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The blue anthocyanin pigments from the blue flowers of *Heliophila coronopifolia* L. (Brassicaceae)

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ABSTRACT

Six acylated delphinidin glycosides (pigments **1**–**6**) and one acylated kaempferol glycoside (pigment **9**) were isolated from the blue flowers of cape stock (*Heliophila coronopifolia*) in Brassicaceae along with two known acylated cyanidin glycosides (pigments **7** and **8**). Pigments **1**–**8**, based on 3-sambubioside-5-glucosides of delphinidin and cyanidin, were acylated with hydroxycinnamic acids at 3-glycosyl residues of anthocyanidins. Using spectroscopic and chemical methods, the structures of pigments **1**, **2**, **5**, and **6** were determined to be: delphinidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(acyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], in which acyl moieties were, respectively, *cis*-*p*-coumaric acid for pigment **1**, *trans*-caffeic acid for pigment **2**, *trans*-*p*-coumaric acid for pigment **5** (a main pigment) and *trans*-ferulic acid for pigment **6**, respectively. Moreover, the structure of pigments **3** and **4** were elucidated, respectively, as a demalonyl pigment **5** and a demalonyl pigment **6**. Two known anthocyanins (pigments **7** and **8**) were identified to be cyanidin 3-(6-*p*-coumaroyl-sambubioside)-5-(6-malonyl-glucoside) for pigment **7** and cyanidin 3-(6-feruloyl-sambubioside)-5-(6-malonyl-glucoside) for pigment **8** as minor anthocyanin pigments. A flavonol pigment (pigment **9**) was isolated from its flowers and determined to be kaempferol 3-*O*-[6-*O*-(*trans*-feruloyl)-β-glucopyranoside]-7-*O*-cellobioside-4′-*O*-glucopyranoside as the main flavonol pigment.

On the visible absorption spectral curve of the fresh blue petals of this plant and its petal pressed juice in the pH 5.0 buffer solution, three characteristic absorption maxima were observed at 546, 583 and 635 nm. However, the absorption curve of pigment **5** (a main anthocyanin in its flower) exhibited only one maximum at 569 nm in the pH 5.0 buffer solution, and violet color. The color of pigment **5** was observed to be very unstable in the pH 5.0 solution and soon decayed. In the pH 5.0 solution, the violet color of pigment **5** was restored as pure blue color by addition of pigment **9** (a main flavonol in this flower) like its fresh flower, and its blue solution exhibited the same three maxima at 546, 583 and 635 nm. On the other hand, the violet color of pigment **5** in the pH 5.0 buffer solution was not restored as pure blue color by addition of deacyl pigment **9** or rutin (a typical flower copigment). It is particularly interesting that, a blue anthocyanin–flavonol complex was extracted from the blue flowers of this plant with H_2O or 5% HOAc solution as a dark blue powder. This complex exhibited the same absorption maxima at 546, 583 and 635 nm in the pH 5.0 buffer solution. Analysis of FAB mass measurement established that this blue anthocyanin–flavonol complex was composed of one molecule each of pigment **5** and pigment **9**, exhibiting a molecular ion $[M+1]^+$ at 2102 *m/z* ($C_{93}H_{105}O_{55}$ calc. 2101.542). However, this blue complex is extremely unstable in acid solution. It really dissociates into pigment **5** and pigment **9**.

1. Introduction

The Brassicaceae is a large family of natural plant species and includes many important ornamental plants such as stock, wall flower, honesty, sweet alyssum and so on. Moreover, the range of their flower colors includes white, yellow, orange, red, violet and violet-blue, but a clear blue color is lacking except for that of *Heliophila* (cape stock in English). *Heliophila coronopifolia*, grown as an ornamental plant with attractive blue flowers, is endemic to cape areas.

In continuing the work on flower color variations caused by acylated anthocyanins in the Brassicaceae, the distribution of structurally complicated pelargonidin and cyanidin glycosides in the

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flowers of *Matthiola incana* (Saito et al., 1995, 1996), *Orychophragonus violaceus* (Honda et al., 2005), *Cheiranthus cheiri*, *Lobularia maritima* and *Lunaria annua* (Tatsuzawa et al., 2006, 2007, 2010), *Malcolmia maritima* (Tatsuzawa et al., 2008a), and *Iberis umbellata* (Saito et al., 2008) were previously reported. To the best of our knowledge, no report in the literature has described an anthocyanin study of the blue flowers of *H. coronopifolia*. Therefore, a study was carried out to elucidate the structures of anthocyanin pigments of the blue flowers of this plant. The anthocyanins in this flower were anticipated to be heavily acylated with molecules of hydroxycinnamic acids and malonic acid. In this paper, the structural elucidation of six new acylated delphinidin 3-sambubioside-5-glucoside and one acylated kaempferol glycoside isolated from the blue flowers of *H. coronopifolia* are reported, as well as a blue anthocyanin–flavonol complex comprising one molecule each of delphinidin 3-*p*-coumaroylsambubioside-5-malonylglucoside and kaempferol 3-feruloylglucoside-7-cellobioside-4′-glucoside.

2. Results and discussion

Eight anthocyanin peaks (pigments **1**–**8**) and one flavonol peak (pigment **9**) were observed in the MAW (methanol–acetic acid–water; 4:1:5, v/v/v) extract or 5% HOAc (acetic acid–water; 5:95, v/v) extract from the blue flowers of *H. coronopifolia* as major peaks by the HPLC analysis with monitoring at 530 nm for anthocyanins and at 350 nm for flavonols (Figs. 1 and 2). The proportions of anthocyanin peaks were 6.1% (pigment **1**), 6.4% (pigment **2**), 0.5% (pigment **3**), 1.1% (pigment **4**), 43.1% (pigment **5**), 29.2% (pigment **6**), 5.3% (pigment **7**) and 3.3% (pigment **8**), based on a percentage of the total absorbance of anthocyanin peaks. From the extract with 5% HOAc, these pigments were isolated and purified using the process described in a previous report (see Tatsuzawa et al., 2007, 2008a and Section 4.3.). The chromatographic and spectroscopic properties of these anthocyanins are summarized in Table 1, and also those of the flavonol glycoside were described in Section 4.4.2.

Acid hydrolysis of six anthocyanins (pigments **1**–**6**) resulted in delphinidin, glucose, xylose, and hydroxycinnamic acids in which *p*-coumaric acid was detected, respectively, in the hydrolysates of pigments **1**, **3** and **5**, caffeic acid in that of pigment **2**, and ferulic acid in those of pigments **4** and **6**. In addition, malonic acid was detected in the hydrolysates of pigments **1**, **2**, **5** and **6**. Similarly, acid hydrolysis of pigments **7** and **8** resulted in cyanidin, glucose, xylose, malonic acid and hydroxycinnamic acids (*p*-coumaric acid for pigment **7** and ferulic acid for pigment **8**).

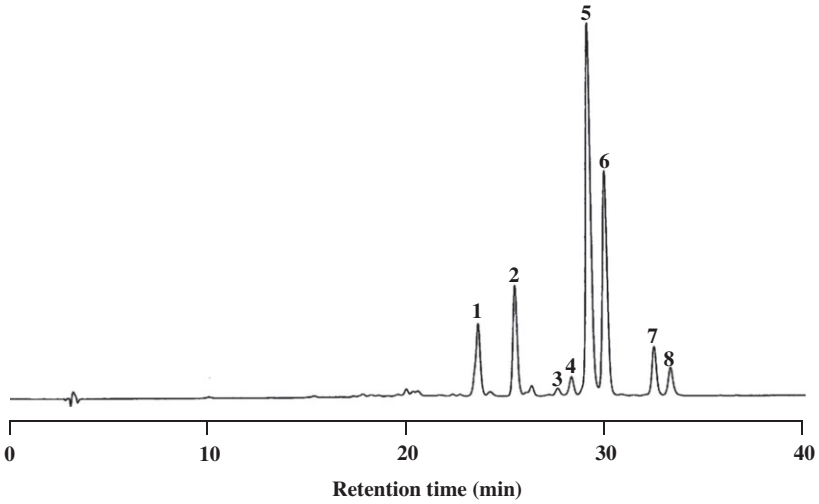


Fig. 1. HPLC profile for anthocyanins (530 nm) in the blue flower extract of *Heliophila coronopifolia* L. Pigments **1**–**8** are same as in Table 1.

