spectroscopic data (Table 1). The elemental components of pigment **3**–**5** were confirmed by measurement of their high-resolution FAB-MS, and the mass data obtained are summarized in Section 4.5. The structures of these pigments (**3**–**5**) were further elucidated based on the analysis of their ^{1}H NMR spectra (500 MHz in CF_3COOD–DMSO-d_6, 1:9), including 2D COSY, 2D NOESY, NOEDIF spectroscopic experiments (Table 2).

2.2.1. Pigment 3

The ^{1}H NMR of pigment **3** was similar to that of pigment **1** except for signals of malonic acid and 5-glucose (Glu B) moieties. The proton chemical shifts of the methylene (–CH_2–) group of Glu B were shifted to a lower magnetic field at δ 4.41 and 4.08, and also those of the methylene functionality of malonic acid was observed at δ 3.36, indicating that Glc B was acylated with malonic acid at 6-OH of Glu B. Thus, the structure of pigment **3** was determined to be cyanidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside] (Fig. 1), which is a new anthocyanin in plants. The structure was further confirmed based on the analysis of its ^{13}C NMR spectra (Section 4.6.).

2.2.2. Pigment 4

The ^{1}H NMR spectrum of pigment **4** was similar to that of pigment **2** except for signals of malonic acid and 5-glucose (Glu B) moieties. The proton chemical shifts of the methylene functionality of Glc B were shifted to a lower magnetic field at δ 4.42 and 3.97, and that of malonic acid was observed at δ 3.53, supporting the view that the 6-OH group of Glu B was acylated with the malonic acid moiety in pigment **4** as that of pigment **3**. Therefore, pigment **4** was determined to be cyanidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(*cis*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside] (Fig. 1), which is a new anthocyanin in plants.

2.2.3. Pigment 5

The proton chemical shifts of pigment **5** were assigned as shown in Table 2. These values were similar to those of pigment **3**, except for those of the hydroxycinnamic acid moiety, which was ferulic instead of *p*-coumaric acid. The linkages of the sugar and acyl groups in this pigment were confirmed by analyses of ROESY and NOEDIF experiments as described above. Consequently, the structure of pigment **5** was determined to be cyanidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(*trans*-feruloyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside] (Fig. 1), which is a new plant anthocyanin.

2.3. Pigments 6–9 isolated from the flowers of Lobularia maritima

Five pigments (**1**, **6**–**9**) were isolated from the purple-violet flowers of *L. maritima* 'Easter Bonnet', and their proportions in the MAW extract were 14.4% (pigment **1**), 10.5% (pigment **6**), 4.2% (pigment **7**), 3.5% (pigment **8**)

and 19.2% (pigment **9**), respectively, by the same procedure as for the case of *C. cheiri*.

The FAB mass spectra of pigments **1**, **6**–**9** gave their molecular ions at 889, 1051, 1213, 1051 and 1065 *m*/*z*, respectively, in agreement with the masses calculated for $C_{41}H_{45}O_{22}$, $C_{47}H_{55}O_{27}$, $C_{56}H_{61}O_{30}$, $C_{50}H_{51}O_{25}$, and $C_{51}H_{53}O_{25}$. These mass values as well as the results of alkaline and acid hydrolyses of these pigments indicated are following: pigment **1** was composed of cyanidin, with one molecule each of xylose and *p*-coumaric acid, and two molecules of glucose; pigment **6** was also a cyanidin with one molecule each of xylose and *p*-coumaric acid, and three molecules of glucose; pigment **7** was constituted from cyanidin with one molecule each of xylose, *p*-coumaric acid and caffeic acid, and three molecules of glucose; pigment **8** was composed of cyanidin with one molecule each of xylose, *p*-coumaric acid and caffeic acid, and two molecules of glucose; pigment **9** was also cyanidin with one molecule each of xylose, *p*-coumaric acid and ferulic acid, and two molecules of glucose. On alkaline hydrolysis, only one deacylanthocyanin, cyanidin 3-sambubioside-5-glucoside, was detected from their hydrolysates by HPLC analysis. The chromatographic data and spectroscopic properties of these pigments are shown in Table 1. Pigment **1** was identified to be a known pigment described in Section 2.1, and its structure was determined to be cyanidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-glucoside in comparison with an authentic sample isolated from *C. cheiri*. The structures of pigments **6**–**9** were elucidated by the analyses of their ^{1}H NMR spectra, including 2D COSY, 2D NOESY and NOEDIF spectroscopic experiments.

