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Acylated cyanidin 3-sambubioside-5-glucosides in the flowers of *Erysimum* cultivars (Brassicaceae)

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Abstract

Two malonylated anthocyanins were isolated and determined to be cyanidin 3-(*trans-p*-coumaroyl)-sambubioside-5-(malonyl)-glucoside and cyanidin 3-(*cis-p*-coumaroyl)-sambubioside-5-(malonyl)-glucoside as major pigments along with cyanidin 3-(*trans-p*-coumaroyl)-sambubioside-5-glucoside, cyanidin 3-(*cis-p*-coumaroyl)-sambubioside-5-glucoside, and cyanidin 3-sambubioside-5-glucoside from the 10 cultivars of *Erysimum* by chemical and spectroscopic methods. Regarding the flower color variation in these cultivars, decrease of the hue values (b*/a*) and increase of color chart numbers of these cultivars were responsible for the increase of malonylated anthocyanins.

Introduction

Erysimum including *Cheiranthus* is a genus of Brassicaceae and has approximately 80 species of annuals, biennials, or woody-based perennials [1]. *Erysimum* cultivars are suitable as garden plants for walls, path edgings, and beds. Recently, complicated complex anthocyanins have been detected in the flowers of brassicaceous plants, such as acylated 3-sambubioside-5-glucosides of pelargonidin, cyanidin, and delphinidin in *Arabis*, *Aubrieta, Cheiranthus, Heliophila, Hesperis, Ionopsidium, Lobularia, Lunaria, Matthiola,* and *Orychophragmus* [2- 15], acylated 3-(3^X-glucosylsambubioside)-5-glucosides of cyanidin in *Ionopsidium* and *Malcolmia* [15, 16], and acylated 3-sophoroside-5-glucosides of pelargonidin, cyanidin and peonidin in *Raphanus*, *Iberis*, and *Moricandia* [17-23]. There is only one previous report on acylated anthocyanin structures from *Cheiranthus cheiri* (Synonym: *Erysimum cheiri*). Cyanidin 3-(*trans-p*-coumaroyl)-sambubioside-5-glucoside and cyanidin 3-(*cis-p*-coumaroyl)-sambubioside-5-glucoside were

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detected in the red flowers of *C. cheiri* (Syn. *E. cheiri*) 'Vega Rose Red' as major anthocyanins [10].

Materials and Methods

General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using seven mobile phases: BAW (*n*-BuOH-HOAc-H_2O, 4:1:2, v/v/v), BuHCl (*n*-BuOH-2N HCl, 1:1, v/v, upper layer), AHW (HOAc-HCl-H_2O, 15:3:82, v/v/v), 1% HCl for anthocyanins, Forestal (HOAc-HCl-H_2O, 30:3:10, v/v/v) for anthocyanidins and BAW, EAA (EtOAc-HOAc-H_2O, 3:1:1, v/v/v) and EFW (EtOAc-HCOOH-H_2O, 5:2:1, v/v/v) for sugars and organic acids with UV light and aniline hydrogen phthalate spray reagent [24].

Analytical HPLC was performed on a LC 10A system (Shimadzu), using a Waters C18 (4.6 × 250 ㎜) column at 40 ℃ 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) with 5 min of re-equilibration at 20% solvent B, for anthocyanins, anthocyanidins and hydroxycinnamic acids (method 1). The other eluant for malonic acid was applied as an isocratic elution of solvent A for 10 min and monitored at 210 nm [20] (method 2).

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 determined on a JEOL JMS-700 Mass spectrometer operating in the positive ion mode using 1:1 mixture of dithiothreitol and 3-nitrobenzyl alcohol as a matrix. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were measured on a JEOL AL-400 MHz NMR spectrometer using CF_3COOD-DMSO-d_6 (1:9) as a solvent. Chemical shifts are reported on the δ-scale from tetramethylsilane as the internal standard, and coupling constants (J) are in Hz.

