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Tetra-acylated cyanidin 3-sophoroside-5-glucosides from the flowers of *Iberis umbellata* L. (Cruciferae)

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ABSTRACT

The structures of 11 acylated cyanidin 3-sophoroside-5-glucosides (pigments **1**–**11**), isolated from the flowers of *Iberis umbellata* cultivars (Cruciferae), were elucidated by chemical and spectroscopic methods. Pigments **1**–**11** were acylated with malonic acid, *p*-coumaric acid, ferulic acid, sinapic acid and/or glucosylhydroxycinnamic acids.

Pigments **1**–**11** were classified into four groups by the substitution patterns of the linear acylated residues at the 3-position of the cyanidin. In the first group, pigments **1**–**3** were determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(acyl)-β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], in which the acyl moiety varied with none for pigment **1**, ferulic acid for pigment **2** and sinapic acid for pigment **3**. In the second one, pigments **4**–**6** were cyanidin 3-*O*-[2-*O*-(2-*O*-(acyl)-β-glucopyranosyl)-6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], in which the acyl moiety varied with none for pigment **4**, ferulic acid for pigment **5** and sinapic acid for pigment **6**. In the third one, pigments **7**–**9** were cyanidin 3-*O*-[2-*O*-(2-*O*-(acyl)-β-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], in which the acyl moiety varied with none for pigment **7**, ferulic acid for pigment **8**, and sinapic acid for pigment **9**. In the last one, pigments **10** and **11** were cyanidin 3-*O*-[2-*O*-(2-*O*-(acyl)-β-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], in which acyl moieties were none for pigment **10** and ferulic acid for pigment **11**.

The distribution of these pigments was examined in the flowers of four cultivars of *I. umbellata* by HPLC analysis. Pigment **1** acylated with one molecule of *p*-coumaric acid was dominantly observed in purple-violet cultivars. On the other hand, pigments (**9** and **11**) acylated with three molecules of hydroxycinnamic acids were observed in lilac (purple-violet) cultivars as major anthocyanins. The bluing effect and stability on these anthocyanin colors were discussed in relation to the molecular number of hydroxycinnamic acids in these anthocyanin molecules.

1. Introduction

Many complicated anthocyanins acylated with organic acids have been isolated from the flowers of *Matthiola incana* (Saito et al., 1995, 1996), *Orychophragonus violaceus* (Honda et al., 2005), *Lobularia maritima*, *Cheiranthus cheiri* and *Lunaria annua* (Tatsuzawa et al., 2006, 2007), and *Malcolmia maritima* (Tatsuzawa et al., 2008). These anthocyanins were acylated with one or more hydroxycinnamic acids, and their color stability and blueness depends on the numbers of hydroxycinnamic acids present (Honda and Saito, 2002). As described previously, there are two different glycosyl patterns at the 3-position of anthocyanidins isolated from the plants of the Cruciferae, such as 3-sophoroside-5-glucosides and 3-sambubioside-5-glucosides (Tatsuzawa et al., 2006). Recently, a 3-glucosylsambubioside pattern was newly identified in the flowers of Virginia stock (Tatsuzawa et al., 2008).

Distribution of 3-sophoroside-5-glucosides of anthocyanidins is highly restricted to the plants of only two genus *Brassica* and *Raphanus* in this family (Harborne, 1967; Andersen and Jordheim, 2006). Furthermore, as the characteristic features of these anthocyanins, their 3-sophoroside residue are acylated with hydroxycinnamic acids or glucosylhydroxycinnamic acids at the 6-position of the inner glucose. The other hydroxycinnamic acids are linked

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to the either 2- and/or 6-positions of the outer glucose, and, rarely, acylated at the 3-position of the outer glucose in these anthocyanins (Andersen and Jordheim, 2006; Idaka et al., 1987b).

In our previous studies, we isolated 33 acylated anthocyanidin 3-sambubioside-5-glucosides from the flowers in the Cruciferae (Honda et al., 2005; Saito et al., 1995, 1996; Tatsuzawa et al., 2006, 2007, 2008). As a separate work from the previous papers, we wish to report the isolation and structure elucidation of 11 new acylated cyanidin 3-sophoroside-5-glucosides from the flowers of *Iberis umbellata* cultivars, and discussed the color stability and bluing effect of these anthocyanins.

2. Results and discussion

2.1. Anthocyanins of Iberis umbellata

Eleven major anthocyanin peaks were observed in the acetic acid extracts from the flowers of four *I. umbellata* cultivars on high performance liquid chromatography (HPLC) (Table 1 and Fig. 1). The relative frequencies of their occurrence in four *I. umbellata* cultivars were 2.5–26.6% (pigment **1**), 1.7–9.4% (pigment **2**), 7.6–18.1% (pigment **3**), 1.1–2.7% (pigment **4**), 4.6–10.4% (pigment **5**), 3.5–20.1% (pigment **6**), 1.9–4.6% (pigment **7**), 4.9–14.7% (pigment **8**), 1.6–17.7% (pigment **9**), 1.7–4.7% (pigment **10**) and 1.7–8.3% (pigment **11**). These 11 anthocyanin pigments (**1**–**11**) were isolated from the flowers of its four cultivars with 5% HOAc as solvent, and purified using Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column chromatography (CC), preparative HPLC and TLC according to the procedures described previously (Honda et al., 2005; Tatsuzawa et al., 2006). The chromatographic and spectroscopic properties of these pigments are summarized in Table 2.

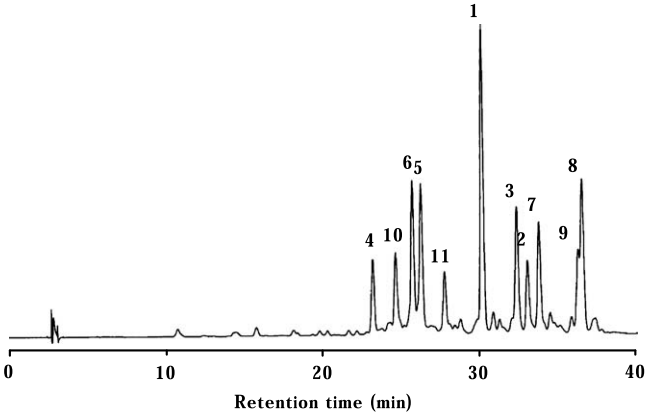

Fig. 1. HPLC profile (530 nm) of anthocyanins present in the extract from flowers of *Iberis umbellata* cv. Purple Flash. (1) pigment **1**, (2) pigment **2**, (3) pigment **3**, (4) pigment **4**, (5) pigment **5**, (6) pigment **6**, (7) pigment **7**, (8) pigment **8**, (9) pigment **9**, (10) pigment **10**, (11) pigment **11**. Structures of pigments are shown in Fig. 2 and Table 2.

Acid hydrolysis of pigments **1**–**11** resulted in cyanidin, glucose, and *p*-coumaric acid. Moreover, ferulic acid was detected in the hydrolysates of pigments **2**, **5**, **7**–**11**, and also sinapic acid was detected in the hydrolysates of pigments **3**, **6** and **9**, respectively. The acylations with malonic acid were presumed to occur in **1**–**11** by their measurements of FAB mass spectra (Table 2). Alkaline hydrolysis of pigments **1**–**11** resulted in only one deacylanthocyanin, whose structure was identified to be cyanidin 3-sophoroside-5-glucoside by direct comparison of TLC and HPLC with authentic sample obtained from *Ipomoea purpurea* anthocyanins by alkaline hydrolysis (Saito et al., 1995b). Its structure was confirmed by the analysis of its NMR spectra (see in Section 2.2 and Tables 5 and 6). In the alkaline hydrolysates, 4-glucosyl-*p*-coumaric acid was detected in those of pigments **4**–**11** and also 4-glucosyl-ferulic acid was detected in those of pigments **10** and **11** except for free hydroxycinnamic acids. Both 4-glucosyl-*p*-coumaric acid and 4-glucosyl-ferulic acid were identified by direct comparison with the authentic samples, 4-glucosyl-*p*-coumaric acid, obtained from anthocyanins of *Lobularia maritima* and 4-glucosyl-ferulic acid, obtained from anthocyanins of *Orychophragonus violaceus* by alkaline hydrolyses (Tatsuzawa et al., 2006; Honda et al., 2005).

FAB mass measurement of pigments **1**–**11** gave their molecular ions $[M]^+$ (m/z) at 1005 (pigment **1**), 1181 (pigment **2**), 1211 (pigment **3**), 1167 (pigment **4**), 1343 (pigment **5**), 1373 (pigment **6**), 1343 (pigment **7**), 1519 (pigment **8**), 1549 (pigment **9**), 1505 (pigment **10**) and 1681 (pigment **11**), respectively. The elemental components of pigments **1**–**11** were confirmed by the measurements of their high-resolution FAB mass spectra, and their chemical compositions based on HR-FAB MS were determined as shown in Table 3.

By partial hydrolysis of pigments **1**–**11**, four intermediary pigment products, cyanidin 3-sophoroside-5-glucoside, cyanidin 3-[2-(glucosyl)-6-(*p*-coumaroyl)-glucoside]-5-glucoside, cyanidin 3-[2-(2-(feruloyl)-glucosyl)-glucoside]-5-glucoside and cyanidin 3-[2-(2-(sinapoyl)-glucosyl)-glucoside]-5-glucoside were detected in their hydrolysates as the main products by HPLC analysis as well as cyanidin 3-sophoroside, cyanidin 3,5-diglucoside and cyanidin 3-glucoside (Table 4). As other major intermediary products, pigment **1** was detected in the hydrolysate of pigment **4**. Similarly, pigment **2** from the hydrolysate of pigment **5**, pigment **3** from pigment **6**, pigments **1** and **4** from pigment **7**, pigments **2** and **5** from pigment **8**, pigment **3** from pigment **9**, pigments **1**, **4** and **7** from pigment **10**, and pigments **2** and **8** from pigment **11**, were also detected, respectively (Table 4).