2.3.1. Pigment *6*

The ^{1}H NMR spectrum of pigment **6** was very similar to that of pigment **1** except for the signals of the Glu C moiety, and the proton chemical shifts of pigment **6** were assigned as shown in Table 2. In the NOEDIF experiment, strong NOEs were observed at 3- and 5-H of *p*-coumaric acid by irradiation at H-1 of Glu C, indicating that the latter was attached to the 4-OH group of *p*-coumaric acid through a glycosidic bond. Therefore, the structure of pigment **6** was determined to be cyanidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) (Fig. 1), which is a new pigment in plants.

2.3.2. Pigment *7*

The ^{1}H NMR spectrum of pigment **7** was very similar to that of pigment **6** except for the signals of the xylose moiety and an additional caffeic acid moiety (Table 2). By analysis of the 2D COSY spectrum, it was revealed that 2-OH of xylose was acylated with a hydroxycinnamic acid, since the H-2 signal of xylose was shifted to a lower field at δ 4.65 (t, $J = 8.9$ Hz). Moreover, NOEs were observed at H-α and -β of caffeic acid by irradiation at H-2 of the xylose moiety. Thus, 2-OH of the xylose moiety was acylated with caffeic acid in this pigment. Consequently, pigment **7** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) (Fig. 1), which is a new plant anthocyanin.

2.3.3. Pigment *8*

The ^{1}H NMR spectrum of pigment **8** was similar to that of pigment **7** except for the absence of the signals corresponding to Glu C at 4-OH of *p*-coumaric acid. The linkages and/or positions of the attachments of the sugar and acyl groups in this pigment were confirmed by using 2D COSY, 2D NOESY and NOEDIF experiments as described above (see section 2.1.). Therefore, pigment **8** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-caffeoyl)-β-D-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) (Fig. 1), which is a new anthocyanin in plants.

2.3.4. Pigment *9*

The ^{1}H NMR spectrum of pigment **9** was also similar to that of pigment **8**. In this spectrum, the signal (δ 3.87) of the OCH_3 moiety at the 3 position of ferulic acid was observed (Table 2). The resonance (δ 3.87) of OCH_3 was correlated to the 2-H signal of ferulic acid by the analysis of 2D COSY and 2D NOESY spectra. The linkages of the sugar and acyl groups were also confirmed by analysis of 2D NOESY spectrum and NOEDIF experiments. Therefore, the structure of pigment **9** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-D-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) (Fig. 1), which is a new anthocyanin in plants.

3. Concluding remarks

From a chemotaxonomical point of view, anthocyanins have unusual chemical structures which provide the best kind of "taxonomic marker". Moreover, anthocyanins are the most important group of plant pigments concerned with flower color. As flower color has evolved, there has been a tendency for plants to produce more complex pigments. In structural terms, the trend has diverted from the simple anthocyanin towards more complex methylated, glycosylated and acylated derivatives (Harborne, 1963). In the Cruciferae, there are two typical anthocyanin glycoside patterns such as anthocyanidin 3-sophoroside-5-glucoside and 3-sambubioside-5-glucoside (Harborne, 1967; Honda et al., 2005). In this study, the latter anthocyanin pattern was restrictively taken up in the Cruciferae. As a dominant characteristic in the Cruciferae, the system controlling acylation is rather unique as explained as follows (Table 3 and Fig. 1); the first acylation is at 6-OH of Glu A in the 3-sambubiose residue with hydroxycinnamic acid, the second one is at 2-OH of xylose with hydroxycinnamic acid, and the third one is at 6-OH of the 5 glucose residue with malo-

