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Acylated cyanidin glycosides from the pale-violet flowers of *Ionopsidium acaule* (Desf.) Rchb. (Brassicaceae)

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ABSTRACT

Three new acylated cyanidin 3-sambubioside-5-glucosides (**1**–**3**) and one new acylated cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside (**4**) were isolated from the pale-violet flowers of *Ionopsidium acaule* (Desf.) Rchb., together with one known anthocyanin. These new pigments were determined by chemical and spectroscopic methods to be cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (**1**), cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-xylopyranosyl)-6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (**2**), cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-xylopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (**3**) and cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-3-*O*-(β-glucopyranosyl)-β-xylopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (**4**).

These polyacylated cyanidin glycosides are responsible for the pale-violet flower color of *I. acaule*, and the contribution of the number of hydroxycinnamic acid residues to the bluing effect was discussed.

1. Introduction

Ionopsidium acaule (Desf.) Rchb. (Violet cress in English) is a plant species native to Portugal, and its small and fragrant flowers are cultivated as a stemless tufted annual garden plant with pale-violet flowers. In the continuing work on flower color variation due to acylated anthocyanins of the ornamental plants in the Brassicaceae, we have already reported the distributions of structurally complicated and acylated anthocyanins in the flowers of *Arabis blepharophylla* (Ito et al., 2013), *Aubrieta* × *cultorum* (Tatsuzawa et al., 2012a), *Cheiranthus cheiri*, *Lobularia maritima*, *Lunaria annua* (Tatsuzawa et al., 2006, 2007, 2010), *Heliophila coronopifolia* (Saito et al., 2011), *Hesperis matronalis* (Tatsuzawa, 2012), *Iberis umbellata* (Saito et al., 2008), *Malcolmia maritima* (Tatsuzawa et al., 2008a), *Matthiola incana* (Saito et al., 1995, 1996; Tatsuzawa et al., 2012c), *Moricandia ramburii* (Tatsuzawa et al., 2012b), *Moricandia arvensis* (Tatsuzawa et al., 2013), *Orychophragmus violaceus* (Honda et al., 2005), and *Raphanus sativus* (Tatsuzawa et al., 2008b). As a part of our continuing work, we are interested in the structures of the floral anthocyanins of *I. acaule*, since anthocyanins of this plant have not been thoroughly studied till now. In this paper, we wish to report the structure elucidation of acylated cyanidin 3-sambubioside-5-glucosides and acylated cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside in the pale-violet flowers of *I. acaule*.

2. Results and discussion

Five major anthocyanin peaks were observed in the 5% HOAc (acetic acid: water = 5: 95, v/v) extract from the flowers of *I. acaule* on high performance liquid chromatography (HPLC) (Fig. 1). The relative frequencies estimated by HPLC were pigment **1** (6.7%), pigment **2** (9.1%), pigment **3** (47.7%), pigment **4** (7.6%) and pigment **A** (9.4%) in the extract.

Five anthocyanins (pigments **1**–**4** and **A**), isolated from the flowers of *I. acaule* as above, were purified using Diaion HP-20 (Mitsubishi Chemicals Ion Exchange Resins) column chromatography (CC), preparative HPLC and thin layer chromatography (TLC) according to the procedure described previously (Tatsuzawa, 2012). The chromatographic and spectroscopic properties of these

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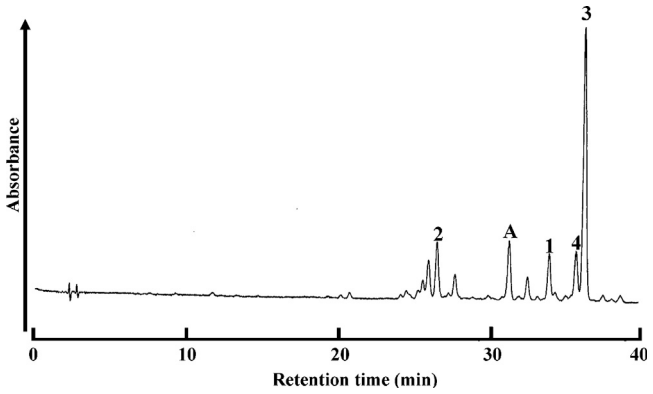