From the above results, the structures of pigments **1**–**11** were presumed to be based on cyanidin 3-[2-(2-(acyl-I)-glucosyl)-6-(acyl-II)-glucoside]-5-[(malonyl)-glucoside], in which the acyl-I moiety was none, ferulic acid and/or sinapic acid and acyl-II moiety was *p*-coumaric acid, glucosyl-*p*-coumaric acid, feruloyl-glucosyl-

Table 1
Distribution of anthocyanins in the flower extracts of *I. umbellata* cultivars

Cultivar	Floral[a] color	Hue[b] b^*/a^*	Anthocyanin (as %)[c]										
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Lilacina	PV82D	−0.79	2.5	1.7	7.6	1.7	5.5	13.6	1.9	4.9	11.3	4.7	8.3
Candycane lilac	PV81D	−0.78	3.7	2.4	10.5	1.1	4.6	20.1	2.8	6.9	17.7	3.0	7.6
Purple flash	PV81A	−0.57	26.6	8.8	13.5	1.8	9.2	3.5	3.3	14.7	1.6	1.8	2.7
Candycane purple	PV81A	−0.57	20.4	9.4	18.1	2.7	10.4	11.1	4.6	8.0	4.5	1.7	1.7

Percentage of total absorbance of all detected anthocyanins at 530 nm in HPLC analysis.
Anthocyanin pigment number and HPLC condition are same as in Table 2.
R_fs of these peaks are shown in Table 2.
[a] Royal Horticultural Society Colour Chart.
[b] Obtained from C.I.E. diagram Hunter values.
[c] The extract solution: dried flowers (ca. 3 mg) of each cultivar were immersed in the MAW solution (1 mL) at room temperature for 2 h and extracted.

Table 2
Chromatographic and spectroscopic data for anthocyanins of *I. umbellata* cultivars[a]

Anthocyanin[b]	R_f values (×100)				Spectral data in 0.1% HCl–MeOH				HPLC R_t (min)	FAB-MS $[M]^+$
	BAW	BuHCl	1% HCl	AHW	λ_{max} (nm)	E_{acyl}/E_{max} (%)	E_{440}/E_{max} (%)	$AlCl_3$		
1	44	17	39	70	529, 316, 297, 281	91	12	+	29.9	1005
2	55	41	30	69	537, 321, 294, 284	163	19	+	32.9	1181
3	52	36	29	70	534, 320, 297, 283	141	19	+	32.2	1211
4	29	3	53	81	531, (307), (294), 282	96	14	+	23.1	1167
5	42	13	39	76	535, (313), 296, (285)	111	13	+	26.2	1343
6	40	10	41	79	537, (309), 296, (285)	109	14	+	25.7	1373
7	30	3	21	51	532, (307), (291), 283	107	14	+	33.5	1343
8	45	16	19	51	536, (313), 296, 284	131	13	+	36.1	1519
9	43	14	26	59	536, (313), 297, (286)	126	13	+	35.9	1549
10	19	2	43	76	537, (308), (295), 282	112	14	+	24.6	1505
11	34	4	39	79	539, (312), (295), 284	138	14	+	27.7	1681
Deacylanthocyanin	24	3	46	70	525, 279	–	13	+	11.0	773

[a] The composition of solvents is given in Section 4.1.
[b] Pigment **1**: cyanidin 3-[2-(glucosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **2**: cyanidin 3-[2-(2-(feruloyl)-glucosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **3**: cyanidin 3-[2-(2-(sinapoyl)-glucosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **4**: cyanidin 3-[2-(glucosyl)-6-(4-(glucosyl)-*p* -coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **5**: cyanidin 3-[2-(2-(feruloyl)-glucosyl)-6-(4-(glucosyl)-*p* -coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **6**: cyanidin 3-[2-(2-(sinapoyl)-glucosyl)-6-(4-(glucosyl)-*p* -coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **7**: cyanidin 3-[2-(glucosyl)-6-(4-(6-(feruloyl)-glucosyl)-*p* -coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **8**: cyanidin 3-[2-(2-(feruloyl)-glucosyl)-6-(4-(6-(feruloyl)-glucosyl)-*p* -coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **9**: cyanidin 3-[2-(2-(sinapoyl)-glucosyl)-6-(4-(6-(feruloyl)-glucosyl)-*p* -coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **10**: cyanidin 3-[(2-(glucosyl)-6-(4-(6-(4-glucosyl)-feruloyl)-glucosyl)-*p* -coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]. Pigment **11**: cyanidin 3-[2-(2-(feruloyl)-glucosyl)-6-(4-(6-(4-(glucosyl)-feruloyl)-glucosyl)-*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside].

Table 3
The estimated molecular formulae of acylated anthocyanins from *I. umbellata* cvs and their chemical composition based on HR-FAB MS

Anthocyanin	HR-FAB MS			Chemical composition[a]					
	Mf	Calc.	$[M]^+$ found	Cy	Glu	Mal	pC	Fer	Sin
1	$C_{45}H_{49}O_{26}$	1005.2512	1005.2515	1	3	1	1		
2	$C_{55}H_{57}O_{29}$	1181.2986	1181.2988	1	3	1	1	1	
3	$C_{56}H_{59}O_{30}$	1211.3091	1211.3113	1	3	1	1		1
4	$C_{51}H_{59}O_{31}$	1167.3040	1167.3059	1	4	1	1		
5	$C_{61}H_{67}O_{34}$	1343.3514	1343.3489	1	4	1	1	1	
6	$C_{62}H_{69}O_{35}$	1373.3619	1373.3586	1	4	1	1		1
7	$C_{61}H_{67}O_{34}$	1343.3514	1343.3523	1	4	1	1	1	
8	$C_{71}H_{75}O_{37}$	1519.3987	1519.3978	1	4	1	1	2	
9	$C7_{72}H_{77}O_{38}$	1549.4093	1549.4097	1	4	1	1	1	1
10	$C_{67}H_{77}O_{39}$	1505.4042	1505.4023	1	5	1	1	1	
11	$C_{77}H_{85}O_{42}$	1681.4516	1681.4557	1	5	1	1	2	

$[M]^+$ and Mf are molecular ion mass values observed, and estimated molecular formulae as flavylium forms of anthocyanins isolated from *I. umbellata* cvs based on HR-FAB mass data.
Anthocyanin numbers are same to those in Table 2.
On the alkaline hydrolysis of these pigments, glucosyl-*p*-coumaric acid was detected in the hydrolysates of pigments **4**–**11**, and also glucosyl-ferulic acid was detected in those of pigments **10** and **11**.
[a] Cy:Glu:Mal:pC:Fer:Sin = molecular numbers of their components: Cy = cyanidin; Glu = glucose; Mal = malonic acid; pC = *p*-coumaric acid; Fer = ferulic acid; Sin = sinapic acid.

Table 4
Main intermediary anthocyanin products of *Iberis* anthocyanin pigments **1**–**11** by the partial acid hydrolysis

	Main intermediary products[a]											
	Cy3S5G	Cy3PS5G	Cy3FS5G	Cy3SS5G	Pigment **1**	Pigment **2**	Pigment **3**	Pigment **4**	Pigment **5**	Pigment **6**	Pigment **7**	Pigment **8**
Pigment **1**	○	○			•							
Pigment **2**	+	+	○		+	•						
Pigment **3**	+	+		○	+		•					
Pigment **4**	○	○			○			•				
Pigment **5**	+	+	○		+	○			•			
Pigment **6**	+	+		○	+		○			•		
Pigment **7**	○	○			○			+			•	
Pigment **8**	+	+	○		+	○			+			•
Pigment **9**	+	+		○	+		+			+		
Pigment **10**	○	○			○			+			○	
Pigment **11**	+	+	○		+	○			+			○

Pigments **1**–**11** are the same as in Table 2.
[a] For key to abbreviation: Methods see Section 4.4.4; the relative frequency of intermediary products by the partial acid hydrolysis: value < 10% = "○", value < 2% = "+", • = no reaction residue. Percent of total absorbance of all detected anthocyanins at 530 nm by HPLC analysis. Cy3S5G: cyanidin 3-sophoroside-5-glucoside; Cy3PS5G: cyanidin 3-(2-glucosyl-6-*p*-coumaroyl-glucoside)-5-glucoside; Cy3FS5G: cyanidin 3-[2-(2-feruloyl-glucosyl)-glucoside]-5-glucoside; Cy3SS5g: cyanidin 3-[2-(2-sinapoyl-glucosyl)-glucoside]-5-glucoside.

p-coumaric acid, and/or glucosyl-feruloyl-glucosyl-*p*-coumaric acid (Fig. 2). The structures of these pigments were further elucidated on the basis of analyses on their ^{1}H and ^{13}C NMR spectra [500 MHz for ^{1}H and 125.78 MHz for ^{13}C spectra in CF_3CO_2D–DMSO-d_6 (1:9) or CF_3CO_2D-CD_3OD (1:9)], including 2D COSY, 2D NOESY, HMQC and HMBC spectra as follows.

2.2. Deacylanthocyanin and demalonyl pigment **1**

The FAB mass spectrum of cyanidin 3-sophoroside-5-glucoside (deacylanthocyanin of *Iberis* anthocyanins) gave a molecular ion $[M]^+$at 773 *m*/*z* (calc. for $C_{33}H_{41}O_{21}$, 773.2140), indicating that deacylanthocyanin is composed of cyanidin with three molecules of glucose. The ^{1}H NMR spectrum (500 MHz in CF_3CO_2D–DMSO-d_6, 1:9) of deacylanthocyanin exhibited six aromatic protons being identified as those of a cyanidin moiety (Saito et al., 1995b) (Table 5). The anomeric protons of sugar moieties were assigned at δ 5.60 (*d*, *J* = 7.4 Hz, H-1 of Glc A), δ 5.15 (*d*, *J* = 7.7 Hz, H-1 of Glc B), and δ 4.68 (*d*, *J* = 7.7 Hz, H-1 of Glc C), and the glucose moieties of Glc A, B and C were determined to be in the β-glucopyranose form based on their observed *J* values. By application of the NOESY experiment, the long-range NOEs between H-1 of Glc A and H-4 (δ 8.85) of cyanidin, H-1 of Glc B and H-6 (δ 7.13) of cyanidin, and H-2 (δ 4.06) of Glc A and H-1 of Glc C were observed (Fig. 2) suggesting 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 Glc C forming sophorose. Therefore, deacylanthocyanin was determined to be cyanidin 3-*O*-[2-*O*-(β-glucopyranosyl)-β-glucopyranoside]-5-(β-glucopyranoside). This structure was also confirmed by analysis of its ^{13}C NMR spectra (Table 6).