Table 3
Variations of acyl groups in acylated cyanidin 3-sambubioside-5-glucoside in the family Cruciferae and their bluing effects on the flower color

Plant species (flower color)[a]	Acyl residue[f]		
	R_1	R_2	R_3
Cheiranthus cheiri (Red 54A)	*trans*-, *cis*-*p*-coumaroyl		
Lunaria annua (Purple 78A)	*trans*-, *cis*-*p*-coumaroyl *trans*-feruloyl		malonyl
Matthiola incana[b] (Purple 76-Violet 84)	*trans*-*p*-*coumaroyl* *trans*-caffeoyl *trans*-feruloyl	*trans*-sinapoyl	malonyl
Sinapis alba[c]	*trans*-feruloyl	*trans*-sinapoyl	malonyl
Arabidopsis thaliana[d]	glucosyl-*trans*-*p*-coumaroyl *trans*-*p*-coumaroyl	*trans*-sinapoyl	malonyl
Lobularia maritima (Purple-Violet 81A)	glucosyl-*trans*-*p*-coumaroyl *trans*-*p*-coumaroyl	*trans*-caffeoyl *trans*-feruloyl	
Orychophragonus violaceus[e] (Violet-Blue 90C)	glucosyl-*trans*-*p*-coumaroyl glucosyl-*trans*-feruloyl glucosyl-*trans*-sinapoyl	glucosyl-*trans*-caffeoyl glucosyl-*trans*-caffeoyl	malonyl

[a] R.H.S. color chart.
[b] Saito et al. (1995).
[c] Takeda et al. (1988).
[d] Bloor and Abrahams (2002).
[e] Honda et al. (2005).
[f] R_1, R_2, and R_3 are the same as those of Fig. 1.

nic acid. Moreover, the acyl groups at 6-OH of Glu A and 2-OH-xylose were modified with a variety of additional glucosylation and acylation patterns, as observed in the acylated pigments of *O. violaceus* and *Arabidopsis thaliana* (see Table 3). The complexity of acyl groups is considered to be responsible for an advanced character in the Cruciferae (Harborne, 1963, 1967; Honda and Saito, 2002). As shown in Table 3, the acyl groups of *O. violaceus* are very complex and show the most bluish color in this family. But the pigments of *C. cheiri* are simply acylated with only one molecule of *p*-coumaric acid at 6-OH of Glu A (Fig. 1 and Table 3), and show a red color.

4. Experimental

4.1. General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using nine mobile phases: BAW (*n*-BuOH–HOAc–H_2O, 4:1:2), BuHCl (*n*-BuOH–2N HCl, 1:1, upper layer), AHW (HOAc–HCl–H_2O, 15:3:82), 1% HCl and Forestal (HOAc–HCl–H_2O, 30:3:10) for anthocyanins, and BAW, APW (EtOAc–pyridine–H_2O, 15:7:5), EAA (EtOAc–HCOOH–H_2O, 5:2:1), BEW (*n*-BuOH–EtOH–H_2O, 4:1:2.2) and 15% HOAc for sugars and organic acid with UV light and aniline hydrogen phthalate spray reagent (Harborne, 1984).

Analytical HPLC was performed on 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 MPS-2400 (Shimadzu) in 0.1% HCl–MeOH (from 200 to 700 nm). FAB mass 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 measured at 500 MHz for ^1H spectra and at 125.78 MHz for ^{13}C spectra in DMSO-d_6–CF_3COOD (9:1). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants are in Hz.