Fig. 1. HPLC profile of anthocyanin pigments in the pale-violet flowers of *I. acaule*. 1: pigment **1**, 2: pigment **2**, 3: pigment **3**, 4: pigment **4**, and A: pigment **A**.

pigments are summarized in Sections 3.4.3–3.4.7. The pigment **A** was easily identified to be cyanidin 3-*O*-[2-*O*-(xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-glucoside]-5-*O*-[6-*O*-(malonyl)-glucoside] by direct comparison of its TLC, UV–vis, HPLC and HR-FAB mass data with the authentic anthocyanin obtained from *Lunaria annua* (Tatsuzawa et al., 2006).

Acid hydrolyses of pigments **1**–**4** yielded cyanidin, glucose, xylose, malonic acid, *p*-coumaric acid and ferulic acid as their acid hydrolysates, respectively.

Alkaline hydrolysis of pigments **1**–**3** yielded cyanidin 3-sambubioside-5-glucoside and the same treatment of pigment **4** yielded cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside as their deacylanthocyanins, respectively. Both deacylanthocyanin structures were identified by direct comparison of their TLC, UV–vis and HPLC with cyanidin 3-sambubioside-5-glucoside prepared from *Lobularia* anthocyanins (Tatsuzawa et al., 2007) and cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside from *Malcolmia* anthocyanins (Tatsuzawa et al., 2008a). Moreover, 4-*O*-glucosyl-*p*-coumaric acid, ferulic acid, and malonic acid were identified by direct comparison of its TLC and HPLC with the authentic samples obtained from anthocyanins of *Lobularia maritima* and commercial standards (Wako Pure Chemical Industrials, Ltd., Japan) (Tatsuzawa et al., 2007).

2.1. Pigments **1** and **2**

The FAB mass spectra of pigments **1** and **2** gave their molecular ions at 1313 and 1313 *m/z*, in agreement with the mass calculated for $C_{60}H_{65}O_{33}$ and $C_{60}H_{65}O_{33}$, respectively. The elemental compositions of both pigments (**1** and **2**) were confirmed by measuring their high-resolution FAB mass spectra; calc. for both $C_{60}H_{65}O_{33}$ require: 1313.3408. Found: 1313.3376 for pigment **1** and 1313.3422 for pigment **2**, respectively. These values indicated that pigments **1** and **2** were composed of cyanidin with three molecules of glucose and one molecule each of *p*-coumaric acid, malonic acid and ferulic acid, respectively. The ^{1}H NMR spectrum of pigment **1** was identical with that of cyanidin 3-*O*-[2-*O*-(xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-glucoside]-5-*O*-[6-*O*-(malonyl)-glucoside] (Lunaria pigment 3) isolated from the flowers of *Lunaria annua* (Tatsuzawa et al., 2006) except for the additional signals of ferulic acid (II) and Glc C moieties in pigment **1** (Table 1 and Fig. 2). Moreover, the ^{1}H NMR spectrum of pigment **2** was identical with that of cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-glucoside]-5-*O*-[6-*O*-(malonyl)-glucoside] (Hesperis pigment 2) isolated from the flowers of *Hesperis matronalis* (Tatsuzawa, 2012) except for the additional signals of Glc C moiety in pigment **2** (Table 1 and Fig. 2). The olefinic protons of both ferulic acids (II) and (III) exhibited large coupling constants (J = 15.9 and 15.9 Hz for ferulic acid (II), 15.9 Hz and 15.9 Hz for ferulic acid (III)), supporting that both ferulic acids have the *trans* configurations. Moreover, the anomeric protons of sugar moieties were assigned at δ5.68 (*d*, J = 7.6 Hz, H-1 of Glc A in pigment **1**), δ5.69 (*d*, J = 7.1 Hz, H-1 of Glc A in pigment **2**), δ5.05 (*d*, J = 8.0 Hz, H-1 of Glc B in pigment **1**), δ5.16 (*d*, J = 7.1 Hz, H-1 of Glc B in pigment **2**), δ5.05 (*d*, J = 7.4 Hz, H-1 of Glc C in pigment **1**), δ4.94 (*d*, J = 7.7 Hz, H-1 of Glc C in pigment **2**), δ4.72 (*d*, J = 7.3 Hz, H-1 of xylose in pigment **1**) and δ5.12 (*d*, J = 8.6 Hz, H-1 of xylose in pigment **2**). Based on the observed coupling constants (Table 1), all sugars were assumed to have β-pyranose forms. In this stage, the structures of pigments **1** and **2** were presumed to be feruloylglucosylcyanidin 3-*O*-[2-*O*-(β-xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucoside]-5-*O*-[6-*O*-(malonyl)-β-glucoside]. In the NOESY spectra of pigments **1** and **2**, NOEs between H-1 of Glc C and H-2,6 and H-3,5 of *p*-coumaric acid (I) were observed (Fig. 2) supporting that OH-4 of *p*-coumaric acid (I) was glycosylated with Glc C in both pigments **1** and **2**. The chemical shifts (δ4.24 H-6a and 4.41 H-6b) of methylene protons of Glc C in pigment **1** and (δ4.64) also that of methine proton of xylose in pigment **2** were shifted to a lower magnetic field than those (δ3.52 and 3.72) of the methylene protons of Glc C in pigment **2** and that (δ3.01) of the methine proton of xylose in pigment **1**, respectively (Table 1). These results indicated that pigment **1** was acylated with ferulic acid (II) at OH-6 of Glc C, and pigment **2** was acylated with ferulic acid (III) at OH-2 of xylose, respectively (Fig. 2). Moreover, two characteristic proton signals shifted to a lower magnetic field were assigned to the methylene protons of Glc B (δ4.09 and 4.35 of pigment **1** and δ4.11 and 4.34 of pigment **2**). On the basis of these results, hydroxyl groups of the sugar moieties of pigments **1** and **2**, and OH-6 of Glc B were assumed to be acylated with malonic acid, respectively.