The demalonyl pigment **1** (cyanidin 3-[2-(glucosyl)-6-(*p*-coumaroyl)-glucoside]-5-glucoside) was obtained by the process previously described (Tatsuzawa et al., 2008 and see Section 4.4.3.). The FAB mass spectrum of the demalonyl pigment **1** gave a molecular ion $[M]^+$ at 919 *m*/*z* (calc. for $C_{42}H_{47}O_{23}$, 919.2508) indicating, that the demalonyl pigment **1** was composed of cyanidin with three molecules of glucose and one molecule of *p*-coumaric acid. The ^{1}H NMR spectrum of this pigment was identical with that of deacylanthocyanin except for the signals of Glc A and *p*-coumaric acid moieties (Table 5). In the ^{1}H NMR spectrum of Glc A of demalonyl pigment **1**, H-6a (δ 4.36) and H-6b (δ 4.44) were shifted into the lower magnetic field than those (δ 3.59 and 3.77) of deacylanthocyanin, due to an acylation of Glc A with *p*-coumaric acid at its OH-6 group. The olefinic proton signals of *p*-coumaric acid (I) exhibited large coupling constants (δ 6.29, *d*, *J* = 15.9 Hz and 7.40, *d*, *J* = 15.9 Hz) with the *trans* configuration (Table 5 and Fig. 2). Therefore, the structure of demalonyl pigment **1** was determined to be cyanidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-(β-glucopyranoside), which has been reported in the plant of *Brassica oleracea* (Idaka et al., 1987).

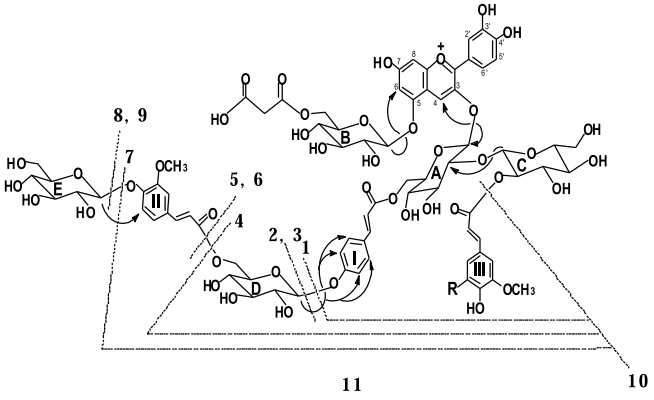

Fig. 2. Iberis acylated anthocyanins from the purple-violet flowers of *I. umbellata*. Observed main NOEs are indicated by arrows. Pigments: Group I – 1 = pigment **1**, 2 = pigment **2** R = H, 3 = pigment **3** R = OCH_3; Group II – 4 = pigment **4**, 5 = pigment **5** R = H, 6 = pigment **6** R = OCH_3; Group III – 7 = pigment **7**, 8 = pigment **8** R = H, 9 = pigment **9** R = OCH_3; Group IV – 10 = pigment **10**, 11 = pigment **11** R = H.

2.3. Pigments **1**–**3**

The FAB mass spectra of pigments **1**–**3** gave their molecular ions at 1005, 1181 and 1211 *m*/*z*, respectively, in agreement with the masses calculated for $C_{45}H_{49}O_{26}$ (calc., 1005.2512), $C_{55}H_{57}O_{29}$ (calc., 1181.2986) and $C_{56}H_{59}O_{30}$ (calc., 1211.3091). These values indicated that this pigments **1**–**3** were composed of cyanidin with three molecules of glucose and one molecule each of *p*-coumaric acid and malonic acid. Moreover, one molecule of ferulic acid was contained in pigment **2**, and also one molecule of sinapic acid was contained in pigment **3** (Table 3). By partial hydrolysis experiments as shown in Table 4, cyanidin 3-sophoroside-5-glucoside and cyanidin 3-[2-(glucosyl)-6-(*p*-coumaroyl)-glucoside]-5-glucoside (demalonyl pigment **1**) were detected in the hydrolysate of pigment **1** as the major intermediary pigment products. Thus, the structure of pigment **1** was presumed to be malonyl cyanidin 3-[2-(glucosyl)-6-(*p*-coumaroyl)-glucoside]-5-glucoside. The structure of pigment **1** was further elucidated on the basis of the analysis of its ^{1}H and ^{13}C NMR spectra as described in Section 2.2. The ^{1}H NMR spectrum of pigment **1** was superimposed on that of demalonyl pigment **1** except for the signals of 5-glucose (Glc B) and malonic acid moieties (Table 5-1). The proton signals δ 4.07 (H-6a) and 4.41 (H-6b) of the methylene of Glc B were shifted to the lower magnetic field than those (δ 3.25–3.85) of demalonyl pigment **1** (see in Section 4.4.3). The proton signals of $-CH_2-$ of malonic acid were additionally observed at δ 3.37. Therefore, the structure of pigment **1** was determined to be cyanidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new anthocyanin in plants.

As shown in Table 4, pigment **1** was detected in the partial hydrolysates of pigments **2** and **3** as the intermediary pigment product as well as the deacylanthocyanin and demalonyl pigment **1**. Therefore, the structures of pigments **2** and **3** were presumed to be feruloyl pigment **1** for pigment **2** and sinapoyl pigment **1** for pigment **3**, which were further supported from the results of their FAB mass values and acid hydrolysis (Tables 3 and 4).

The ^{1}H NMR spectra of pigments **2** and **3** were identical with those of pigment **1** except for the signals of Glc C and ferulic acid (III) moieties in pigment **2** and the signals of Glc C and sinapic acid (III) moieties in pigment **3** (Fig. 2 and Table 5-1). The proton signals of H-2 of Glc C in pigment **2** (δ 4.68) and pigment **3** (δ 4.71) were clearly shifted to a lower field than that of pigment **1** (δ 3.00), indicating that the OH-2 of Glc C was acylated with ferulic acid (III) in pigment **2**, and also the OH-2 of Glc C was acylated with sinapic acid (III) in pigment **3**. The olefinic proton signals of both hydroxycinnamic acids exhibited large coupling constants (ferulic acid (III) δ 6.45, *J* = 15.9 Hz and δ 7.52, *J* = 15.9 Hz; sinapic acid (III) δ 6.51, *J* = 15.9 Hz and δ 7.53, *J* = 15.9 Hz), supporting the presence of *trans* configuration. Consequently, the structure of pigment **2** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new anthocyanin in plants, and the structure of pigment **3** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is also a new anthocyanin in plants. The structure of pigment **2** was confirmed by the analysis of its ^{13}C NMR spectrum (Table 6-1).

Table 5-1
^{1}H NMR spectroscopic data of acylated anthocyanins (Deacyl pigment and pigments 1–5) from the flowers of *I. umbellata*