4.2. Plant materials

Seeds of rose-red flowers of *C. cheiri* 'Vega', purple flowers of *L. annua* and purple-violet flowers of *L. maritima* 'Easter bonnet violet' were purchased from Takii Co. Ltd (Kyoto). The seeds were sown in August 2004, and the plants grown in a greenhouse of Takushoku University, Hokkaido Jr. College. The fresh plants were collected in December 2004 (*C. cheiri* and *L. maritima*) and June 2005 (*L. annua*), and dried overnight at 40 °C, and kept in a refrigerator at about 4 °C. These flowers exhibited Rose-Red [Red 54A by Royal Hoticultural Society (R.H.S)], color chart and its chromaticity value ($b^*/a^* = 0.34$) for *C. cheiri* 'Vega', purple (Purple 78A and $b^*/a^* = -0.39$) for *L. annua* and Purple-violet (Purple-violet 81A and $b^*/a^* = -0.52$) for *L. maritima* 'Easter bonnet violet'. The chromaticity values were recorded on a spectrophotometer CM-2022 (MINOLTA Co., Ltd.).

4.3. Extraction and purification of anthocyanins

Each dried flower (ca. 25 g) of *C. cheiri*, *L. annua* and *L. maritima* was immersed in 5% HOAc–H_2O (2 L; HOAc–H_2O, 1:19) at room temp. for 5 h and extracted. The three extracts were subjected to Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) CC, respectively, and washed with H_2O. The pigments were eluted from these CCs with 5% HOAc–MeOH (500 ml). After concentration, the eluates were fractionated with paper chromatography (PC) using BAW. The crude fractionated pigments obtained 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 18 min from 55% to 80% solvent B in solvent A. Each fraction was transformed to a Diaion HP-20 column, and its anthocyanins were eluted with 5% HOAc–MeOH from the column, and concentrated anthocyanin residues were dissolved in a small volume of 5% HOAc–EtOH, respectively, followed by addition of an excess of Et_2O. Anthocyanin powders, pigment **1** (ca. 20 mg) and pigment **2** (ca. 10 mg) from *C. cheiri*, pigment **3** (*ca*. 10 mg), pigment **4** (ca. 5 mg), and pigment **5** (*ca*. 6 mg) from *L. annua*, and pigment **1** (ca. 5 mg), pigment **6** (ca. 6 mg), pigment **7** (ca. 5 mg), pigment **8** (ca. 5 mg), and pigment **9** (ca. 10 mg) from *L. maritima*.

4.4. Analyses of anthocyanin

Characterization of pigments **1**–**9** was carried out according to the procedure of the standard methods (Harborne, 1984; Honda et al., 2005), and their data are summarized in Table 1. Acid hydrolysis of pigments **1**–**9** (ca. 3 mg) was carried out with 2 N HCl (15 ml) at 100 °C for 1 h to give cyanidin, glucose, xylose, *p*-coumaric acid, caffeic acid, ferulic acid, and malonic acid. Those compounds were confirmed by direct comparison of TLC and HPLC with the authentic samples. Alkaline hydrolysis of pigments **1**–**9** (ca. 3 mg) was carried out with 2 N NaOH solution (20 mL) under N_2 gas at ambient temperature for 15 min to provide only one deacylated anthocyanin, whose structure was identified to that of cyanidin 3-sambubioside-5-glucoside by the analyses of TLC and HPLC with the authentic sample obtained from *Matthiola* violet anthocyanin (Saito et al., 1995). 4-Glucosyl-*p*-coumaric acid was detected in the alkaline hydrolysate, and identified by comparison of TLC and HPLC with the authentic sample obtained from Ternatins of *Clitoria ternatea* by the same alkaline hydrolysis (Terahara et al., 1990).

4.5. High-resolution FABMS

Pigment **1**: HR-FABMS calc. for $C_{41}H_{45}O_{22}$: 889.2403. Found: 889.2403.

Pigment **2**: HR-FABMS calc. for $C_{41}H_{45}O_{22}$: 889.2403. Found: 889.2437.

Pigment **3**: HR-FABMS calc. for $C_{44}H_{47}O_{25}$: 975.2406. Found: 975.2405.