Plant materials

The plants of *E.* 'Artist Paint Box' (voucher specimen No. IUM 19307), *E.* 'Bowles's Mauve' (IUM 19262), *E. cheiri* 'Vega Rose Red' (IUM 19287), *E. cheiri* 'Vega Scarlet' (IUM 19284), *E.* 'Monet's Moment' (IUM 19312), *E.* 'Spring Perty Joy' (IUM 19310), *E.* 'Spring Perty Perty' (IUM 19304), and *E.* 'Spring Perty Rouge' (IUM 19311) were purchased from the Takii Seed Co. Ltd. (Japan) and grown at the experimental farm of Iwate University (Figure 1). Moreover, the plants of *E.* 'Cotswold Gem' (IUM 19281) and *E.* 'Pastel Patchwork' (IUM 19283) were purchased from the World Fantasy Garden Ltd. (Japan) and grown at the experimental farm of Iwate University (Figure 1). Fresh flowers *E.* 'Artist Paint Box' [Purple 75A by RHS color chart and its chromaticity values (L*= 61.22, b*/a*= -3.86/15.86 = -0.24) by CM-700d Spectro Color Meter (Konica-Minolta Co. Ltd., Japan)], *E.* 'Bowles's Mauve' [Purple N78C, L*= 60.25, b*/a*= -20.14/19.16 = -1.05], *E. cheiri* 'Vega Rose Red' [Red 53A, L*= 41.70, b*/a*= 3.61/17.93 = 0.20], *E. cheiri* 'Vega Scarlet' [Orange-Red N34B, L*= 43.90, b*/a*= 11.66/18.82

= 0.62], *E.* 'Monet's Moment' [Red-Purple 71B, L*= 50.71, b*/a*= 1.30/18.88 = 0.07], *E.* 'Spring Perty Joy', [Purple N78C, L*= 50.90, b*/a*= -18.82/25.67 = -0.73], *E.* 'Spring Perty Perty' [Red-Purple 73B, L*= 62.45, b*/a*= -1.22/15.59 = -0.08], *E.* 'Spring Perty Rouge' [Red 46B, L*= 45.40, b*/a*= 4.71/15.03 = 0.31], *E.* 'Cotswold Gem' [Purple N78C, L*= 60.35, b*/a*= -14.64/18.29 = -0.80], and *E.* 'Pastel Patchwork' [Purple 77C, L*= 62.10, b*/a*= -6.21/11.88 = -0.52] were collected in spring. Voucher specimens are deposited at the Iwate University Museum (IUM). Flowers were collected from winter to spring seasons in Iwate, Japan and dried overnight at 40 ℃, and kept in a refrigerator at 4 ℃.

Isolation of anthocyanins

Dried petals (5 g) were immersed in 5% HOAc (acetic acid-H_2O, 5:95, v/v, 500 ml), kept at 4 ℃ for 1 day and extracted. Crude extracted pigments were isolated and purified by Diaion HP-20 Ion Exchange Resins (Mitsubishi Chemical's) column (90㎜×150㎜) chromatography, paper chromatography (developed by BAW: *n*-BuOH-HOAc-H_2O, 4:1:2, v/v/v), and prep. HPLC was performed on an LC 10A system (Shimadzu) using a Waters C18 (19×150㎜) column at 40℃ with a flow rate of 4 ml/min. The eluate was monitored at 530 nm. The eluant was applied to isocratic elution with 60% solvent B in solvent A. Finally, pigments 5 (ca. 5 mg) and 12 (ca. 15 mg) were obtained.

Analyses of anthocyanins

The identification of pigments (5 and 12) were carried out by standard procedures with both alkaline and acid hydrolyses [24]. Acid hydrolysis of pigments (ca. 1 mg each) was carried out with 2N HCl (1 ml) at 100 ℃ for 1h. Alkaline hydrolysis of pigments (ca. 1 mg each) was carried out with 2N NaOH solution (1 ml) under degassed syringe allowed to stand for 15 min. The solution was next acidified with 2N HCl (1.1 ml) and evaporated *in vacuo* to dryness. These products were analyzed by standard procedures [24]. The data of TLC (R_f values), HPLC (R_t-min), UV-Vis (λ_{max}), and FABMS spectra are shown in below.