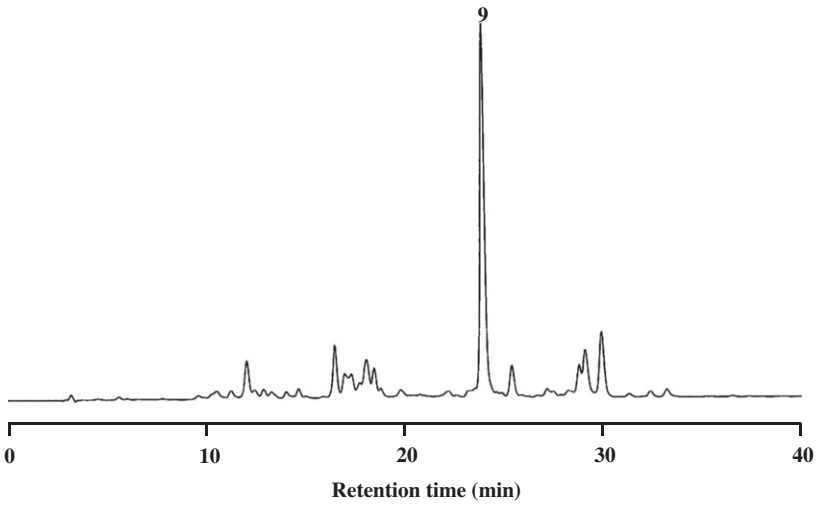


Fig. 2. HPLC profile (350 nm) for flavones and flavonols in the blue flower extract of *H. coronopifolia* L.

Table 1
Chromatographic and spectral properties of acylated anthocyanins isolated from the flowers of *Heliophila coronopifolia*.

Anthocyanins[a]	Rf value(100×)				Spectral data in 0.1% HCl–MeOH					Based on FAB-MS[b]							
	BAW	BuHCl	1%HCl	AHW	λ_{max} (nm)	E_{acyl}/E_{max}	E_{440}/E_{max}	$AlCl_3$	(min)	$[M]^+$	Dp	Cy	Glc	Xyl	pC	Caf	Fer	Mal
Pigment **1**	31	39	47	67	544,308,(304),277	96	11	+	23.4	991	1		2	1	1			1
Pigment **2**	18	24	28	53	542,331,302,279	68	11	+	25.0	1007	1		2	1		1		1
Pigment **3**	34	31	25	54	542,(307),300,281	113	12	+	27.2	905	1		2	1	1			
Pigment **4**	26	26	28	54	543,325,300,282	105	11	+	27.8	934	1		2	1			1	
Pigment **5**	31	40	34	62	542,308,(303),281	87	11	+	28.9	991	1		2	1	1			1
Pigment **6**	26	31	31	58	542,326,301,280	67	11	+	29.7	1021	1		2	1		1		1
Pigment **7**	36	46	30	62	530,315,296,282	64	12	+	32.1	975		1	2	1	1			1
Pigment **8**	32	37	28	58	529,326,295,281	56	12	+	33.0	1005		1	2	1			1	1
Dp 3-sambu-5-glc	9	7	27	55	538,274	–	11	+	10.1	759	1		2	1				
Cy 3-sambu-5-glc	14	12	34	61	526,278	–	13	+	13.1									
Lunaria anthocyanin 3	36	46	30	62	530,315,296,282	64	12	+	32.1									
Lunaria anthocyanin 5	32	37	28	58	529,326,295,281	56	12	+	33.0									

[a] Pigment **1**: Delphinidin 3-[2-(xylosyl)-6-(*cis*-*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]; $C_{44}H_{47}O_{26}$ calc. 991.2356, found 991.2341. Pigment **2**: Delphinidin 3-[2-(xylosyl)-6-(*trans*-caffeoyl)-glucoside]-5-[6-(malonyl)-glucoside]; $C_{44}H_{47}O_{27}$ calc. 1007.2305. found 1007.2293. Pigment **3**: Delphinidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-glucoside; $C_{41}H_{45}O_{23}$ calc. 905.2352. found 905.2396. Pigment **4**: Delphinidin 3-[2-(xylosyl)-6-(*trans*-feruloyl)-glucoside]-5-glucoside; $C_{42}H_{47}O_{24}$ calc. 935.2457. found 935.2454. Pigment **5**: Delphinidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]; $C_{44}H_{47}O_{26}$ calc. 991.2356. found 991.2371. Pigment **6**: Delphinidin 3-[2-(xylosyl)-6-(*trans*-feruloyl)-glucoside]-5-[6-(malonyl)-glucoside]; $C_{45}H_{49}O_{27}$ calc. 1021.2461. found 1021.2455. Pigment **7**: Cyanidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **8**: Cyanidin 3-[2-(xylosyl)-6-(*trans*-feruloyl)-glucoside]-5-[6-(malonyl)-glucoside]. Dp 3-sambu-5-glc: Delphinidin 3-sambubioside-5-glucoside. Cy 3-sambu-5-glc: Cyanidin 3-sambubioside-5-glucoside. Lunaria anthocyanin 3: Cyanidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Lunaria anthocyanin 5: Cyanidin 3-[2-(xylosyl)-6-(*trans*-feruloyl)-glucoside]-5-[6-(malonyl)-glucoside].

[b] $[M]^+$ = molecular ion mass values. Dp (delphinidin), Glc (glucose), Xyl (xylose), pC (*p*-coumaric acid), Caf (caffeic acid), Fer (ferulic acid), Mal (malonic acid) = molecular counts of their components.

Alkaline hydrolysis of pigments **1**–**6** resulted in the same deacylated delphinidin glycoside and hydroxycinnamic acids. Similar hydrolysis of pigments **7** and **8** gave the same deacylated cyanidin glycoside with *p*-coumaric acid in the hydrolysate of pigment **7** and ferulic acid in that of pigment **8**. The deacylated cyanidin glycoside was identified to be cyanidin 3-sambubioside-5-glucoside by the analysis of HPLC in comparison with the authentic specimen obtained from anthocyanins of *L. annua* (Tatsuzawa et al., 2006). The structures of pigments **7** and **8** were identified to be cyanidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] for pigment **7**, and to be cyanidin 3-[2-(xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside] for pigment **8** by analyses using chromatographic and spectroscopic methods in comparison with both authentic pigments isolated from *L. annua* (Tatsuzawa et al., 2006) (Table 1).

Structure elucidations of these new flavonoid pigments were performed based on analyses of their ^{1}H NMR spectra (500 MHz) and ^{13}C NMR (125.78 MHz) in CD_3OD-DCl (9:1) for anthocyanins and DMSO-d_6 for flavonol, including 2D COSY, NOESY, ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC spectra, as described herein.

2.1. Structural determination of new anthocyanins

2.1.1. Deacylanthocyanin of pigments ***1*–*6***

The FAB mass spectrum of the deacylanthocyanin of pigments **1**–**6** gave a molecular ions $[M]^+$ at 759 *m/z* (calc. $C_{32}H_{39}O_{21}$, 759.198) assuming that the deacylanthocyanin is composed of delphinidin with two molecules of glucose and one molecule of xylose. The ^{1}H NMR spectrum of this deacylanthocyanin exhibited five aromatic proton signals assigned for those of a delphinidin moiety (Saito et al., 2007) (Table 2). The anomeric protons of the sugar moieties were assigned at δ5.57 (*d*, *J* = 7.3 Hz, H-1 of Glc A), 5.19 (*d*, *J* = 7.9 Hz, H-1 of Glc B) and 4.70 (*d*, *J* = 7.6 Hz, H-1 of xylose). Those sugars were assumed to adopt β-pyranose forms based on their observed *J*-values (Table 2). By application of its NOESY experiment, the long range NOEs between H-1 of Glc A and H-4 (δ9.00) of delphinidin, H-1 of Glc B and H-6 (δ7.05) of delphinidin, and H-2 (δ4.03) of Glc A and H-1 of xylose were observed (Fig. 3), indicating that the OH-3 and OH-5 of delphinidin are glycosylated with Glc A and Glc B, respectively, and also OH-2 of Glc A is bonded with xylose at the anomeric position forming sambubiose. Therefore, this deacylanthocyanin was determined to be delphinidin 3-*O*-[2-*O*-(β-xylopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside.