	Deacyl pigment[a]	Pigment **1**[a]	Pigment **2**[a]	Pigment **3**[a]	Pigment **4**[a]	Pigment **5**[b]
Cyanidin						
4	8.85 *s*	8.73 *s*	8.73 *s*	8.76 *s*	8.75 *s*	8.68 *s*
6	7.13 br *d* (1.5)	7.03 br *s*	6.99 *d* (1.9)	7.01 br *s*	7.05 br *s*	6.81 br *s*
8	7.01 br *d* (1.5)	6.99 br *s*	6.95 *d* (1.9)	6.95 br *s*	6.97 br *s*	6.61 br *s*
2′	8.09 br *d* (2.1)	8.04 br *s*	7.95 *d* (2.5)	7.96 br *s*	8.04 *d* (2.2)	7.78 br *s*
5′	7.11 *d* (8.6)	7.09 *d* (8.6)	7.16 *d* (8.9)	7.17 *d* (8.9)	7.09 *d* (8.7)	6.98 *d* (8.8)
6′	8.28 *dd* (2.1,8.6)	8.26 br *d* (8.6)	8.38 *dd* (2.5,8.9)	8.39 br *d* (8.9)	8.26 *dd* (2.2,8.7)	8.15 br *d* (8.8)
p-Coumaric acid (I)						
2, 6		7.33 *d* (8.0)	7.30 *d* (8.9)	7.30 *d* (7.9)	7.47 *d* (8.9)	7.14 *d* (8.6)
3, 5		6.72 *d* (8.0)	6.72 *d* (8.9)	6.74 *d* (7.9)	6.98 *d* (8.9)	6.89 *d* (8.6)
α		6.27 *d* (15.9)	6.24 *d* (15.9)	6.24 *d* (15.9)	6.37 *d* (15.9)	6.12 *d* (15.9)
β		7.38 *d* (15.9)	7.35 *d* (15.9)	7.35 *d* (15.9)	7.45 *d* (15.9)	7.20 *d* (15.9)
Ferulic acid (II)						
2						
5						
6						
α						
β						
OCH_3						
Ferulic acid or sinapic acid (III)						
2			7.26 *d* (1.9)	6.97 *s*		6.84 *s*
5			6.83 *d* (8.5)			6.60 *m*
6			7.09 *dd* (1.9, 8.5)	6.97 *s*		6.74 br *d*(8.0)
α			6.45 *d* (15.9)	6.51 *d* (15.9)		6.09 *d* (15.9)
β			7.52 *d* (15.9)	7.53 *d* (15.9)		7.32 *d* (15.9)
OCH_3			3.85 *s*	3.84 *s*		3.73 *s*
OCH_3				3.84 *s*		
Malonic acid						
CH_2		3.37 *s*	3.36 *s*	3.37 *s*	3.37 *s*	3.29 *s*
Glucose (A)						
1	5.60 *d* (7.4)	5.68 *d* (7.3)	5.69 *d* (7.3)	5.72 *d* (7.3)	5.68 *d* (7.3)	5.60 *d* (7.7)
2	4.06 *dd* (7.4,8.9)	4.12 *dd* (7.3,8.0)	4.17 *dd* (7.3,8.3)	4.19 *dd* (7.3,8.9)	4.12 *dd* (7.3,8.3)	4.04 *dd* (7.7,7.9)
3	3.67 *t** (8.9)	3.74 *t** (9.2)	3.68 *m*	3.71 *t** (8.9)	3.74 *t** (8.9)	3.71 *m*
4	3.40 *t** (8.9)	3.49 *t** (9.5)	3.41 *t** (9.5)	3.46 *m*	3.51 *m*	3.42 *m*
5	3.56 *m*	4.03 *m*	3.98 *m*	4.00 *m*	4.01 *m*	3.84 *m*
6a	3.59 *m*	4.31 *dd* (7.5,11.9)	4.29 *dd* (7.7,12.9)	4.28 *dd* (7.9,10.7)	4.33 *dd* (7.0,10.7)	4.27 *m*
6b	3.77 *d* (10.7)	4.44 *d* (11.9)	4.39 *d* (12.9)	4.41 *m*	4.44 *d* (10.7)	4.42 *d* (11.0)
Glucose (B)						
1	5.15 *d* (7.7)	5.18 *d* (7.7)	5.19 *d* (7.3)	5.21 *d* (7.8)	5.17 *d* (7.7)	5.08 *d* (8.0)
2	3.50 *dd* (7.7,8.9)	3.57 *t** (8.3)	3.57 *dd* (7.3,8.9)	3.59 *dd* (7.7,8.6)	3.56 *dd* (7.7,8.6)	3.67 *m*
3	3.41 *t** (8.9)	3.42 *t** (8.9)	3.43 *m*	3.44 *m*	3.41 *t** (9.2)	3.46 *m*
4	3.30 *t** (9.2)	3.27 *t** (9.2)	3.19 *m*	3.30 *t** (9.2)	3.28 *t** (9.2)	3.41 *m*
5	3.50 *m*	3.79 *m*	3.81 *m*	3.83 *m*	3.79 *m*	3.69 *m*
6a	3.50 *m*	4.07 *dd* (6.7,11.9)	4.06 *dd* (7.5,11.6)	4.09 *dd* (6.1, 11.6)	4.13 *m*	4.17 *dd* (5.5,12.2)
6b	3.75 *d* (10.5)	4.41 *d* (11.9)	4.41 *d* (11.6)	4.43 *m*	4.39 *d* (11.0)	4.44 *d* (12.2)
Glucose (C)						
1	4.68 *d* (7.7)	4.70 *d* (7.7)	5.20 *d* (8.2)	5.20 *d* (7.8)	4.70 *d* (7.9)	5.12 *d* (8.3)
2	2.98 *t** (8.3)	3.00 *t** (8.6)	4.68 *dd* (8.2,9.2)	4.71 *t** (8.9)	3.00 *dd* (7.9,8.9)	4.77 *dd* (8.3,8.9)
3	3.09 *t** (8.9)	3.12 *t** (9.2)	3.43 *m*	3.43 *t** (9.2)	3.12 *t** (9.2)	
4	3.03 *t** (9.2)	3.05 *t** (9.2)	3.27 *t** (9.2)	3.22 *t** (9.2)	3.04 *t** (9.2)	
5	2.68 *m*	2.74 *m*	3.09 *m*	3.11 *m*	2.75 *m*	3.19–3.90
6a	3.18 *m*	3.19 *dd* (5.8, 11.3)	3.45 *m*	3.47 *m*	3.20 *m*	
6b		3.16 *d* (11.3)	3.68 *m*	3.70 *m*	3.20 *m*	
Glucose (D)						
1					4.95 *d* (7.7)	4.91 *d* (6.7)
2					3.21 *m*	3.29 *m*
3						
4					3.25–3.41	3.19–3.90
5						
6a					3.51 *m*	
6b					3.73 *m*	3.87 *d* (11.0)

^{1}H NMR (500 MHz), an internal standard of TMS. Coupling constants (*J* in Hz) in parentheses.
s = singlet; br *s* = broad singlet; *d* = doublet; br *d* = broad doublet; *dd* = double doublet; *t** = distoted triplet; *m* = multiplet.
Anthocyanin numbers is same in Table 2 and Fig. 2.

2.4. Pigments **4**–**6**

The FAB mass spectra of pigments **4**–**6** gave their molecular ions at 1167, 1343 and 1373 *m*/*z*, respectively, in agreement with the masses calculated for $C_{51}H_{59}O_{31}$ (MW = 1167.3040), $C_{61}H_{67}O_{34}$ (MW = 1343.3514) and $C_{62}H_{69}O_{35}$ (MW = 1373.3619). These values indicated that pigments **4**–**6** were composed of cyanidin with four molecules of glucose and one molecule each of *p*-coumaric acid

Table 5-2
^{1}H NMR spectroscopic data of acylated anthocyanins (Pigments 6–11) from the flowers of *I. umbellata*

	Pigment **6**[a]	Pigment **7**[a]	Pigment **8**[a]	Pigment **9**[a]	Pigment **10**[a]	Pigment **11**[a]
Cyanidin						
4	8.76 *s*	8.77 *s*	8.74 *s*	8.74 *s*	8.75 *s*	8.72 *s*
6	7.00 br *s*	7.04 br *s*	6.96 br *s*	6.96 br *s*	7.07 br *s*	7.03 br *s*
8	6.96 br *s*	7.02 br *s*	6.93 br *s*	6.92 br *s*	7.01 br *s*	6.92 br *s*
2′	7.95 *d* (1.9)	8.04 *d* (2.1)	7.92 *d* (2.5)	7.92 br *s*	7.99 br *s*	7.89 *d* (2.2)
5′	7.16 *d* (8.6)	7.11 *d* (8.9)	7.16 *d* (8.9)	7.15 *d* (8.9)	7.11 *d* (8.6)	7.15 *d* (8.9)
6′	8.39 *dd* (1.9,8.6)	8.25 *dd* (2.1,8.9)	8.37 br *d* (8.9)	8.36 br *d* (8.9)	8.24 br *d* (8.6)	8.38 br *d* (8.9)
p-Coumaric acid (I)						
2, 6	7.43 *d* (8.7)	7.43 *d* (8.9)	7.39 *d* (8.9)	7.39 *d* (8.9)	7.44 *d* (8.9)	7.41 *d* (8.6)
3, 5	6.99 *d* (8.7)	7.01 *d* (8.9)	7.00 *d* (8.9)	6.99 *d* (8.9)	7.01 *d* (8.9)	6.99 *d* (8.6)
α	6.34 *d* (15.9)	6.34 *d* (15.9)	6.30 *d* (15.9)	6.29 *d* (15.9)	6.43 *d* (15.9)	6.38 *d* (15.9)
β	7.41 *d* (15.9)	7.40 *d* (15.9)	7.36 *d* (15.9)	7.36 *d* (15.9)	7.41 *d* (15.9)	7.40 *d* (15.9)
Ferulic acid (II)						
2		7.23 *s*	7.21 *s*	7.21 *s*	7.26 *s*	7.26 *s*
5		6.74 *d* (8.0)	6.72 *d* (7.9)	6.72 *d* (7.9)	6.93 *d* (8.9)	6.83 *d* (8.0)
6		6.94 br *d* (8.0)	6.92 br *d* (7.9)	6.91 br *d* (7.9)	6.86 br *d* (8.9)	7.09 br *d* (8.0)
α		6.42 *d* (15.9)	6.41 *d* (15.9)	6.41 *d* (15.9)	6.51 *d* (16.2)	6.44 *d* (15.9)
β		7.48 *d* (15.9)	7.47 *d* (15.9)	7.47 *d* (15.9)	7.45 *d* (16.2)	7.52 *d* (15.9)
OCH_3		3.78 *s*	3.77 *s*	3.77 *s*	3.74 *s*	3.84 *s*
Ferulic acid or Sinapic acid (III)						
2	6.96 *s*		7.25 *s*	6.96 *s*		7.29 *s*
5			6.83 *d* (8.3)			6.96 *d* (8.2)
6	6.96 *s*		7.08 br *d* (8.3)	6.96 *s*		6.93 br *d* (8.2)
α	6.49 *d* (15.9)		6.44 *d* (15.9)	6.49 *d* (15.9)		6.53 *d* (15.9)
β	7.53 *d* (15.9)		7.52 *d* (15.9)	7.52 *d* (15.9)		7.46 *d* (15.9)
OCH_3	3.84 *s*		3.85 *s*	3.84 *s*		3.76 *s*
OCH_3	3.84 *s*			3.84 *s*		
Malonic acid						
CH_2	3.37 *s*	3.39 *s*	3.38 *s*	3.37 *s*	3.37 *s*	3.35 *s*
Glucose (A)						
1	5.72 *d* (7.0)	5.69 *d* (7.0)	5.70 *d* (7.0)	5.70 *d* (7.3)	5.67 *d* (7.9)	5.66 *d* (7.7)
2	4.18 *t** (8.0)	4.14 *t** (8.3)	4.18 *m*	4.18 *dd* (7.3,8.3)	4.18 *m*	4.23 *m*
3	3.70 *m*	3.77 *m*	3.71 *m*	3.71 *m*	3.70 *m*	3.68 *m*
4	3.48 *m*	3.52 *t** (9.2)	3.45 *m*	3.48 *m*	3.55 *m*	3.47 *m*
5	3.98 *m*	4.06 *m*	4.01 *m*	4.00 *m*	4.10 *m*	4.02 *m*
6a	4.31 *m*	4.34 *dd* (7.3,12.1)	4.28 *m*	4.29 *dd* (7.4,11.9)	4.40 *m*	4.35 *m*
6b	4.40 br *d* (10.7)	4.51 *d* (12.1)	4.46 *d* (11.0)	4.45 *d* (11.9)	4.51 *d* (11.9)	4.42 *d* (10.7)
Glucose (B)						
1	5.19 *d* (7.3)	5.15 *d* (7.6)	5.16 *d* (7.6)	5.16 *d* (7.6)	5.19 *d* (7.7)	5.19 *d* (8.0)
2	3.56 *m*	3.58 *t** (8.3)	3.57 *t** (8.6)	3.57 *t** (8.5)	3.60 *t** (8.2)	3.57 *dd* (8.0,8.5)
3	3.28 *m*	3.43 *t** (8.9)	3.43 *m*	3.43 *m*	3.44 *m*	3.43 *m*
4	3.21 *t** (7.6)	3.27 *t** (9.2)	3.27 *t** (9.2)	3.27 *t** (9.2)	3.28 *t** (9.2)	3.27 *m*
5	3.69 *m*	3.82 *m*	3.78 *m*	3.78 *m*	3.77 *m*	3.79 *m*
6a	4.13 *m*	4.10 *m*	4.09 *m*	4.10 *dd* (5.5,11.3)	4.10 *m*	4.08 *dd* (6.4,11.6)
6b	4.39 *m*	4.41 *d* (11.3)	4.38 *d* (11.6)	4.37 *d* (11.3)	4.36 *d* (11.3)	4.34 *d* (11.6)
Glucose (C)						
1	5.21 *d* (8.3)	4.72 *d* (7.7)	5.21 *d* (7.7)	5.21 *d* (8.0)	4.68 *d* (7.6)	5.19 *d* (8.3)
2	4.70 *t** (8.7)	3.03 *dd* (7.7,8.9)	4.70 *t** (8.9)	4.70 *t** (8.6)	3.02 *t** (8.2)	4.67 *dd* (8.3,9.2)
3	3.42 *t** (9.2)	3.15 *t** (8.9)	3.43 *m*	3.43 *m*	3.13 *m*	3.41 *m*
4	3.25 *t** (8.8)	3.07 *t** (9.2)	3.22 *t** (9.2)	3.22 *t** (9.2)	3.06 *t** (9.2)	3.20 *t** (9.2)
5	3.12 *m*	2.75 *m*	3.11 *m*	3.11 *m*	2.69 *m*	3.08 *m*
6a	3.40–3.60	3.21 *m*	3.48 *m*	3.47 *m*	3.21 *m*	3.47 *m*
6b		3.18 *d* (13.1)	3.69 *m*	3.71 *m*	3.69 *m*	3.68 *m*
Glucose (D)						
1	4.97 *d* (7.7)	5.05 *d* (7.3)	5.05 *d* (7.4)	5.05 *d* (7.3)	5.07 *d* (7.3)	5.06 *d* (7.7)
2	3.32 *t** (7.3)	3.37 *m*	3.38 *m*	3.41 *m*	3.38 *m*	3.39 *m*
3		3.39 *m*	3.41 *m*	3.37 *m*	3.41 *m*	3.30–3.45
4		3.30 *m*	3.36 *m*	3.33 *m*	3.36 *m*	3.32 *m*
5	3.20–3.90	3.77 *m*	3.77 *m*	3.75 *m*	3.76 *m*	3.74 *m*
6a		4.25 *dd* (6.4,11.9)	4.25 *dd* (6.7,11.0)	4.25 *dd* (6.4,12.3)	4.24 *m*	4.25 *m*
6b		4.48 *d* (11.9)	4.45 *d* (11.0)	4.48 *d* (12.3)	4.51 *d* (11.9)	4.48 *d* (10.4)
Glucose (E)						
1					5.06 *d* (7.0)	5.04 *d* (7.0)
2					3.37 *m*	3.39 *m*
3						
4						
5					3.35–3.90	3.30–3.80
6a						
6b						