Pigment 12: Cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside]

UV-VIS in 0.1 % HCl-MeOH; λ_{max} 529, 311, 296, 281 nm, E_{acyl}/E_{max} (%) = 92, E_{440}/E_{max} (%) = 15 $AlCl_3$ shift +, TLC; R_f-values BAW (*n*-BuOH-HOAc-H_2O, 4:1:2, v/v/v) 0.33, BuHCl (*n*-BuOH-2N HCl, 1:1, v/v, upper phase) 0.09, 1% HCl 0.31, AHW (HOAc-HCl-H_2O, 15:3:82, v/v/v) 0.58, HPLC; R_t (min) 34.2, HR-FABMS; calc. for $C_{44}H_{47}O_{25}$: 975.2406. found: 975.2403. ^{1}H NMR δ cyanidin: 8.75 (*s*, H-4), 7.00 (*d*, *J* = 1.7 Hz, H-6), 7.04 (*d*, *J* = 1.7 Hz, H-8), 8.05 (*d*, *J* = 2.4 Hz, H-2'), 7.07 (*d*, *J* = 8.8 Hz, H-5'), 8.38 (*dd*, *J* = 2.4, 8.8 Hz, H-6'). *trans*-*p*-coumaric acid: 7.32 (*d*, *J* = 8.6 Hz, H-2, 6), 6.73 (*d*, *J* = 8.6 Hz, H-3, 5), 6.27 (*d*, *J* = 15.9 Hz, H-α), 7.37 (*d*, *J* = 15.9 Hz, H-β). malonic acid: 3.37 (*s*, -CH_2-). glucose A: 5.72 (*d*, *J* = 7.6 Hz, H-1), 4.03 (*t*, *J* = 8.4 Hz, H-2), 3.77 (*t*, *J* = 8.3 Hz, H-3), 3.47 (*t*, *J* = 9.5 Hz, H-4), 4.02 (*m*, H-5), 4.30 (*dd*, *J* = 7.4, 11.7 Hz, H-6a), 4.45 (*dd*, *J* = 2.2, 11.7 Hz, H-6b). glucose B: 5.18 (*d*, *J* = 7.6 Hz, H-1), 3.59 (*t*, *J* = 8.5 Hz, H-2), 3.44 (*t*, *J* = 9.5 Hz, H-3), 3.30 (*t*, *J* = 9.5 Hz, H-4), 3.81 (*m*, H-5), 4.09 (*dd*, *J* = 6.1, 11.7 Hz, H-6a), 4.42 (*brd*, *J* = 11.7 Hz, H-6b). xylose: 4.74 (*d*, *J* = 7.8 Hz, H-

1), 3.05 (*t*, *J* = 8.3 Hz, H-2), 3.14 (*t*, *J* = 8.8 Hz, H-3), 3.27 (*m*, H-4), 3.57 (*m*, H-5a), 3.01 (*t*, *J* = 10.4 Hz, H-5b). ^{13}C NMR δ cyanidin: 162.4 (C-2), 144.5 (C-3), 131.6 (C-4), 155.3 (C-5), 105.0 (C-6), 167.7 (C-7), 96.4 (C-8), 155.1 (C-9), 111.7 (C-10), 119.7 (C-1'), 117.9 (C-2'), 146.6 (C-3'), 155.6 (C-4'), 117.0 (C-5'), 128.4 (C-6'). *trans-p*-coumaric acid: 125.2 (C-1), 130.6 (C-2, 6), 115.9 (C-3, 5), 160.1 (C-4), 114.0 (C-7), 145.3 (C-8), 166.9 (C-9). malonic acid: 41.4 (-CH_2-), 167.1 (COO-1), 168.3 (COO-2). glucose A: 98.4 (C-1). 80.7 (C-2), 76.8 (C-3), 70.2 (C-4), 74.1 (C-5), 63.4 (C-6), glucose B: 102.0 (C-1), 73.4 (C-2), 76.0 (C-3), 69.8 (C-4), 74.4 (C-5), 64.2 (C-6). xylose: 104.9 (C-1), 74.6 (C-2), 76.8 (C-3), 69.7 (C-4), 66.3 (C-5).

Pigment 5: Cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*cis-p*- coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside]