2.1.2. Pigments ***2*, *5* and *6***

The molecular ions $[M]^+$ of pigments **2**, **5** and **6** as three major pigments in this plant were observed at 1007 *m/z* ($C_{44}H_{47}O_{27}$ calc. 1007.230) for pigment **2**, at 991 *m/z* ($C_{44}H_{47}O_{26}$ calc. 991.236) for pigment **5**, and at 1021 *m/z* ($C_{45}H_{49}O_{27}$ calc. 1021.246) for pigment **6**, indicating that these three pigments are composed of delphinidin with two molecules of glucose and one molecule each of xylose, malonic acid and hydroxycinnamic acids (caffeic acid for pigment **2**, *p*-coumaric acid for pigment **5** and ferulic acid for pigment **6**). The elemental components of these pigments were confirmed by measuring their high resolution FAB MS (HRMS) (Table 1).

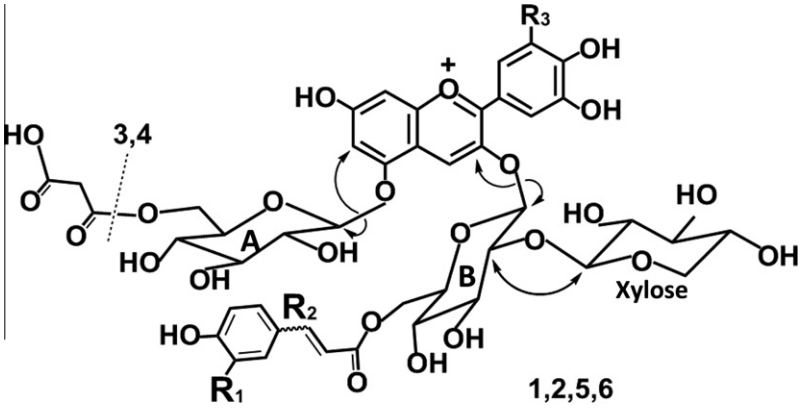
These pigment structures were further elucidated based on the analysis of their NMR spectra. The ^{1}H NMR spectra of these pigments were mutually superimposed, except for the signals of hydroxycinnamic acid moieties, as described above. As shown in Table 2, the proton chemical shifts of the delphinidin moiety with coupling constants were assigned in these three pigments. Their values showed good mutual agreement. Signals of the sugar moieties were observed in the region of δ5.61–3.01, where three characteristic anomeric protons were observed at δ5.56–5.61 for Glc

Table 2
^{1}H NMR spectroscopic data (δ) of acylated anthocyanins isolated from *Heliophila coronopifolia* (500 MHz, CD_3OD-DCl, TMS as an internal standard).

	Deacyl pigments **1**–**6**	Pigment **1**	Pigment **2**	Pigment **3** (demalonyl pigment **5**)	Pigment **4** (demalonyl pigment **6**)	Pigment **5**	Pigment **6**
	^{1}H	^{1}H	^{1}H	^{1}H	^{1}H	^{1}H	^{1}H
Delphinidin							
4	8.98 s	8.50 s	8.80 s	8.83 s	8.78 s	8.74 s	8.80 s
6	7.04 d(1.8)	6.84 d(1.8)	6.95 d(1.8)	6.97 d(1.8)	6.82 b(1.9)	6.91 d(1.8)	6.92 d(1.8)
8	7.09 d(1.8)	6.74 d(1.8)	6.90 d(1.8)	6.87 d(1.8)	6.88 b(1.9)	6.86 d(1.8)	6.88 d(1.8)
2′	7.81 s	7.74 s	7.79 s	7.78 s	7.72 s	7.77 s	7.78 s
6′	7.81 s						
Hydroxycinnamic acid I							
2		7.11 d(8.9)	6.79 d(1.8)	7.26 d(8.9)	6.82 s	7.16 d(8.9)	6.83 brs
3		6.29 d(8.9)		6.74 d(8.9)		6.68 d(8.9)	
5		6.29 d(8.9)	6.68 d(8.6)	6.74 d(8.9)	6.68 d(8.6)	6.68 d(8.9)	6.71 d(8.3)
6		7.11 d(8.9)	6.72 dd(1.8, 8.6)	7.26 d(8.9)	6.82 m	7.16 d(8.9)	6.82 dd(1.9, 8.3)
α		5.72 d(12.9)	6.18 d(15.9)	6.21 d(15.9)	6.13 d(15.9)	6.20 d(15.9)	6.21 d(15.9)
β		6.38 d(12.9)	7.28 d(15.9)	7.35 d(15.9)	7.26 d(15.9)	7.31 d(15.9)	7.34 d(15.9)
–OCH3					3.73 s		3.77 s
Malonic acid							
$-CH_2-$		3.25–3.35	3.24–3.36			3.30 s	3.30 s
Glucose A							
1	5.55 d(7.6)	5.53 d(7.7)	5.56 d(7.4)	5.55 d(7.3)	5.54 d(7.3)	5.61 d(7.3)	5.57 d(7.4)
2	4.02 dd(7.6, 9.2)	4.08 dd(7.7, 8.6)	4.06 t*(8.6)	4.07 dd(7.3, 8.8)	3.99 dd(7.3, 8.4)	4.09 dd(7.3, 8.2)	4.08 dd(7.4, 8.9)
3	3.79 t*(9.2)	3.81 m	3.81 m	3.80 t*(9.2)	3.75 t*(8.9)	3.85 t*(8.9)	3.83 t*(8.9)
4	3.50 t*(9.2)	3.50 m	3.54 m	3.54 m	3.50 t*(9.5)	3.55 t*(9.2)	3.55 t*(8.9)
5	3.67 m	3.96 m	3.96 m	3.92 m	3.87 m	3.99 ddd(2.5, 7.9, 11.9)	3.99 ddd(2.5, 8.4, 9.0)
6a	3.74 m	4.32 brd(11.9)	4.42 dd(8.0, 11.9)	4.38 m	4.34 dd(7.3, 11.9)	4.43 dd(7.9, 11.9)	4.37 dd(8.3, 12.1)
6b	3.93 d(11.9)	4.72 m	4.53 d(11.9)	4.49 dd(3.4, 12.5)	4.44 dd(3.1, 11.9)	4.52 dd(2.8, 11.9)	4.58 dd(2.5, 12.1)
Glucose B							
1	5.16 d(7.6)	5.21 d(7.6)	5.16 d(7.7)	5.17 d(7.7)	5.06 d(7.6)	5.17 d(7.9)	5.15 d(7.9)
2	3.67 dd(7.6, 9.2)	3.73 m	3.75 m	3.73 m	3.64 dd(7.6, 9.2)	3.77 dd(7.9, 9.2)	3.76 dd(7.9, 9.2)
3	3.58 m	3.59 m	3.57 m	3.58 m	3.47 t*(9.2)	3.59 t*(9.2)	3.57 t*(8.0)
4	3.46 t*(9.5)	3.44 m	3.45 m	4.42 m	3.33 t*(9.5)	3.46 t*(9.5)	3.41 m
5	3.55 m	3.77 m	3.79 m	3.71 m	3.48 m	3.82 m	3.80 m
6a	3.74 m	4.15 m	4.25 dd(6.1, 12.2)	3.88 m	3.70–3.77	4.23 dd(5.8, 12.1)	4.10 dd(6.4, 11.3)
6b	3.93 d(11.9)	4.59 brd(12.5)	4.56 d(12.2)	3.96 dd(2.5, 12.2)	3.85 d(11.9)	4.51 d(12.1)	4.52 dd(1.9, 11.3)
Xylose							
1	4.71 d(7.7)	4.67 d(8.0)	4.70 d(7.7)	4.69 d(7.6)	4.62 d(7.6)	4.73 d(7.3)	4.71 d(7.4)
2	3.13 dd(7.7, 9.2)	3.16 m	3.20 dd(7.7, 8.9)	3.16 dd(7.6, 8.9)	3.11 dd(7.6, 8.9)	3.25 dd(7.9, 9.2)	3.22 dd(7.4, 8.9)
3	3.27 t*(9.2)	3.27 m	3.24–3.34	3.20 dd(8.5, 8.9)	3.20 m	3.33 t*(8.8)	3.33 m
4	3.34 m	3.25–3.35	3.35 m	3.33 m	3.23 m	3.39 t*(8.5)	3.37 t*(8.7)
5a	2.95 t(11.6)	2.95 t*(11.3)	3.01 dd(10.4, 11.3)	3.10 dd(10.1, 11.6)	2.93 t*(11.3)	3.05 t*(11.5)	3.03 t*(11.6)
5b	3.58 m	3.54 m	3.62 dd(5.2, 11.3)	3.63 dd(5.2, 11.6)	3.55 dd(5.2, 11.6)	3.68 dd(5.2, 11.5)	3.65 dd(5.2, 11.6)