^{1}H NMR (500 MHz), an internal standard of TMS. Coupling constants (*J* in Hz) in parentheses.
s = singlet; br *s* = broad singlet; *d* = doublet; br *d* = broad doublet; *dd* = double doublet; *t** = distoted triplet; *m* = multiplet.

Table 6-1
^{13}C NMR spectroscopic data of acylated anthocyanins (Deacyl pigment and pigments 1,3,4,5,7) from the flowers of *I. umbellata* in DMSO-d_6/CF_3CO_2D (9:1)

	Deacyl pigment	Pigment **1**	Pigment **3**	Pigment **4**	Pigment **5**[a]	Pigment **7**
Cyanidin						
2	162.6	162.5	161.9	162.6	164.6	162.8
3	144.8	144.5	144.7	144.6	146.8	146.5
4	132.1	131.1	131.7	131.4	133.8	131.6
5	155.1	154.9	154.9	154.9	156.4	155.1
6	104.1	106.2	104.3	104.7	105.9	105.1
7	167.4	167.3	167.1	167.2	168.8	167.6
8	96.1	96.3	96.1	96.3	97.4	96.7
9	155.2	155.5	155.2	155.0	156.5	155.1
10	111.5	111.5	113.9	111.5	115.2	111.2
1′	119.7	119.6	120.0	119.6	121.1	119.8
2′	117.7	117.7	116.7	117.0	118.4	116.9
3′	146.3	146.4	146.7	146.4	149.9	146.5
4′	155.1	155.3	155.0	155.3	157.0	155.5
5′	117.1	117.1	116.9	116.4	117.5	117.3
6′	127.7	127.7	127.7	127.7	129.7	128.8
p-Coumaric acid (I)						
1		125.0	125.1	127.7	121.1	125.9
2		130.4	130.6	130.1	131.0	130.2
3		115.7	115.9	116.0	117.8	115.8
4		159.9	160.1	159.4	160.8	159.3
5		115.7	115.9	116.0	117.8	115.8
6		130.4	130.6	130.1	131.0	130.2
α		113.7	113.8	115.5	115.8	114.7
β		145.2	145.9	144.6	145.8	144.5
COOH		166.7	166.2	166.5	167.4	166.8
Ferulic acid (II)						
1					127.2	123.3
2					111.4	111.7
3					149.2	148.6
4					150.9	149.6
5					116.5	115.8
6					124.1	123.7
α					115.3	114.7
β					146.9	145.3
COOH					168.2	166.6
OCH_3					56.5	55.9
Ferulic acid or sinapic acid (III)						
1			125.0			
2			106.5			
3			138.5			
4			148.4			
5			138.5			
6			106.5			
α			116.1			
β			145.3			
COOH			166.2			
OCH_3			56.4			
OCH_3			56.4			
Malonic acid						
CH_2		41.3	41.0	41.3	41.0	41.0
COOH		166.9	167.0	167.0	168.6	167.1
COOH		168.1	168.3	168.1	169.1	168.2
Glucose (A)						
1	99.6	98.9	98.6	99.0	99.8	99.3
2	81.0	80.9	–	80.8	80.0	81.1
3	76.5	76.3	–	75.9	76.2	74.2
4	69.1	69.9	–	69.7	–	70.3
5	77.4	73.7	–	73.7	74.9	73.4
6	60.5	63.2	64.1	63.4	64.4	63.6
Glucose (B)						
1	101.4	101.8	100.2	101.7	102.0	102.1
2	77.5	73.2	69.8	73.3	74.7	73.4
3	76.0	76.1	–	77.2	76.9	–
4	69.6	69.7	–	76.2	78.0	69.9
5	73.1	74.2	–	74.2	77.7	74.8
6	60.6	64.1	64.2	64.2	63.5	64.3
Glucose (C)						
1	103.7	104.8	102.2	103.8	100.2	104.0
2	74.5	75.9	–	74.6	75.2	74.8
3	76.2	76.9	–	76.1	–	76.1

(*continued on next page*)

Table 6-1 (*continued*)

	Deacyl pigment	Pigment **1**	Pigment **3**	Pigment **4**	Pigment **5**[a]	Pigment **7**
4	69.6	69.7	–	69.5	–	74.0
5	76.8	74.6	73.4	76.7	–	77.1
6	60.6	60.6	64.2	60.6	62.6	60.9
Glucose (D)						
1				100.1	101.6	101.0
2				76.9	74.7	76.4
3				–	–	74.2
4				69.5	–	70.2
5				73.3	–	74.7
6				60.8	62.6	63.7

[a] In CD_3OD/CF_3CO_2D (9:1).

and malonic acid, and one additional molecule of ferulic acid in pigment **5**, and also one additional molecule of sinapic acid in pigment **6** (Table 3). By partial acid hydrolysis, pigments **1**–**3** were detected in the hydrolysates of pigments **4**–**6**, respectively, as the main intermediary pigment products (Table 4).

The ^{1}H NMR spectrum of pigment **4** was identical with that of pigment **1** except for the signals of Glc D moiety (Table 5-1). The resonance of the anomeric proton of Glc D appeared at δ 4.95 and its coupling constant is J = 7.7 Hz. Thus, Glc D must be of β-glucopyranose form. By analysis of its NOESY spectrum, strong long-range NOEs between H-1 of Glc D and H-3 and -5 of *p*-coumaric acid (I) were observed (Fig. 2), indicating that OH-4 of *p*-coumaric acid (I) was glycosylated with Glc D. Therefore, the structure of pigment **4** was determined to be cyanidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β- glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], which is a new anthocyanin in plants. This structure was further confirmed by the measurement of its ^{13}C NMR spectra (Table 6-1).

The ^{1}H NMR spectrum of pigment **5** was identical with that of pigment **4** except for the signals of Glc C and ferulic acid (III) moieties. The chemical shift (δ 4.77) of H-2 of Glc C was shifted to a lower magnetic field than that (δ 3.00) of pigment **4**, supporting that OH-2 of Glc C was acylated with ferulic acid (III) in pigment **5** (Fig. 2). The olefinic proton signals of ferulic acid (III) exhibited large coupling constants (J = 15.9 and 15.9 Hz) suggesting that ferulic acid (III) was in a *trans*-form. Based on these results, the structure of pigment **5** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], which is a new anthocyanin in plants. This structure was also supported by its ^{13}C NMR spectra (Table 6-1).