Thus, the structures of pigments **1** and **2** were determined to be cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucosyl)-*trans*-*p*-coumaroyl)-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (**1**) and cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-xylopyranosyl)-6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-*p*-coumaroyl)-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (**2**), respectively, which are new acylated anthocyanins in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Honda and Saito, 2002).

2.2. Pigment **3**

The FAB mass spectrum of pigment **3** gave its molecular ion $[M]^+$ at 1489 *m/z*, in agreement with its mass calculated for $C_{70}H_{73}O_{36}$. The elemental components were confirmed by measuring its high-resolution FAB MS; calc. for $C_{70}H_{73}O_{36}$ requires: 1489.3882. Found: 1489.3885. This value indicated that pigment **3** was composed of cyanidin with three molecules of glucose, two molecules of ferulic acid and one molecule each of *p*-coumaric acid and malonic acid. By partial acid hydrolysis of pigment **3**, two intermediary pigment products, cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-xylosyl)-6-*O*-(4-*O*-glucosyl-*trans*-*p*-coumaroyl)-glucoside]-5-*O*-glucoside and cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-glucoside]-5-*O*-glucoside were generated and determined as the main products of the hydrolysates by HPLC analysis, in addition to cyanidin 3-*O*-[2-*O*-(xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-glucoside]-5-*O*-glucoside, cyanidin 3-*O*-[2-*O*-(xylosyl)-glucoside]-5-*O*-glucoside, and cyanidin 3-*O*-glucoside. Thus, the structure of pigment **3** was presumed to be feruloylmalonylcyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-xylosyl)-6-*O*-(4-*O*-glucosyl-*trans*-*p*-coumaroyl)-glucoside]-5-*O*-glucoside. Detailed structure of pigment **3** was further elucidated on the basis of analyses on its NMR spectra as follows.

The ^{1}H NMR spectrum of pigment **3** was identical with that of pigment **2** except for the signals of Glc C and ferulic acid (II)

Table 1
NMR spectroscopic data of acylated anthocyanins from the flowers of *I. acaule*.