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

A, at δ5.15–5.17 for Glc B and at δ4.70–4.73 for xylose for these three pigments. Based on the observed coupling constants (Table 2), the nine sugars were assigned to have β-pyranose forms. Chemical shifts of hydroxycinnamic acids were also assigned as shown in Table 2, and identified to be caffeic acid for pigment **2**, *p*-coumaric acid for pigment **5** and ferulic acid for pigment **6**. The signals of six olefinic protons of three hydroxycinnamic acids exhibited large coupling constants (J = 15.9 and 15.9 Hz for caffeic acid, J = 15.9 and 15.9 Hz for *p*-coumaric acid, and J = 15.9 and 15.9 Hz for ferulic acid) indicating to be *trans* configurations (Table 2). The linkages and/or positions of the attachments of the sugar and acyl groups in these pigments were determined by analysis of their COSY and NOESY spectra (Fig. 3). Long range NOEs were observed in the spectrum of each pigment **2**, **5** and **6** between H-1 of Glc A and H-4 of delphinidin, H-1 of Glc B and H-6 of delphinidin, and H-1 of xylose and H-2 of Glc A, supporting that OH-3 and -5 of delphinidin were glycosylated with Glc A and Glc B, respectively. Furthermore, xylose was bonded with Glc A at OH-2 of Glc A forming sambubiose in these pigments (Fig. 3). Based on results of analyses of their COSY spectra, four characteristic methylene proton signals (H-6a and b) being shifted to lower magnetic fields were assigned to those of Glc A (δ4.42 and 4.53 for pigment **2**, δ4.43 and 4.52 for pigment **5**, and δ4.37 and 4.58 for pigment **6**) and Glc B (δ4.25 and 4.56 for pigment **2**, δ4.23 and 4.51 for pigment **5**, and δ4.10 and 4.52 for pigment **6**) in these pigments **2**, **5** and **6**. These results indicated that both OH-6 groups of Glc A and B were acylated with malonic acid and hydroxycinnamic acid in these pigments. However, we were unable to determine the acid moiety of



Pigment	R_1	R_2	R_3
1	H	*cis*	OH
2	OH	*trans*	OH
3	H	*trans*	OH
4	OCH_3	*trans*	OH
5	H	*trans*	OH
6	OCH_3	*trans*	OH
7	H	*trans*	H
8	OCH_3	*trans*	H

Fig. 3. Acylated anthocyanins from *H. coronopifolia* L. Observed main NOEs are indicated by arrows.

OH-6 of Glc A and Glc B, where malonic acid and hydroxycinnamic acid were bonded alternatively, based on analyses of their NOESY spectra and also their HMBC spectra. To determine the position of both acids, demalonyl pigments **5** and **6** were prepared using the procedures described in a previous report (Saito et al., 2007). The structures of demalonyl pigments **5** and **6** were determined (see in the following Section 2.1.3.). The obtained results established that demalonyl pigments **5** and **6** were free from malonic acid at OH-6 of Glc B, and acylated with hydroxycinnamic acid at OH-6 of Glc A. Therefore, the structures of pigments **5** and **6** were determined unambiguously to be delphinidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] for pigment **5**, and to be delphinidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-feruloyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] for pigment **6** (Fig. 3). Both pigments are new anthocyanins in plants (Harborne and Baxter, 1999; Honda and Saito, 2002; Andersen and Jordheim, 2006; Veitch and Grayer, 2008). These structures were confirmed based on analyses of their ^{13}C NMR spectra (Table 3). For pigment **2**, we were unable to prepare its demalonyl pigment **2** because of the small amounts available. Therefore, H_2O_2 degradation of pigment **2** was performed to prepare the acyl sugar residue at the OH-3 of delphinidin, yielding caffeoyl-sambubiose from degradation products by analyses of TLC and HPLC (See in the Section 4.4.6.). Consequently, the structure of pigment **2** was determined to be delphinidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-caffeoyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 3), which is a new anthocyanin in plants.

*2.1.3. Demalonyl pigment **5** (pigment **3**) and demalonyl pigment **6** (pigment **4**)*

Demalonyl pigments **5** and **6** were prepared by hydrolysis of pigments **5** and **6** with 1 N HCl at room temperature for 2 weeks. They were purified with preparative HPLC by the process described in a previous report (Saito et al., 2007). The chromatographic and spectroscopic data of the demalonyl pigments **5** and **6** are presented in Table 1. The analytical results obtained for demalonyl pigment **5** were identical to those of pigment **3**. These results of demalonyl pigment **6** were identical to those of pigment **4**.

The FAB mass spectra of demalonyl pigment **5** and pigment **3** exhibited a molecular ion $[M]^+$ at 905 *m/z* in agreement with the mass $C_{41}H_{45}O_{23}$ (calc. 905.235). The elemental components were confirmed by measuring their high-resolution FAB MS (Table 1). In the ^1H NMR spectrum of the demalonyl pigment **5**, the chemical shifts were almost identical to those of pigment **5**, except for the proton signals of Glc B and malonic acid moieties (Table 2). Particularly, the up-field shifts of methylene protons (δ3.88 and 3.96, H-6a and b of Glc B) were observed in this spectrum in comparison with those of pigment **5** (δ4.23 and 4.51, H-6a and b of Glc B), supporting that the OH-6 of Glc B in demalonyl pigment **5** is free from malonic acid (Fig. 3). Therefore, the structures of demalonyl pigment **5** and pigment **3** were determined to be delphinidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside, which is a new anthocyanin in plants.

The FAB mass spectra of demalonyl pigment **6** and pigment **4** exhibited a molecular ion $[M]^+$ at 934 *m/z*, in agreement with the mass $C_{42}H_{47}O_{24}$ (calc. 935.246). The elemental components were confirmed by measuring their high-resolution FAB MS (Table 1). The ^1H NMR spectrum of the demalonyl pigment **6** was superimposed on that of pigment **6**, except for the proton signals of Glc B and malonic acid moieties (Table 2). By analysis of its COSY spectrum, the chemical shifts of methylene protons of Glc B were shifted to a higher field at δ3.70–3.77 (H-6a) and 3.85 (H-6b) than those (δ4.10, H-6a and δ4.52, H-6b) of pigment **6**, indicating that the OH-6 of Glc B is free from malonic acid in demalonyl pigment **6**. Furthermore, the signals of methylene protons of malonic acid disappeared in the ^1H NMR spectrum of the demalonyl pigment **6**. Therefore, the structures of demalonyl pigment **6** and pigment **4** were determined to be delphinidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-feruloyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside, which is a new anthocyanin in plants.

*2.1.4. Pigment **1***

By analysis of FAB mass spectrum, the molecular ion $[M]^+$ of pigment **1** was observed at 991 *m/z* ($C_{44}H_{47}O_{26}$ calc.991.236), which is identical to that of pigment **5**, indicating that pigment **1** was composed of delphinidin with two molecules of glucose, and

Table 3
^{13}C NMR spectroscopic data of acylated anthocyanins (pigments **5** and **6**) from the flowers *H. coronopifolia* in CD_3OD-DCl (9:1).