UV-VIS in 0.1 % HCl-MeOH; λ_{max} 533, 313, 297, 280 nm, E_{acyl}/E_{max} (%) = 120, E_{440}/E_{max} (%) = 16 $AlCl_3$ shift +, TLC; R_f-values BAW 0.42, BuHCl 0.11, 1% HCl 0.36, AHW 0.57, HPLC; R_t (min) 27.6, HR-FABMS; calc. for $C_{44}H_{47}O_{25}$: 975.2406. found: 975.2418. ^{1}H NMR δcyanidin: 8.60 (s, H-4), 6.90 (*brs*, H-6), 6.91 (*brs*, H-8), 8.04 (*d*, *J* = 2.4 Hz, H-2'), 7.09 (*d*, *J* = 8.8 Hz, H-5'), 8.32 (*dd*, *J* = 2.4, 8.8 Hz, H-6'). *cis-p*-coumaric acid: 7.30 (*d*, *J* = 8.8 Hz, H-2, 6), 6.50 (*d*, *J* = 8.8 Hz, H-3, 5), 5.73 (*d*, *J* = 13.2 Hz, H-α), 6.49 (*d*, *J* = 13.2 Hz, H-β). malonic acid: 3.55 (s, -CH_2-). glucose A: 5.72 (*d*, *J* = 7.1 Hz, H-1), 4.06 (*t*, *J* = 8.5 Hz, H-2), 3.75 (*m*, H-3), 3.36 (*t*, *J* = 9.3 Hz, H-4), 3.98 (*m*, H-5), 4.35 (*m*, H-6a), 4.51 (*brd*, *J* = 11.9 Hz, H-6b). glucose B: 5.23 (*d*, *J* = 7.8 Hz, H-1), 3.55 (*t*, *J* = 8.8 Hz, H-2), 3.38 (*m*, H-3), 3.29 (*m*, H-4), 3.73 (*m*, H-5), 3.98 (*m*, H-6a), 4.43 (*m*, H-6b). xylose: 4.74 (*d*, *J* = 7.8 Hz, H-1), 3.03 (*t*, *J* = 8.2 Hz, H-2), 3.17 (*t*, *J* = 8.8 Hz, H-3), 3.23 (*m*, H-4), 3.52 (*m*, H-5a), 2.97 (*t*, *J* = 10.7 Hz, H-5b).

Cyanidin

UV-Vis: λ_{max} 536, 273 nm, E_{440}/E_{max} = 44%, $AlCl_3$ shift +; TLC: R_f-values (x 100) Forestal 42; HPLC (method 1) : R_t (min) 25.3.

Glucose

TLC: R_f-values (x 100) BAW 24, EAA 18, EFW 49; Color (AHP) Brown.

Xylose

TLC: R_f-values (x 100) BAW 29, EAA 26, EFW 49; Color (AHP) Reddish Brown.

p-Coumaric acid

TLC: R_f-values (x 100) BAW 91 and 91 (*trans* and *cis*), EAA 96 and 96 (*trans* and *cis*), EFW 85 and 85 (*trans* and *cis*) ; Color (Under UV) Violet; HPLC (method 1) : R_t (min) 17.2 and 16.2 (*trans* and *cis*).

Malonic acid

HPLC (method 2) : R_t (min) 4.1.

E. 'Bowles's Mauve'

E. 'Spring Perty Perty'

E. 'Cotswold Gem'

E. 'Monet's Moment'

E. 'Spring Perty Joy'

E. cheiri 'Vega Rose Red'

E. 'Pastel Patchwork'

E. 'Spring Perty Rouge'

E. 'Artist Paint Box'

E. cheiri 'Vega Scarlet'

Results and Discussion

Isolation and identification of anthocyanins

Over 15 anthocyanin peaks were observed in the 5%HOAc-H_2O extract from the flowers of *Erysimum* cultivars by high performance liquid chromatography (HPLC) (Table 1). Three of these peaks have previously been identified in *C. cheiri* (Syn. *E. cheiri*) 'Vega Rose Red' anthocyanins [10] as cyanidin 3-sambubioside-5-glucoside (1 in Table 1), cyanidin 3-(*cis-p*-coumaroyl)-sambubioside-5-glucoside (3 in Table 1), and cyanidin 3-(*trans-p*-coumaroyl)-sambubioside-5-glucoside (10 in Table 1).

In the present paper, we wish to report the results of structural study of additional two acylated cyanidin glycosides [5 in Table 1: (Rt (min) = 27.6) and 12 in Table 1: (Rt (min) = 34.2)] from the flowers of *Erysimum* cultivars. These two anthocyanins were extracted and purified from the dried purple petals of *E.* 'Bowles's Mauve'.

Acid hydrolysis of pigments 5 and 12 gave cyanidin as their anthocyanidin [24]. These anthocyanins contained glucose and xylose as their sugar components according to the results of acid hydrolysis. Moreover, *trans-p*-coumaric acid was detected in the hydrolysate of 12 and *cis-p*-coumaric acid was detected in that of 5 using HPLC, respectively.