	1	**2**	**3**		**4**	
	^{1}H(ppm)[a]	^{1}H(ppm)[a]	^{1}H(ppm)[a]	^{13}C(ppm)[a]	^{1}H(ppm)[a]	^{13}C(ppm)[a]
Cyanidin						
2				162.2		162.2
3				144.9		144.3
4	8.73 s	8.68 s	8.69 s	131.0	8.69 s	131.0
5				154.9		154.8
6	6.99 s	6.95 s	6.99 s	104.7	6.98 s	104.6
7				168.1		167.2
8	7.02 s	7.01 s	6.99 s	96.3	7.00 s	96.2
9				155.1		154.9
10				110.0		111.0
1′				119.6		119.6
2′	7.99 brs	7.98 d(1.7)	7.95 d(2.1)	116.2	7.95 brs	117.4
3′				146.5		146.5
4′				155.5		155.4
5′	7.04 d(8.6)	7.04 d(8.7)	7.04 d(8.9)	117.4	7.04 d(8.6)	116.6
6′	8.36 brd(8.6)	8.48 dd(1.7, 8.7)	8.47 dd(2.1, 8.9)	128.9	8.44 brd(8.6)	128.7
Glucose A						
1	5.68 d(7.6)	5.69 d(7.1)	5.68 d(7.3)	99.9	5.67 d(7.4)	97.6
2	4.00 t(8.4)	4.00 t(7.9)	4.02 t(8.4)	77.2	4.03 t(8.0)	77.4
3	3.75 m	3.59 t(8.6)	3.61 t(8.7)	76.5	3.60 t(8.9)	77.0
4	3.45 t(8.7)	3.39 m	3.40 m	73.2	3.38 t(9.5)	73.2
5	4.00 m	3.93 m	3.95 m	74.8	3.72 m	73.5
6a	4.34 dd(7.0, 11.2)	4.27 m	4.27 dd(5.5, 11.6)	63.4	4.25 m	63.4
6b	4.43 brd(11.2)	4.34 brd(10.1)	4.38 brd(11.6)		4.37 brd(11.3)	
Glucose B						
1	5.14 d(8.0)	5.16 d(7.1)	5.14 d(7.5)	101.9	5.14 d(7.7)	101.8
2	3.52 m	3.50 m	3.51 m	77.2	3.50 t(8.4)	73.4
3	3.39 m	3.39 m	3.36 m	76.4	3.38 t(9.5)	76.5
4	3.51 m	3.27 m	3.83 m	73.2	3.52 m	69.9
5	3.77 m	3.79 m	3.88 m	75.6	3.58 m	68.7
6a	4.09 dd(5.6, 11.0)	4.11 m	4.06 dd(5.8, 11.7)	63.4	4.06 m	64.1
6b	4.35 brd(11.0)	4.34 brd(10.1)	4.33 brd(11.7)		4.34 brd(11.9)	
Glucose C						
1	5.05 d(7.4)	4.94 d(7.7)	5.03 d(7.4)	101.3	5.02 d(7.7)	99.8
2	3.51 m	3.31 m	3.31 m	77.1	3.31 t(8.9)	76.5
3	3.36 m	3.20 t(9.5)	3.24 t(9.5)	69.8	3.23 t(9.5)	77.0
4	3.24 m	3.37 m	3.51 m	69.9	3.38 t(9.5)	73.2
5	3.75 m	3.40 m	3.74 m	73.9	3.72 m	73.9
6a	4.24 dd(5.6, 11.2)	3.52 m	4.24 dd(6.9, 11.0)	63.4	4.21 m	63.4
6b	4.41 brd(11.2)	3.72 brd(11.6)	4.44 brd(11.0)		4.43 brd(11.0)	
Glucose D						
1					4.31 d(7.7)	103.2
2					2.95 t(8.2)	73.3
3					3.11 t(8.9)	76.5
4					3.03 t(9.0)	70.7
5					3.33 m	70.1
6a					3.70 m	61.3
6b					3.50 m	
Xylose						
1	4.72 d(7.3)	5.12 d(8.6)	5.14 d(8.3)	102.9	5.19 d(8.0)	101.3
2	3.01 t(8.2)	4.64 t(8.2)	4.65 t(8.6)	74.0	4.80 t(8.6)	71.9
3	3.14 t(8.9)	3.39 m	3.39 m	74.2	3.69 t(7.9)	83.7
4	3.24 m	3.52 m	3.41 m	70.0	3.40 m	73.2
5a	3.51 m	3.79 m	3.78 m	66.8	3.22 m	65.6
5b	2.96 m	3.27 m	3.21 t(10.0)		3.97 m	
p-Coumaric acid (I)						
1				127.7		127.7
2	7.50 d(8.6)	7.43 d(8.0)	7.39 d(8.6)	130.1	7.40 d(8.8)	130.1
3	7.03 d(8.6)	6.96 d(8.0)	6.97 d(8.6)	115.7	6.97 d(8.8)	116.3
4				159.1		159.0
5	7.03 d(8.6)	6.96 d(8.0)	6.97 d(8.6)	115.7	6.97 d(8.8)	116.3
6	7.50 d(8.6)	7.43 d(8.0)	7.39 d(8.6)	130.1	7.40 d(8.8)	130.1
α	6.35 d(15.9)	6.34 d(15.9)	6.31 d(15.9)	114.5	6.30 d(15.9)	115.6
β	7.38 d(15.9)	7.41 d(15.9)	7.36 d(15.9)	144.4	7.36 d(15.9)	144.3
COOH				167.2		166.5
Ferulic acid (II)						
1				125.7		125.6
2	7.25 brs		7.23 brs	111.0	7.23 brs	111.3
3				148.0		148.0
4				148.1		149.4