	Pigment **5**	Pigment **6**
Delphinidin		
2	164.4	164.6
3	145.8	145.8
4	133.1	133.4
5	156.3	156.4
6	105.9	106.0
7	169.2	169.2
8	97.2	97.3
9	156.6	156.7
10	112.8	113.0
1′	119.8	120.5
2′	113.3	113.0
3′	147.7	147.7
4′	145.9	146.2
5′	147.7	147.7
6′	113.3	113.0
Hydroxycinnamic acid I		
1	126.7	119.9
2	131.4	112.2
3	116.7	149.1
4	161.2	150.6
5	116.7	116.5
6	131.4	124.0
α	147.0	147.3
β	114.7	115.1
–OCH3		56.5
C=O	169.2	169.1
Malonic acid		
–CH2–	49.3	49.2
C=O	173.7	170.5
C=O	168.9	169.1
Glucose A		
1	100.6	99.9
2	82.5	82.4
3	77.9	77.8
4	72.0	72.0
5	75.8	75.5
6	64.6	64.6
Glucose B		
1	102.6	102.8
2	74.8	74.7
3	77.7	77.7
4	71.0	71.0
5	75.9	75.9
6	65.1	65.1
Xylose		
1	106.3	106.2
2	75.4	75.8
3	77.7	77.7
4	70.9	70.9
5	67.1	67.1

Table 4
NMR spectroscopic data of a flavonol from *H. coronopifolia*.

	Pigment **9**		Deacyl pigment **9**	
	^{13}C δ (ppm)	^{1}H δ (ppm)	^{13}C δ (ppm)	^{1}H δ (ppm)
Kaempferol				
2	156.0		156.1	
3	134.2		134.1	
4	177.8		177.8	
5	160.8		160.9	
6	99.2	6.33 s	99.2	6.48 d(2.1)
7	162.7		162.8	
8	94.6	6.78 s	94.5	6.84 d(2.1)
9	156.1		156.2	
10	105.7		105.8	
1′	123.5		123.6	
2′,6′	130.7	8.16 d(8.6)	130.8	8.18 d(8.9)
3′,5′	115.9	7.15 d(8.6)	115.9	7.15 d(8.9)
4′	159.4		159.4	
Ferulic acid				
1	125.4			
2	111.1	7.21 s		
3	147.9			
4	149.3			
5	115.5	6.79 d(8.9)		
6	123.2	6.98 brd(8.0)		
α	113.9	6.26 d(15.9)		
β	145.1	7.37 d(15.9)		
COOH	166.3			
–OCH3	55.7	3.81 s		
Glucose A				
1	101.5	5.45 d(7.7)	101.5	5.43 d(7.7)
2	71.1	3.54 m	71.2	3.54 dd(7.6, 9.5)
3	76.8	3.38 m	76.8	3.42 m
4	68.2	3.67 m	67.9	3.66 m
5	73.1	3.72 m	73.1	3.40 m
6a	63.1	4.08 m	60.2	3.50 m
6b		4.15 d(10.9)		3.72 m
Glucose B				
1	99.2	5.08 d(7.6)	99.4	5.19 d(8.0)
2	77.1	3.33 m	77.1	3.31 m
3	72.8	3.45 m	74.8	3.48 m
4	79.7	3.43 m	79.7	3.44 m
5	75.1	3.60 m	75.1	3.65 m
6a	60.0	3.61 m	60.0	3.61 m
6b		3.76 m		3.76 m
Glucose C				
1	100.1	5.01 d(7.1)	100.1	5.02 d(7.3)
2	73.2	3.28 m	73.2	3.27 m
3	76.5	3.26 m	75.9	3.32 m
4	69.6	3.49 m	69.8	3.50 m
5	74.7	3.47 m	72.8	3.35 m
6a	60.6	3.51 m	60.6	3.34 m
6b		3.71 m		3.68 m
Glucose D				
1	103.1	4.30 d(7.6)	103.1	4.30 d(8.0)
2	73.3	3.04 m	73.3	3.02 t*(8.6)
3	69.6	3.18 m	69.6	3.17 t*(9.2)
4	70.0	3.07 m	70.0	3.07 t*(9.2)
5	76.5	3.20 m	76.5	3.21 m
6a	61.1	3.39 m	61.0	3.29 m
6b		3.68 m		3.44 m

^{1}H: 500 MHz DMSO-d_6, TMS as an internal standard.
^{13}C: 125.78 MHz DMSO-d_6, TMS as an internal standard.
Coupling constants (*J* in Hz) in parentheses.
s = singlet, brs = broad singlet, d = doublet, brd = broad doublet, dd = double doublet, t* = distorted triplet, m = multiplet.

one molecule each of xylose, malonic acid and *p*-coumaric acid. The elemental components were confirmed by measuring its HRMS, and chemical and spectroscopic data of pigment **1** are presented in Table 1. The results of chemical and spectroscopic analyses of pigment **1** were identical to those of an authentic *cis*-derivative of pigment **5**, which was prepared from pigment **5** using the process described previously (Tatsuzawa et al., 2008b and see in Section 4.4.5. and Table 1).

The ^{1}H NMR spectrum of pigment **1** was the same as that of pigment **5** except for signals of *p*-coumaric acid moiety (Table 2). Particularly, the chemical shifts of the olefinic protons of *p*-coumaric acid were shifted to a higher magnetic field at δ5.72 (*d*, *J* = 12.9 Hz, H-α) and δ6.38 (*d*, *J* = 12.9 Hz, H-β) with smaller coupling constants than those (δ6.20, *d*, *J* = 15.9 Hz, H-α and δ7.31, *d*, *J* = 15.9 Hz, H-β) of pigment **5**. These results supported the configuration of *p*-coumaric acid in pigment **1** to be a *cis*-form. Consequently, pigment **1** was determined to be delphinidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*cis*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 3), which is a new anthocyanin in plants.

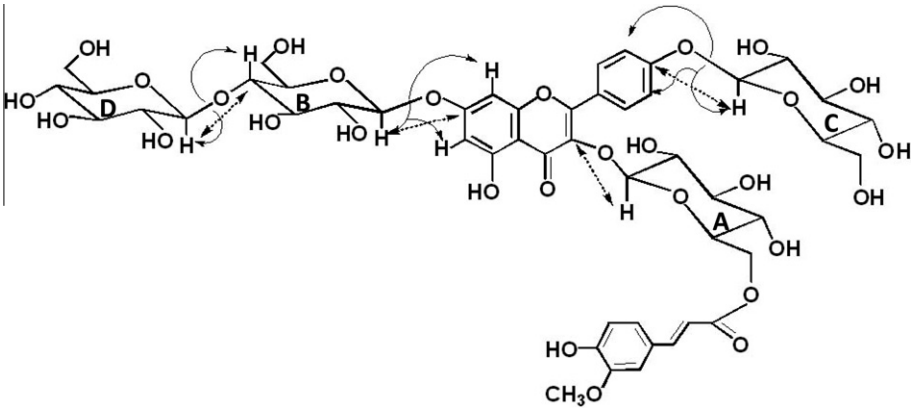


Fig. 4. Acylated kaempferol glycoside (pigment **9**) isolated from the flowers of *H. coronopifolia* L. Observed main NOEs are indicated by arrows. Observed main HMBCs are indicated by dotted arrows.

2.2. Structure of a flavonol (pigment **9**)

As shown in Fig. 2, several flavonol glycosides were detected in 5% HOAc extract from the blue flowers of *H. coronopifolia* using HPLC analysis. From these flavonol glycosides, a dominant pigment (pigment **9**) was isolated and then purified as a pale yellow powder. The chromatographic and spectroscopic properties of pigment **9** are summarized in Section 4.4.2. Acid hydrolysis of pigment **9** resulted in kaempferol, glucose and ferulic acid. Alkaline hydrolysis of pigment **9** resulted in a deacyl kaempferol glycoside and ferulic acid. The FAB mass spectrum of pigment **9** gave a molecular ion $[M+1]^+$ at 1111 *m/z* ($C_{49}H_{58}O_{29}$, calc. 1110.306) indicating that pigment **9** was composed of kaempferol with four molecules of glucose and one molecule of ferulic acid. The chromatographic and spectroscopic properties of deacyl pigment **9** are summarized in Section 4.4.3. The structure of deacyl pigment **9** was presumed to be kaempferol 3,7,4′-tetra-glucoside by its chromatographic and spectroscopic properties (in Section 4.4.2) and also by analysis of the ^{1}H NMR spectrum (Table 4). From the ^{1}H NMR spectrum of deacyl pigment **9**, six aromatic protons of kaempferol and four anomeric protons of glucose were assigned. From their coupling constants, the four glucoses were of β-pyranose form. By analysis of its NOESY spectrum, three long-range NOEs were observed between H-8 and H-6 of kaempferol and H-1 of Glc B, H-3′ and -5′ of kaempferol and H-1 of Glc C, and H-4 of Glc B and H-1 of Glc D, suggesting that kaempferol was glycosylated with Glc B at 7-OH and with Glc C at OH-4′, and that Glc B was bonded with Glc D at OH-4. The glycosylation at OH-3 of kaempferol with Glc A was concluded by the results of spectroscopic properties as described above and confirmed by observation of HMBC connectively from H-1 of Glc A to C-3 of kaempferol as depicted in Fig. 4. Therefore, the structure of deacyl pigment **9** was determined to be kaempferol 3-*O*-(β-glucopyranoside)-7-*O*-[4-*O*-(β-glucopyranosyl)-β-glucopyranoside]-4′-*O*-(β-glucopyranoside). This structure was further confirmed by measurement of its ^{13}C NMR spectrum (Table 4).