The ^{1}H NMR spectrum of pigment **6** was similar to that of pigment **5** changing the signals of ferulic acid (III) to sinapic acid (III) (Tables 5-1 and 5-2). The two aromatic proton signals of sinapic acid (III) were observed at δ 6.96 (H-2 and H-6) with large coupling constants (J = 15.9 and 15.9 Hz) of olefinic proton signals (δ 6.49 H-α and δ 7.53 H-β) indicating a *trans*-sinapic acid. From these results, the structure of pigment **6** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O* -(*trans*-sinapoyl)-β-glucopyranosyl)-6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new anthocyanin in plants.

2.5. Pigments **7**–**9**

The FAB mass spectra of pigments **7**–**9** gave their molecular ions at 1343, 1519 and 1549 *m*/*z*, respectively, in agreement with the mass calculated for $C_{61}H_{67}O_{34}$ (pigment **7**; MW = 1343.3514), $C_{71}H_{75}O_{37}$ (pigment **8**; MW = 1519.3987) and $C_{72}H_{77}O_{38}$ (pigment **9**; MW = 1549.4093). These values indicated that pigments **7**–**9** were composed of cyanidin with four molecules of glucose and one molecule each of *p*-coumaric acid, malonic acid and ferulic acid, respectively. Pigment **8** also contained an additional molecule of ferulic acid and pigment **9** contained an additional molecule of sinapic acid (Table 3). On the partial acid hydrolysis experiment, pigments **4** and **5** were detected in the hydrolysates of pigments **7** and **8**, respectively, and pigments **3** and **6** were detected in that of pigment **9** as the main intermediary pigment products, respectively (Table 4). Therefore, the structures of pigments **7**–**9** were presumed to be a feruloyl pigment **4** for pigment **7**, a feruloyl pigment **5** for pigment **8**, and a feruloyl pigment **6** for pigment **9**. The structures of these pigments were further elucidated on the basis of analysis on their NMR spectra as follows.

The ^{1}H NMR spectrum of pigment **7** was identical with that of pigment **4** except for the signals of ferulic acid (II) and Glc D moieties. The chemical shifts (δ 4.25 H-6a and 4.48 H-6b) of methylene protons of Glc D were shifted to a lower magnetic field than those (δ 3.51 H-6a and 3.73 H-6b) of pigment **4** (Tables 5-1 and 5-2), indicating that OH-6 of Glc D was acylated with ferulic acid (II) in pigment **7**. The olefinic protons of ferulic acid (II) exhibited large coupling constants (J = 15.9 and 15.9 Hz) indicator of a *trans*-form (Table 5-2). Therefore, the structure of pigment **7** was determined to be cyanidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(4-*O*-(6-O-(*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new anthocyanin in plants. This structure was confirmed by the measurement of its ^{13}C NMR spectra (Table 6-1).

The ^{1}H NMR spectra of pigments **8** and **9** were quite similar to that of pigment **7** except for the signals of Glc C and ferulic acid (III) moieties for pigment **8**, and also except for the signals of Glc C and sinapic acid (III) moieties for pigment **9** (Table 5-2). The chemical shifts (δ 4.70 in pigment **8** and δ 4.70 pigment **9**) of H-2 of Glc C in both pigments **8** and **9** were shifted to a lower magnetic field than that of pigment **7** (δ 3.03), indicating that pigments **8** and **9** were acylated with ferulic acid (III) and sinapic acid (III) at OH-2 of Glc C, respectively (Fig. 2). Both hydroxycinnamic acids exhibited large coupling constants (J = 15.9 and 15.9 Hz at ferulic acid, and J = 15.9 and 15.9 Hz at sinapic acid) of both olefinic proton signals, supporting that both hydroxycinnamic acids have the *trans* configurations. Therefore, the structure of pigment **8** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new anthocyanin in plants. The structure was further confirmed by the measurement of its ^{13}C NMR spectra (Table 6-2). The structure of pigment **9** was also determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-β-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new anthocyanin in plants. The structure was also confirmed by the measurement of its ^{13}C NMR spectra (Table 6-2).

Table 6-2
^{13}C NMR spectroscopic data of acylated anthocyanins (Pigments 8, 9, 11) from the flowers of *I. umbellata* in DMSO-d_6/CF_3CO_2D (9:1)

	Pigment **8**	Pigment **9**	Pigment **11**
Cyanidin			
2	162.6	162.6	162.5
3	146.7	146.7	146.8
4	131.8	131.9	131.6
5	155.1	155.0	155.0
6	106.5	106.5	106.5
7	167.5	167.5	167.6
8	96.4	96.4	96.5
9	155.1	155.1	155.0
10	110.0	111.2	111.0
1′	119.9	119.9	119.8
2′	116.9	117.2	115.3
3′	146.7	148.4	148.8
4′	155.6	155.6	155.8
5′	117.3	117.3	117.1
6′	128.8	128.8	129.0
p-Coumaric acid (I)			
1	127.9	127.9	127.9
2	130.2	130.2	130.2
3	116.5	116.6	116.1
4	159.0	159.1	159.1
5	116.5	116.6	116.1
6	130.2	130.2	130.2
α	115.4	116.1	114.3
β	144.5	144.5	144.6
COOH	166.9	166.9	166.7
Ferulic acid (II)			
1	125.9	125.0	128.2
2	111.1	111.6	111.4
3	148.2	148.3	148.6
4	148.4	148.4	149.6
5	115.8	115.8	115.8
6	123.6	123.3	123.2
α	114.8	113.8	113.9
β	144.7	144.7	144.8
COOH	166.2	166.7	166.2
OCH_3	55.8	55.9	56.0
Ferulic acid or sinapic acid (III)			
1	126.1	125.9	126.0
2	111.5	115.9	111.4
3	148.3	138.6	148.3
4	149.6	149.6	149.5
5	115.9	138.6	115.9
6	123.2	115.9	123.2
α	114.8	114.8	113.9
β	145.4	145.4	144.8
COOH	166.7	166.2	166.7
OCH_3	56.0	56.4	56.4
OCH_3		56.4	
Malonic acid			
CH_2	41.0	41.5	41.5
COOH	167.5	167.2	167.1
COOH	168.3	168.3	168.3
Glucose (A)			
1	98.6	98.6	98.8
2	80.0	77.7	–
3	76.5	–	–
4	70.8	70.9	–
5	74.2	74.5	–
6	63.7	63.7	63.8
Glucose (B)			
1	102.1	102.1	102.0
2	73.4	73.4	–
3	74.9	74.9	–
4	69.8	69.8	–
5	74.2	74.3	–
6	64.3	64.7	63.8
Glucose (C)			
1	99.9	99.9	104.7
2	77.7	77.6	–
3	76.0	–	–
4	70.7	–	–
5	74.2	–	–
6	61.5	61.5	60.8
Glucose (D)			
1	100.1	100.1	100.1
2	76.7	76.8	–
3	73.9	73.9	–
4	70.2	70.2	–
5	74.5	74.3	–
6	63.6	63.7	64.1
Glucose (E)			
1			99.7
2			–
3			–
4			–
5			–
6			61.5

2.6. Pigments ***10*** and ***11***

The FAB mass spectra of pigments **10** and **11** gave their molecular ions $[M]^+$ at 1505, and 1681 *m*/*z*, respectively, in agreement with their masses calculated for $C_{67}H_{77}O_{39}$ (pigment **10**; MW = 1505.4040) and $C_{77}H_{85}O_{42}$ (pigment **11**; MW = 1681.4515). These values indicated that pigments **10** and **11** were composed of cyanidin with five molecules of glucose and one molecule each of *p*-coumaric acid, malonic acid and ferulic acid, and pigment **11** contained additional one molecule of ferulic acid (Table 3). By partial acid hydrolysis, pigment **7** was detected in the hydrolysate of pigment **10**, and pigment **8** was detected in that of pigment **11** as the main intermediary pigment products, respectively (Table 4). Thus, the structures of pigments **10** and **11** were presumed to be a glucosyl pigment **7** for pigment **10**, and to be a glucosyl pigment **8** for pigment **11**. The detailed structures of pigments **10** and **11** were elucidated on the basis of analysis on their NMR spectra as follows.

The ^{1}H NMR spectrum of pigment **10** was identical with that of pigment **7** except for the signals of Glc E moiety of pigment **10** (Table 5-2). Since the chemical shifts of anomeric proton of Glc E exhibited at δ 5.06 (*d*, *J* = 7.0 Hz), Glc E of pigment **10** must be of β-glucopyranose form. In the NOESY spectrum of pigment **10**, strong long-range NOE between H-1 of Glc E and H-5 of ferulic acid (II) was observed (Fig. 2), supporting that OH-4 of ferulic acid (II) was glycosylated with Glc E. Therefore, the structure of pigment **10** was determined to be cyanidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new anthocyanin in plants.

The ^{1}H NMR spectrum of pigments **11** was identical with that of pigment **8** except for the signals of Glc E moiety (Table 5-2). The anomeric proton of Glc E was assigned at δ 5.04 (*d*, *J* = 7.0 Hz), and its coupling constant suggested Glc E to be β-glucopyranose.

By analysis of its NOESY spectrum, a strong long-range NOE between H-1 of Glc E and H-5 of ferulic acid (II) was observed to support that OH-4 of ferulic acid (II) was glycosylated with Glc E. Therefore structure of pigment **11** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(4-*O*- (β-glucopyranosyl)-*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new anthocyanin in plants.