Table 1 (*Continued*)

	1	**2**	**3**		**4**	
	^{1}H(ppm)[a]	^{1}H(ppm)[a]	^{1}H(ppm)[a]	^{13}C(ppm)[a]	^{1}H(ppm)[a]	^{13}C(ppm)[a]
5	6.74 d(8.0)		6.71 d(8.5)	115.3	6.71 d(8.8)	115.0
6	6.99 brd(8.0)		6.93 brd(8.5)	123.2	6.93 brd(8.8)	123.2
α	6.49 d(15.9)		6.42 d(15.6)	114.3	6.42 d(15.9)	114.5
β	7.48 d(15.9)		7.46 d(15.6)	145.2	7.46 d(15.9)	144.9
COOH				166.2		166.7
OCH_3	3.78 s		3.77 s	55.7	3.76 s	55.7
Ferulic acid (III)						
1				126.0		126.0
2		7.31 brs	7.32 brs	111.4	7.29 brs	111.5
3				148.0		148.0
4				148.1		149.3
5		6.83 d(7.9)	6.84 d(8.5)	115.8	6.84 d(8.3)	115.7
6		7.15 brd(7.9)	7.16 brd(8.5)	123.1	7.14 brd(8.3)	123.0
α		6.51 d(15.9)	6.52 d(15.9)	114.7	6.47 d(15.9)	115.2
β		7.57 d(15.9)	7.58 d(15.9)	144.3	7.54 d(15.9)	145.2
COOH				166.5		166.0
OCH_3		3.85 s	3.86 s	55.9	3.85 s	55.9
Malonic acid						
CH_2	3.33 s	3.33 s	3.35 s	41.3	3.34 s	41.3
COOH				166.7		166.9
COOH				166.9		168.1

[a] NMR (500 MHz for ^{1}H and 125.78 MHz for ^{13}C) (CF_3CO_2D-DMSO-d_6, 1:9), at 25 °C, an internal standard of TMS; Coupling constants (*J* in Hz) in parentheses.

moieties (Table 1). The chemical shifts (δ4.24 H-6a and 4.44 H-6b) of methylene protons of Glc C was shifted to a lower magnetic field than those (δ3.52 and 3.72) of pigment **2** (Table 1). This result indicated that pigment **3** was acylated with ferulic acid (II) at OH-6 of Glc C (Fig. 2). By analysis of its NOESY spectrum, a weak NOE between H-6a and 6b of Glc C and H-6 of ferulic acid (II) was observed to support that ferulic acid (II) was attached to OH-6 of Glc C. The olefinic protons of *p*-coumaric acid (I), ferulic acid (II) and ferulic acid (III) exhibited large coupling constants (*J* = 15.9 Hz, 15.9 Hz, 15.6 Hz, 15.6 Hz, 15.9 Hz and 15.9 Hz), supporting that these hydroxycinnamic acids have the *trans* configurations. Moreover, the anomeric protons of sugar moieties were assigned at δ5.68 (*d*, *J* = 7.3 Hz, H-1 of Glc A), δ5.14 (*d*, *J* = 7.5 Hz, H-1 of Glc B), δ5.03 (*d*, *J* = 7.4 Hz, H-1 of Glc C) and δ5.14 (*d*, *J* = 8.3 Hz, H-1 of xylose). Based on the observed coupling constants (Table 1), all sugars were assumed to have β-pyranose forms.