The ^{1}H NMR spectrum of pigment **9** was identical to that of deacyl pigment **9**, except for the signals of ferulic acid and Glc A moieties. Eight chemical shifts of ferulic acid were assigned as shown in Table 4. Two olefinic protons of this acid with large coupling constants (J = 15.9 and 15.9 Hz) indicated that the geometry of the olefin was of *trans*-configuration. By analysis of its COSY

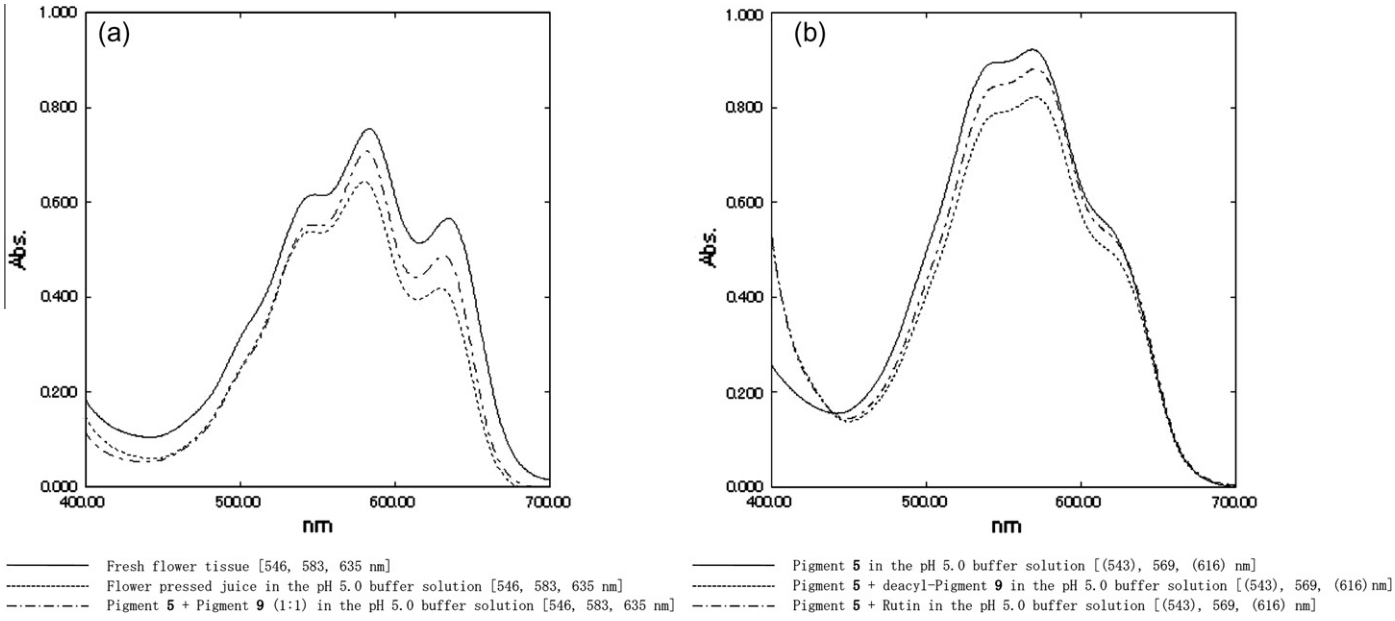


Fig. 5. Visible spectra of *H. coronopifolia* L.

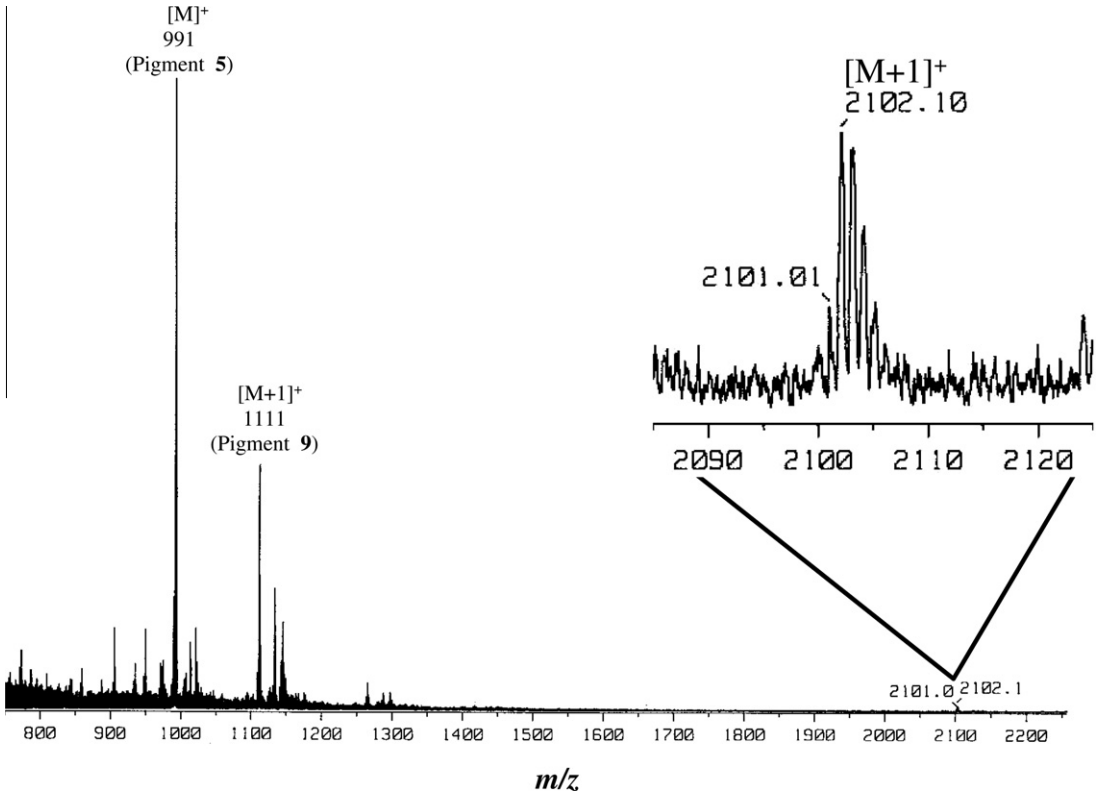


Fig. 6. FABMS spectra of anthocyanin–flavonol (pigments **5** and **9**) complex pigment.

spectrum, the downshifts of methylene protons (δ4.08, H-6a and δ4.15, H-6b) of Glc A were observed, supporting that Glc A was acylated with ferulic acid at the OH-6 group. Furthermore, the glycosylation on OH-7 and OH-4′ of kaempferol with Glc C and Glc D was confirmed by measurement of its NOESY spectrum as described for deacyl-pigment **9**. Consequently, the structure of pigment **9** was determined to be kaempferol 3-*O*-[6-*O*-(*trans*-feruloyl)-β-glucopyranoside]-7-*O*-[4-*O*-(β-glucopyranosyl)-β-glucopyranoside]-4′-*O*-(β-glucopyranoside) (Fig. 4), which is a new kaempferol glycoside in plants (Harborne and Baxter, 1999; Williams, 2006; Veitch and Grayer, 2008). This structure was confirmed further by measuring its ^{13}C NMR spectrum (Table 4). Regarding the glycosylation patterns of flavonol glycosides in the Brassicaceae, kaempferol 3-sophoroside-7-cellobioside has been found in plants of *Brassica oleracea* (Nielsen et al., 1998). However, kaempferol 3-glucoside-7-cellobioside-4′-glucoside is a new pattern.