By alkaline hydrolysis, pigments 5 and 12 yielded cyanidin 3-sambubioside-5-glucoside as their deacyl anthocyanin. The deacyl anthocyanin structure was identified by direct comparison of the analyses of co-TLC and co-HPLC with authentic cyanidin 3-sambubioside-5-glucoside that was prepared from *Lunaria annua* [10]. Moreover, *trans-p*-coumaric acid was detected in the hydrolysate of 12 and *cis-p*-coumaric acid was detected in that of 5 by HPLC, respectively.

The pigments 5 and 12 were presumed to be cyanidin 3-(*cis-p*-coumaroyl)-sambubioside-5-(malonyl)-glucoside and cyanidin 3-(*trans-p*-coumaroyl)-sambubioside-5-(malonyl)-glucoside based on comparisons with authentic samples obtained from *Lunaria annua* [10] by co-TLC, co-HPLC, and UV-VIS spectrometry. Moreover, elemental components of these pigments were confirmed by measuring their high resolution fast atom bombardment mass spectra (HR-FABMS). The structure of these pigments were confirmed by analysis of their ^{1}H (400 MHz) and 2D (COSY and NOESY) NMR spectra in CF_3COOD-DMSO-d_6 (1:9). Chemical shifts were reported relative to a tetramethylsilane (TMS) internal standard (δ), and coupling constants (J) are in Hz. Moreover, the structure of pigment 12 was further confirmed by the measurements of its ^{13}C (100 MHz) and 2D (^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC) NMR spectra (see section Materials and Methods).

Pigment 12

Pigment 12 was presumed to be cyanidin 3-(*trans-p*-coumaroyl)-sambubioside-5-(malonyl)-glucoside when compared with an authentic sample obtained from purple flowers of *L. annua* [10] by co-TLC, co-HPLC, UV-VIS spectrometry (see section experimental).

The molecular ion $[M]^+$ of pigment 12 was observed at m/z 975 ($C_{44}H_{47}O_{25}$) indicating that pigment 12 was composed of cyanidin with two molecules of glucose, one molecule each of xylose, *p*-coumaric acid and malonic acid. The elemental components were confirmed by

measuring its HR-FABMAS, and the structure was elucidated based on the analysis of the ^{1}H and ^{13}C NMR spectra (see section Materials and Methods).

The chemical shifts of 10 aromatic protons of cyanidin and *p*-coumaric acid moieties with their coupling constants were assigned as shown in the section Materials and Methods. A set of one pair of doublet peaks assigned to two olefinic proton signals of *p*-coumaric acid with its large coupling constant (J = 15.9 Hz) showed that the acid was present with the *trans* configuration. The chemical shifts of the sugar moieties were observed in the region of δ 5.72 - 3.01, where the three anomeric protons exhibited at δ 5.72 (*d*, J = 7.6 Hz, Glc A), δ 5.18 (*d*, J = 7.6 Hz, Glc B), δ 4.74 (*d*, J = 7.8 Hz, Xylose). Based on the observed coupling constants, these three sugars were assumed to be in the β-pyranose forms. The linkages and/or positions of the attachments of the sugar and acyl groups were determined based on 2D COSY and NOESY experiments.

By application of the NOESY experiment, the NOEs between H-1 of Glc A and H-4 (δ 8.75) of cyanidin, H-1 of Glc B and H-6 (δ 7.00) of cyanidin, and H-2 (δ 4.03) of Glc A and H-1 of Xyl were observed supporting that OH-3 and OH-5 of cyanidin are glycosylated with Glc A and Glc B, respectively, and also OH-2 of Glc A is bonded with xylose forming sambubiose (Figure 2).

Four characteristic proton signals shifted to a lower magnetic field were also assigned to the methylene protons of Glc A (δ 4.30 and δ 4.45, H-6a and b) and Glc B (δ 4.09 and δ 4.42, H-6a and b). Thus, two hydroxyl groups of the sugar moieties, OH-6s of Glc A and B, were assumed to be acylated with two molecules of acids. In the NOESY spectrum, the correlations between H-6a, b of Glc A and H-2, 6 of *p*-coumaric acid were observed, establishing

Table 1 : Flower colors and anthocyanins in 10 cultivars of *Erysimum*.