Therefore, the structure of pigment **3** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-β-xylopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucosyl)-*trans*-*p*-coumaroyl)-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new acylated anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Honda and Saito, 2002). This structure was confirmed by the measurement of its ^{13}C NMR spectrum (Table 1).

2.3. Pigment **4**

The FAB mass spectrum of pigment **4** gave its molecular ion $[M]^+$ at 1651 *m/z*, in agreement with its mass calculated for $C_{76}H_{83}O_{41}$. The elemental components were confirmed by measuring its high-resolution FAB MS; calc. for $C_{76}H_{83}O_{41}$ requires: 1651.4410. Found: 1651.4465. This value indicated that pigment **4** was composed of cyanidin with four molecules of glucose, two molecules of ferulic acid and one molecule each of xylose, *p*-coumaric acid and malonic acid. By alkaline hydrolysis, pigment **4** yielded cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside as the deacyl anthocyanin as described previously. The ^{1}H NMR spectrum of pigment **4** was identical with that of pigment **3** except for the signals of xylose and Glc D moieties (Table 1). The chemical shift (δ3.69 H-3) of a methine proton of xylose was

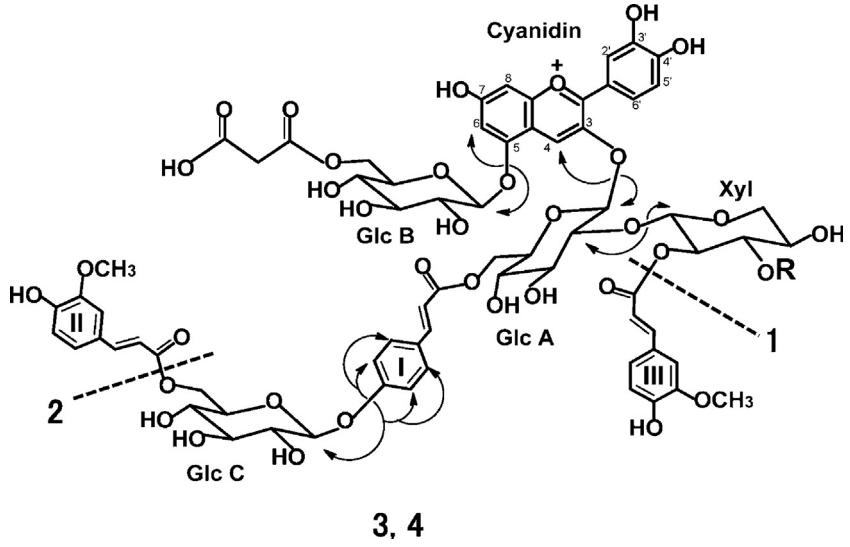

Fig. 2. New acylated anthocyanins from the flowers of *I. acaule*. Observed NOE's are indicated by arrows. **1**–**3**: R = H, **4**: R = Glc D.

shifted to a lower magnetic field than that (δ3.39 H-3) of xylose in pigment **3** (Table 1). This result indicated that pigment **4** was glycosylated with Glc D at OH-3 of xylose (Fig. 2). The olefinic protons of *p*-coumaric acid (I), ferulic acid (II) and ferulic acid (III) exhibited large coupling constants (*J* = 15.9 Hz, 15.9 Hz, 15.6 Hz, 15.6 Hz, 15.9 Hz and 15.9 Hz), supporting that these hydroxycinnamic acids have the *trans* configurations. Moreover, the anomeric protons of sugar moieties were assigned at δ5.67 (*d*, *J* = 7.4 Hz, H-1 of Glc A), δ5.14 (*d*, *J* = 7.7 Hz, H-1 of Glc B), δ5.02 (*d*, *J* = 7.4 Hz, H-1 of Glc C), δ4.31 (*d*, *J* = 7.7 Hz, H-1 of Glc D) and δ5.19 (*d*, *J* = 8.3 Hz, H-1 of xylose). Based on the observed coupling constants (Table 1), all sugars were assumed to have β-pyranose forms. By analysis of NOESY spectrum of pigment **4**, main NOEs were identical with those of pigment **3** except for the additional NOE between H-1 of Glc D and H-3 of xylose.