Pigment **4**: HR-FABMS calc. for $C_{44}H_{47}O_{25}$: 975.2406. Found: 975.2405.

Pigment **5**: HR-FABMS calc. for $C_{45}H_{49}O_{26}$: 1005.2512. Found: 1005.2498.

Pigment **6**: HR-FABMS calc. for $C_{47}H_{55}O_{27}$: 1051.2931. Found: 1051.2948.

Pigment **7**: HR-FABMS calc. for $C_{56}H_{61}O_{30}$: 1213.3248. Found: 1213.3268.

Pigment **8**: HR-FABMS calc. for $C_{50}H_{51}O_{25}$: 1051.2719. Found: 1051.2728.

Pigment **9**: HR-FABMS calc. for $C_{51}H_{53}O_{25}$: 1065.2876. Found: 1065.2922.

4.6. ^{13}C NMR data of pigments *1*, *2* and *3* (125.78 MHz, DMSO-d_6–CF_3COOD, 1:9, an internal standard of TMS)

Pigment **1**: δ Cyanidin 162.4 (C-2), 144.4 (3), 131.7 (4), 155.5 (5), 105.2 (6), 167.7 (7), 96.4 (8), 155.2 (9), 111.8 (10), 118.8 (1′), 117.9 (2′), 146.6 (3′), 155.5 (4′), 116.9 (5′), 128.3 (6′); *p*-Coumaric acid 125.2 (1), 130.7 (2), 116.0 (3), 160.2 (4), 116.0 (5), 130.7 (6), 113.9 (α), 145.4 (β), 166.9 (CO); Glucose A 98.5 (1), 80.7 (2), 76.7 (3), 70.1 (4), 74.1 (5), 63.3 (6); Glucose B 102.5 (1), 77.9 (2), 76.3 (3), 74.5 (4), 73.5 (5), 61.1 (6); Xylose 104.8 (1), 74.5 (2), 76.8 (3), 69.7 (4), 66.3 (5).

Pigment **2**: δ Cyanidin 162.1 (C-2), 144.5 (3), 130.6 (4), 155.2 (5), 103.9 (6), 167.7 (7), 96.0 (8), 155.2 (9), 111.4 (10), 119.6 (1′), 117.9 (2′), 146.4 (3′), 155.1 (4′), 116.9 (5′), 128.1 (6′); *p*-Coumaric acid 125.7 (1), 132.9 (2), 114.8 (3), 159.0 (4), 114.8 (5), 132.9 (6), 114.8 (α), 144.6 (β), 166.2 (CO); Glucose A 98.0 (1), 80.8 (2), 76.7 (3), 69.7 (4), 74.5 (5), 63.4 (6); Glucose B 101.8 (1), 77.6 (2), 76.0 (3), 74.6 (4), 73.2 (5), 60.7 (6); Xylose 104.8 (1), 74.5 (2), 76.8 (3), 69.5 (4), 66.3 (5).

Pigment **3**: δ Cyanidin 162.4 (C-2), 144.4 (3), 131.4 (4), 155.0 (5), 105.0 (6), 167.5 (7), 96.3 (8), 155.2 (9), 111.6 (10), 119.6 (1′), 117.8 (2′), 146.5 (3′), 155.4 (4′), 116.9 (5′), 128.3 (6′); *p*-Coumaric acid 125.1 (1), 130.5 (2), 115.8 (3), 160.0 (4), 115.8 (5), 130.5 (6), 113.9 (α), 145.2 (β), 167.0 (CO); Glucose A 98.3 (1), 80.6 (2), 76.7 (3), 70.1 (4), 74.0 (5), 63.4 (6); Glucose B 102.0 (1), 73.3 (2), 75.9 (3), 69.6 (4), 74.3 (5), 64.2 (6); Xylose 104.8 (1), 74.5 (2), 76.7 (3), 69.7 (4), 66.2 (5); Malonic acid 41.3 (–CH_2–), 166.8 (CO), 168.2 (CO).

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