2.7. The color stability and bluing effect of Iberis anthocyanins

As shown in Fig. 3, the color stability of *Iberis* anthocyanins was compared with each other. Each pigment was dissolved in buffer

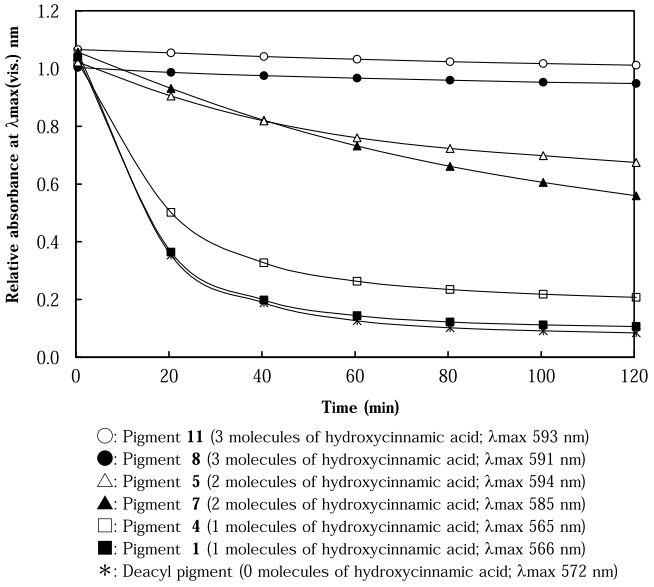

Fig. 3. Stability of Iberis acylated anthocyanins in buffer solution (pH 6.86, path length 10 mm, ca. 25 °C). Anthocyanin number is same in Table 2 and Fig. 2. (○): Pigment **11** (3 molecules of hydroxycinnamic acid, λ_{max} 593 nm); (●) pigment **8** (3 molecules of hydroxycinnamic acid, λ_{max} 591 nm); (△) pigment **5** (2 molecules of hydroxycinnamic acid, λ_{max} 594 nm); (▲) pigment **7** (2 molecules of hydroxycinnamic acid, λ_{max} 585 nm); (□) pigment **4** (1 molecules of hydroxycinnamic acid, λ_{max} 565 nm); (■) pigment **1** (1 molecules of hydroxycinnamic acid, λ_{max} 566 nm); (∗) deacyl pigment (0 molecules of hydroxycinnamic acid, λ_{max} 572 nm).

solution (phosphate–citrate buffer pH 6.9), kept at room temperature (ca. 25 °C), and measured at intervals of 20 min. The stabilities of these pigments appeared to increase with increasing content of hydroxycinnamic acids. Thus, pigments **8** and **11** (three molecules of hydroxycinnamic acids) were more stable than pigments **5** and **7** (two molecules of hydroxycinnamic acids). Pigments **4** and **1** were very unstable as well as that of cyanidin 3-sophoroside-5-glucoside (Fig. 3).

The bluing color effect of pigments **1**–**11** was also increased with increasing content of hydroxycinnamic acids as shown in Table 2. The λ_{max} values of VIS region in 0.1% HCl–MeOH solution were 529 and 531 nm at pigments **1** and **4** (one molecule of hydroxycinnamic acid), 532–537 nm at pigments **2**, **3**, **5**, **6**, **7** and **10** (two molecules of hydroxycinnamic acids) and 536–539 nm at pigments **8**, **9** and **11** (three molecules of hydroxycinnamic acids) (Table 2). These results indicated that the intramolecular co-pigmentation between cyanidin and hydroxycinnamic acids, which might be formed in these polyacylanthocyanins, of *I. umbellata* is similar to that from the flowers of *Ipomoea* (*Pharbitis*), *Petunia* and so on (Honda and Saito, 2002).

3. Concluding remarks

In this study, anthocyanins of *I. umbellata* were proved to be composed from polyacylated anthocyanins as its main anthocyanins, and those of *I. umbellata* are classified into the group of acylated anthocyanidin 3-sophoroside-5-glucoside pattern as new members. To the best of our knowledge, the plants containing anthocyanins of this group are *Brassica*, *Iberis* and *Raphanus* in the Cruciferae.

On the acylation pattern of these anthocyanins, pigments **10** and **11** are acylated at the 6-position of inner glucose in the 3-sophoroside with a long-linear side chain of glucosyl-feruloyl-glucosyl-*p*-coumaric acid. This long-linear side chain is comparable to that of glucosyl-caffeoyl-glucosyl-caffeic acid in the Orychophragonus violet-blue anthocyanins whose long-linear side chains were acylated with at the 2-position of xylose moiety in their 3-sambubiose residues (Honda et al., 2005). Orychophragonus violet-blue anthocyanins were isolated from the flowers of *Orychophragonus violaceus* in the Cruciferae. Similar acylated anthocyanins with glucosyl-hydroxycinnamoyl-glucosyl-hydroxycinnamic acids or hydroxycinnamoyl-glucosyl-hydroxycinnamic acids at the 3-glycoside residues were recognized to be present in the plants of *Ipomoea* (*Pharbitis*) and *Evolvulus* (Convolvulaceae), *Petunia* (Solanaceae), *Phacelia* (Hydrophyllaceae) and *Triteleia* (Liliaceae) (Andersen and Jordheim, 2006; Honda and Saito, 2002; Mori et al., 2006). These complicating structures of acyl groups are considered to be an advanced character in the Cruciferae (Harborne, 1967; Honda and Saito, 2002).

4. Experimental

4.1. 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), BuHCl (*n*-BuOH–2 N HCl, 1:1, upper layer), AHW (HOAc–HCl–H_2O, 15:3:82), 1% HCl for anthocyanins, 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 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. The eluant was applied as a linear gradient elution for 40 min from 20% to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O).

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

High resolution FAB mass (HR-FAB MS) spectra were obtained in the positive ion mode using the magic bullet (5:1 mixture of dithiothreitol and dithioerythritol) as a matrix.

NMR spectra were determined at 500 MHz for ^1H spectra and at 125.78 MHz for ^{13}C spectra in CF_3COOD–DMSO-d_6 (1:9). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants are in Hz.

4.2. Plant materials and flower colors

Seeds of *I. umbellate* cultivars 'Candycane Lilac' and 'Candycane Purple' were purchased from Murakami Seed Co. Ltd. (Ibaraki, Japan) and those of 'Lilacina' and 'Purple Flash' were purchased from Fukukaen Nursery & Bulb Co. Ltd. (Nagoya, Japan), and Sakata Co. Ltd. (Yokohama, Japan), respectively. Seeds were planted in greenhouses at the experimental farm of Minami-Kyushu University. The flower colors of these plants were recorded by comparing directly with the Royal Horticultural Society (R.H.S.) color chart and their chromaticities were recorded on a SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co. Ltd.). These values are shown in Table 1. The fresh flowers were collected from winter to spring seasons in Japan, and dried overnight at 40 °C, and kept in a refrigerator at about 4 °C.

4.3. Isolation of anthocyanins

Dried mixed flowers (ca. 300 g) of four cultivars were immersed in 5% HOAc–H_2O (5 L) at room temperature overnight, and extracted. The extract was passed through a Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column (90∅ × 150 mm), on

which acylated anthocyanins were adsorbed. Then, the column was thoroughly washed with 5% HOAc–H_2O (10 L), and eluted with 5% HOAc–MeOH (500 mL) to recover the anthocyanins. After concentration, the crude anthocyanins were roughly separated into four bands with paper chromatography (PC; ADVANTEC 590, 60 × 60 cm) using BAW. The pigments of the above four bands were further purified by TLC (15% HOAc) and prep. HPLC. Prep. HPLC was performed on a Waters C18 (19∅ × 150 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 60 min from 40% to 85% solvent B in solvent A. Each fraction was transformed to a Diaion HP-20 column, on which pigments were adsorbed. Pigments were eluted with 5% HOAc–MeOH followed by addition of an excess of Et_2O and then dried. The anthocyanin pigments finally obtained were as follows; pigment **1** (ca. 50 mg), pigment **2** (ca. 5 mg), pigment **3** (ca. 5 mg), pigment **4** (ca. 5 mg), pigment **5** (ca. 15 mg), pigment **6** (ca. 25 mg), pigment **7** (ca. 15 mg), pigment **8** (ca. 15 mg), pigment **9** (ca. 10 mg), pigment **10** (ca. 7 mg), pigment **11** (ca. 35 mg) and deacylanthocyanin (ca. 5 mg).

4.4. Analyses of anthocyanin

Dried petals (ca. 10 mg) of each cultivar were extracted with 0.5 mL MAW (MeOH–HOAc–H_2O, 4:1:5, v/v/v). The anthocyanin quantitative analysis of these extracts was performed by HPLC 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 for anthocyanin pigments. Solvent system used were linear gradient elution for 40 min from 20% to 85% solvent B in solvent A. The identification of anthocyanins was carried out by standard procedures involving deacylation with acid, and hydrolyses with alkaline and acid (Harborne, 1984). The data of TLC (R_f value), HPLC (R_t (min)), UV–Vis (λ_{max}), and FAB-MS spectra are shown in Tables 2 and 3, and ^1H and ^{13}C NMR spectroscopic data are shown in Tables 5 and 6 (see in Sections 4.4.1,4.4.2,4.4.3,4.4.4).

4.4.1. Anthocyanin pigments *1*–*11*

Characterization of pigments **1**–**11** are as follows: all anthocyanins are dark purple-red powders; for UV–Vis, TLC and HPLC, see Table 2; for HR-FAB MS, see Table 3; for ^1H and ^{13}C NMR spectroscopic assignments, see Tables 5 and 6.

4.4.2. Deacylanthocyanin and 4-*O*-glucosylhydroxycinnamic acids

Mixed pigments (ca. 20 mg) were individually dissolved in 2 N NaOH (2 mL) using a degassed syringe with each allowed to stand for 15 min. Each solution was then acidified with 2 N HCl and evaporated in vacuo to dryness, with the resulting residue dissolved in 1% HCl–MeOH and applied on TLC (BAW) to yield a deacylanthocyanin (ca. 5 mg), 4-*O*-glucosyl-*p*-coumaric acid (ca. 1 mg), and also small amounts of 4-*O*-glucosyl-ferulic acid as described previously (Honda et al., 2005; Tatsuzawa et al., 2006).