Cultivars	RHS. CC	b*/a*	HPLC data of anthocyanins (as%)															
			1[a]	2[f]	3[b]	4[f]	5[c]	6[f]	7[f]	8[f]	9[f]	10[d]	11[f]	12[e]	13[f]	14[f]	15[f]	16[f]
		(hue) Rt(min)	14.3	19.0	26.8	27.3	27.6	28.0	28.6	29.3	29.7	32.6	33.2	34.2	34.7	34.9	35.5	36.5
E. 'Bowles's Mauve'	Purple N78C	-1.05	0.6	0.5	0.8	1.0	8.4	0.1	0.3		0.5	4.6	1.6	42.6	11.0	5.5	16.1	2.8
E. 'Cotswold Gem'	Purple N78C	-0.80	0.2	0.4	0.1	0.8	12.8	0.1	0.5	0.2	0.2	3.8	0.8	56.8	2.4	9.7	5.5	3.3
E. 'Spring Perty Joy'	Purple N78C	-0.73	0.3	1.0	0.4	1.8	4.3		0.3		0.6	4.4	2.9	38.4	3.9	16.6	5.3	7.2
E. 'Pastel Patchwork'	Purple 77C	-0.52	2.2	4.2	0.9	1.4	6.1	0.1	0.5	0.1	0.4	3.7	2.0	37.4	5.2	15.6	10.2	6.2
E. 'Artist Peint Box'	Purple 75A	-0.24	5.2	0.1	4.0		0.9	0.1	0.6		0.2	24.3	19.0	25.2	7.9	2.6	1.3	0.5
E. 'Spring Perty Perty'	Red-Purple 73B	-0.08	0.7	0.5	2.7	0.7	6.9		1.1	0.1	1.4	22.7	6.2	39.1		12.8		1.3
E. 'Monet's Moment'	Red-Purple 71B	0.07	1.0	0.1	2.2		9.7		0.4	0.3	0.1	15.1	2.8	47.4	4.8	5.8	2.8	
E. cheiri 'Vega Rose Red'	Red 53A	0.20	1.2		4.5				0.3			76.6	3.2	2.9		6.6	0.3	0.2
E. 'Spring Perty Rouge'	Red 46B	0.31	1.4		4.9		0.2		0.3			51.7	20.2	10.1	2.2	4.4	1.1	0.3
E. cheiri 'Vega Scarlet'	Orange-Red N34B	0.62	1.0		4.8				0.4			67.8	8.5	4.6		6.6	0.7	0.1

[a]cyanidin 3-sambubioside-5-glucoside [10]
[b]cyanidin 3-(*cis-p*-coumaroyl)-sambubioside-5-glucoside [10]
[c]cyanidin 3-(*cis-p*-coumaroyl)-sambubioside-5-(malony1)-glucoside
[d]cyanidin 3-(*trans-p*-coumaroyl)-sambubioside-5-glucoside [10]
[e]cyanidin 3-(*trans-p*-coumaroyl)-sambubioside-5-(malony1)-glucoside
[f]Unknown anthocyanins

the acylation acid at C-6 OH (Glc A) as *p*-coumaric acid (Figure 2).

In the HMBC spectrum, the correlations between the anomeric proton of Glc A and C-3 carbon (δ 144.5) of cyanidin, the anomeric proton of Glc B and C-5 carbon (δ 155.3) of cyanidin, the anomeric proton of Xyl and C-2 carbon (δ 80.7) of Glc A, methine proton of H-2 of Glc A and C-1 carbon (δ 104.9) of Xyl, and methylene proton of Glc B and COOH carbon (δ 167.1) of malonic acid were observed, establishing the glycosylation or acylation group at C-3 OH (cyanidin), C-5 OH (cyanidin), C-2 OH (Glc A), COOH (malonic acid), and C-1 OH (Xyl) as Glc A, Glc B, Xyl, Glc B, and Glc A, respectively (Figure 1). Consequently, the structure of pigment 12 was determined to be cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Figure 1). Which is a new anthocyanin in genus *Erysimum* (Synonym: *Cheiranthus*), although this pigment has been found in the flowers of *Arabis*, *Aubrieta*, *Heliophila*, *Hesperis*, *Lunalia*, and *Matthiola* (family: Brassicaceae) [3, 5-8, 10, 13, 14, 25].

Pigment 5

The molecular ion $[M]^+$ of pigment 5 was observed at *m/z* 975 ($C_{44}H_{47}O_{25}$) indicating that pigment 5 was composed of cyanidin with two molecules of glucose, one molecule each of xylose, *p*-coumaric acid and malonic acid. The elemental components were confirmed by measuring its HR-FABMAS, and the structure was elucidated based on the analysis of the ^{1}H NMR spectra (see section Materials and Methods).