2.3. Flower color and an anthocyanin–flavonol complex pigment in blue flowers of H. coronopifolia

As shown in Fig. 5, the blue petal of *H. coronopifolia* and its fresh flower juice in the pH 5.0 buffer solution exhibited three characteristic absorption maxima at 546, 583 and 635 nm in the visible region of 400–700 nm. These three absorption maxima are similar to those of platyconin (533, 570 and 618 nm) and cinerarin (537, 575 and 622 nm) in the same buffer solution (Honda and Saito, 2002). These pigments are composed from delphinidin glycosides with two molecules of caffeic acid as platyconin and also with three molecules of caffeic acid as cinerarin. However, anthocyanins of *H. coronopifolia* delphinidin 3-sambubioside-5-malonylglucoside were acylated, respectively, with only one molecule of hydroxycinnamic acids. A main anthocyanin of this plant, pigment **5**, exhibits only one absorption maximum (569 nm) in the pH 5.0 solution, and its color is unstable in the solution and soon decayed as usually observed for non-acylated anthocyanins. Particularly, the extra band at 635 nm in the visible region is not observed in pH 5.0 solution of delphinidin 3-sambubioside-5-diglucoside.

In order to recover the original blue color for the solution of pigment **5**, copigments such as rutin (Asen et al., 1972) and deacyl pigment **9** (kaempferol 3,4′-diglucoside-7-cellubioside) were added to the pH 5.0 solution of pigment **5**. However, only the violet color at an absorption maximum (near 570 nm) was produced as the usually observed co-pigmentation between delphinidin 3,5-diglycoside and flavone and/or flavonol (Asen et al., 1972; Chen and Hrazdina, 1981) (Fig. 5b).

On the other hand, the stable and blue color solution of pigment **5** with a strong extra absorption maximum at 635 nm is restored, as observed for the fresh flower juice by addition of pigment **9**, which is acylated with ferulic acid, as shown in Fig. 5a. The absorption pattern of this solution showing maxima at 546, 583 and 635 nm is very similar to those of polyacylated anthocyanins (Honda and Saito, 2002). Consequently, it might be considered that both the hydroxycinnamic acid residues of pigment **9** (ferulic acid) and of pigment **5** (*p*-coumaric acid) mutually collaborate for the formation of the stable and blue anthocyanin–flavonol complex, which exhibits three characteristic absorption maxima as those of platyconin and cinerarin.

At the same time, a blue anthocyanin–flavonol complex powder was isolated from the blue flowers of this plant with water or 5% HOAc solution (see in Sections 4.3. and 4.5.). This blue powder was confirmed to be constituted of pigments **5** and **9** in a ratio of 1:1 by analysis of TLC and HPLC, and also exhibited the same three absorption maxima at 546, 583 and 635 nm as those of the fresh flowers of this plant (Fig. 5). By analysis of the FAB mass spectrum, the anthocyanin–flavonol complex gave a molecular ion $[M+1]^+$ at 2102.1 *m/z* (calc. $C_{93}H_{105}O_{55}$, 2101.542) with fragment ion 991 $[M]^+$ of pigment **5** and 1111 $[M+1]^+$ of pigment **9** (Fig. 6). Therefore, it was revealed that, in the blue flower of *H. coronopifolia*, delphin-

idin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] (pigment **5**) and kaempferol 3-[6-(feruloyl)-glucoside]-7-cellobioside-4′-glucoside (pigment **9**) play an important role in forming the blue copigmentation complex by interaction of the hydroxycinnamic acid residues of both pigments.

3. Concluding remarks

Six acylated delphinidin glycosides were isolated along with two known acylated cyanidin glycosides from the blue flowers of cape stock (*H. coronopifolia*). From the chemotaxonomical point of view, this report is the first to describe the distribution of delphinidin glycosides in Brassicaceae (Harborne, 1967; Timberlake and Bridle, 1975; Hrazdina, 1982; Harborne and Grayer, 1988; Strack and Wray, 1994; Andersen and Jordheim, 2006). These anthocyanins have rather simple acylation patterns and more common glycosylation patterns than those of polyacylated anthocyanins of *M. incana* (Saito et al., 1995, 1996), *O. violaceus* (Honda et al., 2005), *Iberis umbellata* (Saito et al., 2008), and others in this family. Among six new acylated anthocyanins, the structure of pigment **5** was determined to be delphinidin 3-*p*-coumaroylsambubioside-5-malonylglucoside as a main anthocyanin.

At the same time, a new flavonol, kaempferol 3-feruloylglucoside-7-cellobioside-4′-glucoside (pigment **9**), was isolated from this blue flower as a dominant flavonol. This flavonol is responsible for producing the blue flower color in this plant as a copigment. Moreover, a blue anthocyanin–flavonol complex powder was isolated from this blue flower using water. By FAB mass spectrographic analysis, this blue complex exhibited a molecular ion $[M+1]^+$ at 2102 *m*/*z* ($C_{93}H_{105}O_{55}$ calc. 2101.542) composed from pigments **5** and **9** (in the ratio of 1:1). Therefore, we propose that the blue flower color of *H. coronopifolia* is formed mainly by strong intermolecular copigmentation between delphinidin 3-*p*-coumaroylsambubioside-5-malonylglucoside and kaempferol 3-feruloylglucoside-7-cellobioside-4′-glucoside.

4. Experimental

4.1. General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using ten 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 (HCl–H_2O, 3:97), 5% HOAc (HOAc–H_2O, 5:95), 15% HOAc (HOAc–H_2O, 15:85) and H_2O for anthocyanins and flavonols, 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 × 250 mm) column at 40 °C with a flow rate of 1 mL/min and monitoring at 530 nm for anthocyanins and at 350 nm for flavonols by photodiode array detector. 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). The spectral absorption of flowers was directly measured on the intact tissue using a recording spectrophotometer operating as a double-beam instrument (Type: MPS-2450) (Yokoi and Saito, 1973; Saito, 1967).

High resolution FAB mass (HR 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 DCl-CD_3OD (1:9) for anthocyanins and DMSO-d_6 for flavonols. Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants are in Hz.

4.2. Plant materials

Seeds of *H. coronopifolia* L. (Synonym *H. longifolia* DC.) were purchased from Takii Seed Co., Ltd. (Kyoto, Japan), and grown in greenhouses at Iwate University (Morioka, Iwate, Japan) and Minamikyushu University (Takanabe, Miyazaki, Japan). Flowers were collected during winter to spring seasons. The flowers exhibited a blue color [Blue 99C by Royal Horticultural Society (R.H.S.) Color Chart, and its chromaticity value $b*(-49.82)$/$a*(31.64) = -3.46$ and $L*(47.83)$] was recorded on a SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co., Ltd.). The flowers collected were dried at 40 °C overnight and stored at −20 °C in the refrigerator until needed.

4.3. Isolation of flower pigments

Dried blue flowers (ca. 300 g) of *H. coronopifolia* were immersed in 5% HOAc (30L) at room temp. for 5 h. The extract was passed through a Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column (90 × 150 mm), on which acylated pigments were absorbed. The column was thoroughly washed with 5% HOAc (15L), and eluted with 5% HOAc–MeOH (1L) to recover the pigments. After concentration, eluates were separated and purified with paper chromatography using BAW.

4.3.1. Pigments ***5*** *and* ***9***

For a further purification of the major pigments **5** and **9**, the crude blue anthocyanin pigment powder (*ca.* 80 mg) was subjected to HPLC on a Waters C18 (4.6 × 250 mm) at 40 °C with a flow rate of 1 ml/min and monitoring at 330 nm. The solvent used was as follows: a linear gradient elution for 15 min from 55% to 70% solvent B in solvent A. By this procedure, major pigments **5** and **9** were completely separated from the blue anthocyanin pigment powder as the two different fractions. Each fraction was transformed to a Diaion HP-20 column, on which pigments were adsorbed. Pigments **5** and **9** were eluted with 5% HOAc–MeOH followed by addition of excess of Et_2O, and then dried. Purified pigment **5** (*ca.* 30 mg) and pigment **9** (*ca.* 30 mg) were obtained.