4.4.2.1. Deacylanthocyanin (cyanidin 3-sophoroside-5-glucoside). Dark purple-red powders: for UV–Vis and TLC see Table 1; For ^1H and ^{13}C NMR spectra, see Tables 5 and 6, HR-FAB MS calc. for 773.2140 ($C_{33}H_{41}O_{21}$). Found 773.2126.

4.4.2.2. 4-O-Glucosyl-p-coumaric acid. UV–Vis in MeOH (λ_{max}) 314, 296, (240), (217) nm; TLC (R_f ×100) BAW 86, BuHCl 75, 1% HCl 19, AHW 51; color dark-blue (UV), dark-violet (UV + NH_3 gas); HPLC (R_t (min)) 7.1.

4.4.2.3. 4-O-Glucosyl-ferulic acid. UV–Vis in MeOH (λ_{max}) 321, (298), 233, (214) nm; TLC (R_f ×100) BAW 83, BuHCl 72, 1% HCl 13, AHW 44; color green-blue (UV), violet (UV + NH_3 gas); HPLC (R_t (min)) 9.6.

4.4.3. Demalonyl pigment *1*

Pigment **1** (ca. 10 mg) was dissolved in 1 N HCl solution (2 mL) and allowed to stand at room temperature for 2 weeks as described previously (Tatsuzawa et al., 2008). Pigment **1** was almost demalonylated in this solution within this period. Demalonylated pigment **1** was then absorbed on the resin column of Diaion HP-20, and was eluted with 5% HOAc–MeOH from the column. After evaporation in vacuo, the concentrate residue was dissolved in a small volume of 5% HOAc–MeOH followed by addition of excess Et_2O, from which solids were then dried in vacuo to give demalonyl pigment **1** powder (ca. 4 mg). Demalonyl pigment **1** was already reported to be present in *B. oleracea* (Idaka et al., 1987a).

UV–Vis (in 0.1% HCl–MeOH), λ_{max} 527, 315, 297, 282 nm, E_{acyl}/E_{max} (%) 143, E_{440}/E_{max} (%) 13, +$AlCl_3$ + shift; TLC (R_f ×100) BAW 50, BuHCl 22, 1% HCl 32, AHW 65; HPLC (R_t (min)) 27.6; FAB-MS *m/z* 919 $[M]^+$ (calc. for $C_{42}H_{47}O_{23}$); HR-FAB MS calc. for 919.2508. Found 919.2568; ^1H NMR (500 MHz, CF_3CO_2D–DMSO-d_6 = 1:9, an internal standard of TMS); δ cyanidin: 8.78 (*s*, H-4), 7.04 (br *s*, H-6), 7.01 (br *s*, H-8), 8.06 (*d*, *J* = 2.5 Hz, H-2′), 7.11 (*d*, *J* = 8.6 Hz, H-5′), 8.25 (*dd*, *J* = 2.5 and 8.6 Hz, H-6′). *p*-Coumaric acid: 7.41 (2H, *d*, *J* = 8.6 Hz, H-2 and -6), 6.80 (2H, *d*, *J* = 8.6 Hz, H-3 and -5), 6.29 (*d*, *J* = 15.9 Hz, H-α), 7.40 (*d*, *J* = 15.9 Hz, H-β). Glucose A: 5.70 (*d*, *J* = 7.4 Hz, H-1), 4.13 (*m*, H-2), 3.78 (*m*, H-3), 3.52 (*m*, H-4), 4.04 (*m*, H-5), 4.36 (*m*, H-6a), 4.44 (br *d*, *J* = 11.0 Hz, H-6b). Glucose B: 5.10 (*d*, *J* = 8.0 Hz, H-1), 3.58 (*m*, H-2), 3.42 (*m*, H-3), 3.25–3.85 (rest of the sugar protons). Glucose C: 4.71 (*d*, *J* = 8.0 Hz, H-1), 3.03 (*m*, H-2), 3.14 (*t*, *J* = 8.9 Hz, H-3), 3.07 (*m*, H-4), 2.76 (*m*, H-5), 3.20 (*m*, H-6a), 3.30 (*m*, H-6b).

4.4.4. Partial acid hydrolysis of anthocyanin pigments

Pigments **1**–**11** (each ca. 1 mg) were dissolved in each 0.5 mL of 2 N HCl, and hydrolyzed by heating on a water bath (ca. 90 °C) for 10 min. The partial hydrolysates were quickly analyzed by HPLC with authentic cyanidin glycosides. As shown in Table 4, cyanidin 3-sophoroside-5-glucoside (deacylanthocyanin), cyanidin 3-[2-(glucosyl)-6-(*p*-coumaroyl)-glucoside]-5-glucoside (demalonyl pigment **1**), cyanidin 3-[2-(2-sinapoyl-glucosyl)-glucoside]-5-glucoside and cyanidin 3-[2-(2-feruloyl-glucosyl)-glucoside]-5-glucoside were detected in the hydrolysates as the main intermediary pigment products, respectively. As authentic samples, cyanidin 3-sophoroside-5-glucoside (deacylanthocyanin) and demalonyl pigment **1** were obtained by the processes described above. Cyanidin 3-[2-(2-sinapoyl-glucosyl)-glucoside]-5-glucoside was isolated from red cabbage (Idaka et al., 1987b). Cyanidin 3-[2-(2-feruloyl-glucosyl)-glucoside]-5-glucoside was obtained from the partial hydrolysates of pigments **2**, **5**, **8** and **11**.

4.4.4.1. Cyanidin 3-[2-(2-sinapoyl-glucosyl)-glucoside]-5-glucoside. UV–Vis (in 0.1% HCl–MeOH), λ_{max} 528, 321, 295, 281 nm, E_{acyl}/E_{max} (%) 74, E_{440}/E_{max} (%) 14, +$AlCl_3$ + shift; TLC (R_f ×100) BAW 33, BuHCl 7, 1% HCl 38, AHW 70; HPLC (R_t (min)) 15.3; FAB-MS *m/z* 979 $[M]^+$ (calc. for $C_{44}H_{51}O_{25}$); HR-FAB MS calc. 979.2719. Found 979.2705; ^1H NMR (500 MHz, CF_3CO_2D–DMSO-d_6 = 1:9, an internal standard of TMS); δ cyanidin: 8.89 (*s*, H-4), 6.69 (br *s*, H-6), 7.04 (br *s*, H-8), 7.98 (br *s*, H-2′), 7.16 (*d*, *J* = 8.9 Hz, H-5′), 8.40 (br *d*, *J* = 8.9 Hz, H-6′). Sinapic acid: 6.93 (2H, *s*, H-2 and -6), 6.45 (*d*, *J* = 15.9 Hz, H-α), 7.48 (*d*, *J* = 15.9 Hz, H-β), 3.85 (*s*, 2 × OCH_3). Glucose A: 5.58 (*d*, *J* = 7.6 Hz, H-1), 4.09 (*dd*, *J* = 7.6 and 9.2 Hz, H-2), 3.59 (*m*, H-3), 3.31 (*t*, *J* = 9.2 Hz, H-4), 3.55–3.85 (H-5, H-6a and -6b). Glucose B: 5.14 (*d*, *J* = 7.7 Hz, H-1), 3.51 (*m*, H-2), 3.42 (*m*, H-3), 3.50–3.60 (H-4), 3.31 (*t*, *J* = 9.2 Hz, H-5), 3.55–3.85 (H-6a and -6b). Glucose C: 5.20 (*d*,

J = 7.9 Hz, H-1), 4.67 (*dd*, *J* = 7.9 and 9.5 Hz, H-2), 3.37 (*m*, H-3), 3.19 (*m*, H-4), 3.08 (*m*, H-5), 3.55–3.85 (H-6a and -6b).

4.4.4.2. Cyanidin 3-[2-(2-feruloyl-glucosyl)-glucoside]-5-glucoside. UV–Vis (in 0.1% HCl–MeOH), λ_{max} 529, (322), 296, 282 nm, E_{acyl}/E_{max} (%) 110, E_{440}/E_{max} (%) 15, +$AlCl_3$ + shift; TLC (R_f ×100) BAW 39, BuHCl 8, 1% HCl 36, AHW 69; HPLC (R_t (min)) 15.5; FAB-MS *m*/*z* 949 $[M]^+$ (calc. for $C_{43}H_{49}O_{24}$, 949.2613); ^1H NMR (500 MHz, CF_3CO_2D–DMSO-d_6 = 1:9, an internal standard of TMS); δ cyanidin: 8.89 (*s*, H-4), 6.99 (br *s*, H-6), 7.05 (br *s*, H-8), 8.00 (br *s*, H-2′), 7.17 (*d*, *J* = 8.9 Hz, H-5′), 8.41 (br *d*, *J* = 8.9 Hz, H-6′). Ferulic acid: 7.24 (br *s*, H-2), 6.82 (*d*, *J* = 8.3 Hz, H-5), 7.07 (br *d*, *J* = 8.3 Hz, H-6), 6.42 (*d*, *J* = 15.9 Hz, H-α), 7.50 (*d*, *J* = 15.9 Hz, H-β), 3.84 (*s*, OCH_3). Glucose A: 5.58 (*d*, *J* = 8.0 Hz, H-1), 4.10 (*m*, H-2), 3.57 (*m*, H-3), 3.39 (*m*, H-4), 3.55–3.90 (H-5, H-6a and -6b). Glucose B: 5.14 (*d*, J = 7.3 Hz, H-1), 3.51 (*m*, H-2), 3.39 (*m*, H-3), 3.30 (*m*, H-4), 3.54 (*m*, H-5), 3.55–3.90 (H-6a and -6b). Glucose C: 5.19 (*d*, *J* = 8.0 Hz, H-1), 4.66 (*m*, H-2), 3.41 (*m*, H-3), 3.18 (*m*, H-4), 3.09 (*m*, H-5), 3.55–3.90 (H-6a and -6b).

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