The ^{1}H NMR spectrum of pigment 5 was superimposed on that of pigment 12 except for signals of a *p*-coumaric acid moiety. In particular, the chemical shifts of the olefinic protons were shifted to a higher magnetic field at δ 5.73 and 6.49 with smaller coupling constants (J = 13.2 and 13.2 Hz) in comparison with those of pigment 12 (δ 6.27, 15.9 Hz and δ 7.37, 15.9 Hz). Thus the configuration of *p*-coumaric acid was confirmed to be *cis*, pigment 5 was determined to be cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*cis*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Figure 2). Which is a new anthocyanin in genus *Erysimum* (Synonym: *Cheiranthus*), although this pigment has been found in the flowers of *Arabis* and *Lunalia* (family: Brassicaceae) [6, 10].

Four acylated cyanidin 3-sambubioside-5-glucosides were isolated from the flowers of *Erysimum* (including *Cheiranthus*) cultivars. From the chemotaxonomical point of view, there are two glycosidic patterns in the flowers of plants in the Brassicaceae at OH-3 of anthocyanins, such as 3-sambubioside (including 3-(3^X-glucosylsambubioside)) [2-16] and 3-sophoroside [17-23]. The floral anthocyanins of *Erysimum* cultivars are grouped into the former pattern. Moreover, the distribution of cyanidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] has been reported in seven genera of this family, *Arabidopsis*, *Arabis, Aubrieta*, *Heliophila*, *Hesperis*, *Lunaria*, and *Matthiola* [3, 5-8, 10, 13, 14, 25]. Therefore, *Erysimum* is the eighth genus known to contain cyanidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside].

When comparing anthocyanin distributions, 10 cultivars of *Erysimum* analyzed in this study contained cyanidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] (5 and 12) and/or cyanidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-glucoside (3 and 10)

as major anthocyanins (Table 1). Regarding the flower color variation in these cultivars, decrease of the hue values (b*/a*) and increase of color chart numbers of these cultivars (Table 1) were responsible for the increase of malonylated anthocyanins (5 and 12). Therefore, an increase of malonylated anthocyanins is considered to be one of the contributing factors for bluing effect in *Erysimum* flower coloration.

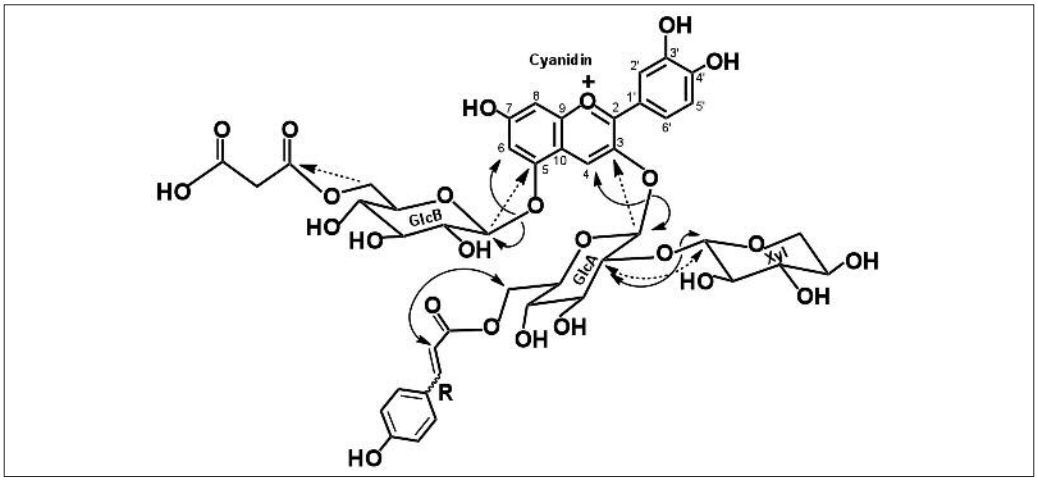

Figure 2 : Two additional acylated cyanidin 3-sambubioside-5-glucosides were isolated from the flowers of *Erysimum* cultivars. (12, R = *trans*, 5, R = *cis*). Observed main NOEs in pigments 12 and 5 are indicated by arrows.
Observed main HMBCs in pigment 12 are indicated by dotted arrows.

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