4.3.2. Pigments ***1***, ***2***, ***3***, ***4***, ***6***, ***7*** *and* ***8***

The residual extract solutions in which pigments **5** and **9** were removed were collected and passed through a Diaion HP-20 column in order to obtain minor pigments (**1**–**4**, **6**–**8**). The column was thoroughly washed with 5% HOAc and the pigments were eluted with 5% HOAc–MeOH from the column. After concentration, the eluates were separated and purified by prep. HPLC. Prep. HPLC was performed on a Waters C18 (4.6 × 250 mm) column at 40 °C with a flow rate of 1 ml/min and monitoring at 530 nm. The solvent used was as follows; a linear gradient elution for 20 min from 55% to 70% solvent B in solvent A. Each fraction was transformed to a Diaion HP-20 column, on which pigments were absorbed. These pigments were eluted with 5% HOAc–MeOH, followed by addition of excess of Et_2O, and then dried. The purified pigments were obtained as follows; pigment **1** (*ca.* 5.0 mg), pigment **2** (*ca.* 10.0 mg), pigment **3** (*ca.* 1.0 mg), pigment **4** (*ca.* 2.0 mg), pigment **6** (*ca.* 20.0 mg), pigment **7** (*ca.* 2.0 mg) and pigment **8** (*ca.* 1.5 mg).

4.4. Analyses of anthocyanins

The identification of anthocyanins was carried out by standard procedures involving deacylation, demalonylation, partial

acid-hydrolysis with acid, both alkaline and acid hydrolyses, and H_2O_2 oxidation (Harborne, 1984; Saito et al., 2008).

*4.4.1. Anthocyanin pigments **1**–**8***

Characterization of pigments **1**–**8** are as follows: anthocyanin pigments **1**–**6** are dark violet powders, and pigments **7** and **8** are dark purple-red powders: for UV–Vis, TLC, HPLC and HR-FAB MS, see Table 1: for ^1H and ^{13}C NMR spectroscopic assignments, see Table 3 and 4. The structure determination of pigments **7** and **8** was described in the report (Tatsuzawa et al., 2006).

*4.4.2. Flavonol pigment (pigment **9**)*

Pale yellow powder: for ^1H and ^{13}C NMR spectroscopic assignments, see Table 4. TLC: Rf (100×), BAW 17, BuHCl 12, 1% HCl 11, AHW 48, UV: λmax(nm) MeOH 326, (299), 267; +NaOMe 365, (299), 267; +$AlCl_3$ 360, 324, 237; +$AlCl_3$/HCl 360, 318, 238; +NaOAc 326, (299), 267; +NaOAc/H_3BO_3 326, (299), 267. HPLC: Rt(min) 24.1. HR-FAB MS: calc $C_{49}H_{59}O_{29}$ 1111.3142. Found 1111.3163; for ^1H and ^{13}C NMR spectroscopic assignments, see Table 4.

*4.4.3. Deacyl pigments **1**–**9***

A deacyl cyanidin glycoside was obtained from pigments **7** and **8**, a deacyl delphinidin glycoside was obtained from pigments **1**–**6**, and also a deacyl kaempferol glycoside was obtained from pigment **9** by the procedure of alkaline hydrolysis (Tatsuzawa et al., 2006).

4.4.3.1. Deacyl cyanidin glycoside. Dark purple-red powder; for TLC, HPLC, and UV–Vis, see Table 1.

4.4.3.2. Deacyl Delphinidin glycoside. Dark violet powder; for TLC, HPLC, UV–Vis and FAB MS, see Table 1, for ^1H NMR spectroscopic assignments, see Tables 2 and 4.

4.4.3.3. Deacyl pigment 9. Yellow powder; for ^1H and ^{13}C NMR spectroscopic assignments, see Table 4. TLC: Rf (100×), BAW 13, BuHCl 12, 1% HCl 5, AHW 21, UV: λmax(nm) MeOH 342, 316, 267; +NaOMe (352), 342, (315), (280), 268; +$AlCl_3$ (380), 333, (300), 274; +$AlCl_3$/HCl (380), 331, (300), 274; +NaOAc 341, 321, 266; +NaOAc/H_3BO_3 341, 321, 266. HPLC: Rt(min) 10.2. HR-FAB MS: calc. $C_{39}H_{51}O_{26}$ 935.2669. Found 935.2629; for ^1H and ^{13}C NMR spectroscopic assignments, see Table 4.

*4.4.4. Demalonyl pigments **5** and **6** (pigments **3** and **4**)*

Pigments **5** and **6** (each ca. 8 mg) were dissolved in 1 N HCl solution (each 10 mL), respectively, and allowed to stand at room temperature for 2 weeks as described previously (Saito et al., 2007). Both pigments **5** and **6** were almost demalonylated in these solutions within this period. Demalonylated pigments **5** and **6** were then absorbed on the resin columns of Diaion HP-20, respectively, and were eluted with 5% HOAc–MeOH from the columns. After evaporation in vacuo, both concentrate residues demalonyl pigments **5** and **6** were dissolved in small volumes of 5% HOAc–MeOH, respectively, followed by addition of excess Et_2O, from which precipitated solids were then dried in vacuo to give demalonyl pigment **5** (ca. 4 mg) and demalonyl pigment **6** (ca. 4 mg). By analysis of chemical and spectroscopic methods, demalonyl pigment **5** was identical with pigment **3** and demalonyl pigment **6** was identical with pigment **4** as shown in Table 1. Demalonyl pigments **5** and **6**; Dark violet powders; for TLC, HPLC, UV–Vis, and FAB MS, see Table 1; for ^1H NMR spectra, see Table 2.

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

Pigment **5** (ca. 3 mg) was dissolved in 5% HOAc–MeOH (2 ml), and exposed for sunlight for half an hour to transform into the *cis*-isomer in about 40% (Tatsuzawa et al., 2008b). The isomer was isolated and purified by TLC and HPLC to give the purified isomer of pigment **5** (ca. 1 mg). By analysis of chemical and spectroscopic methods, this isomer was identical with pigment **1** as shown in Table 1. The isomer of pigment **5**; dark violet powder; for TLC, HPLC, UV–Vis, see Table 1.

4.4.6. Caffeoyl sambubiose

Pigment **2** (ca. 1 mg) was dissolved in H_2O (1 mL), and 35% H_2O_2 (1 mL) was added to this solution, and the solution was kept standing for 60 min at 25 °C. A small amount of manganese dioxide was added to the solution to decompose excess H_2O_2 and stood overnight (Saito et al., 1995). After removal of manganese dioxide by syringe filter, the solution was saturated with NH_3 for 60 min and added EtOH (8 mL). The solution was evaporated to dryness to give brown residues. From this residue of pigment **2**, caffeoylsambubiose (0.6 mg) was obtained. On the acid hydrolysis of this caffeoylsambubiose, by 2 N HCl at 80–100 °C for 60 min, caffeic acid, glucose and xylose were detected in its hydrolysate.

Caffeoylsambubiose; TLC (100×) BAW 40, 15% HOAc 60, AHW 71; color pale blue (under UV).

4.5. Isolation of a blue anthocyanin–flavonol complex

Dried blue flowers (ca. 10 g) of *H. coronopifolia* were immersed in H_2O (500 ml) at room temp. for 3 h. The extract was treated with EtOH (1.5 L) and concentrated to a dark blue syrup. The blue pigment syrup was dissolved in H_2O (50 ml), and was passed through a Sephadex LH-20 column with H_2O. The blue fraction was collected, and concentrated to dryness, which was further purified with TLC. A blue anthocyanin–flavonol complex band of TLC was eluted with H_2O and dried. A dark blue anthocyanin–flavonol complex powder (3 mg) was obtained.

A dark blue powder which was composed with pigment **5** and pigment **9** in a ratio of 1:1; Visible spectra in pH 5.0 buffer solution, λ_{max} 546, 583 and 635 nm; TLC Rf (100×), BAW 31 (pigment **5**) and 17 (pigment **9**), BuHCl 40 (pigment **5**) and 12 (pigment **9**), 1% HCl 34 (pigment **5**) and 11 (pigment **9**), AHW 62 (pigment **5**) and 48 (pigment **9**), and Water 88 (blue spot of anthocyanin–flavonol complex) {92 (pigment **5**) and 36 (pigment **9**)}; HPLC Rt(min) 28.9 and 24.1. FAB MS *m/z* 2102 $[M+1]^+$ (calc. for $C_{93}H_{105}O_{55}$, 2101.542).

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