Therefore, the structure of pigment **4** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-3-*O*-(β-glucopyranosyl)-β-xylopyranosyl)-6-*O*-(4-*O*-(6-*O*-(*trans*-feruloyl)-β-glucosyl)-*trans*-*p*-coumaroyl)-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 2), which is a new acylated anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Honda and Saito, 2002). This structure was confirmed by the measurement of its ^{13}C NMR spectrum (Table 1).

2.4. Flower color and anthocyanins in I. acaule

Anthocyanins based on cyanidin are responsible for the flower colors of the taxa listed in Table 2 with the exception of *Heliophila coronopifolia* and *Moricandia ramburii*. Both the chromophore and intramolecular copigmentation effects related to the numbers of hydroxycinnamic acid residues present, contributed to color (Brouillard, 1994; Honda and Saito, 2002). In these plants, the main anthocyanins, exhibited red to violet flower colors, were acylated with one or two molecules of hydroxycinnamic acids. On the other hand, the main anthocyanins showing purple-violet to violet-blue flower colors were acylated with two or three molecules of hydroxycinnamic acids (Table 2). Based on the consideration of these results, the flower color of *I. acaule* having three molecules of hydroxycinnamic acids, is more blue than those of other genera bearing one or two molecules of the hydroxycinnamic acids (Table 2) as the almost same results as observed in common polyacylated anthocyanins (Honda and Saito, 2002).

As typical examples, absorption spectra of floral pigments and fresh petals are indicated for *I. acaule*, *Orychophragmus violaceus*, *Matthiola incana* and *Cheiranthus cheiri* (Figs. 3 and 4). Their λ_{max} values of fresh petals are 544 nm for *Cheiranthus cheiri*, 554 and 583sh nm for *Matthiola incana*, 521sh, 557 and 592sh nm for *Orychophragmus violaceus*, and 522sh, 556 and 591sh nm for *I. acaule*. The λ_{max} (521sh, 557 and 592sh nm) of *Orychophragmus violaceus* are very similar to those (522sh, 556 and 591sh nm) of *I. acaule* (Fig. 3). Furthermore, as shown in Fig. 4, absorption spectral curves of *Orychophragmus* anthocyanin 3 (Honda et al., 2005) and pigment **3** of *I. acaule* in pH 5.6 buffer solutions exhibit very similar curves, and give λ_{max} values at 556, 593sh nm for the *Orychophragmus* anthocyanin 3 and 554, 591 nm for the pigment **3**. Both anthocyanins were acylated with three molecules of hydroxycinnamic acids. Particularly, the absorbance at 591–593 nm of both pigments are very sharp and strong than those of other plants. These results indicate that both absorption spectral curves of *Ionopsidium* anthocyanin **3** and *Orichophragmus* anthocyanin are

Table 2
Flower colors and acylated cyanidin glycosides with hydroxycinnamic acids in the flowers of the Brassicaceous plants.

Species	R.H.S.CC	Deacyl anthocyanin	Hydroxycinnamic acids	Molecule number of hydroxycinnamic acid residues
Cheiranthus cheiri[a]	Red 54A	Cyanidin 3-sambubioside-5-glucoside	*p*-Coumaric acid	1
Arabis blepharophylla[b]	Red-purple 72C	Cyanidin 3-sambubioside-5-glucoside	*p*-Coumaric acid, sinapic acid	1
Aubrieta xcultorum[c]	Purple 78A	Cyanidin 3-sambubioside-5-glucoside	*p*-Coumaric acid, sinapic acid	1
Lunaria annua[a]	Purple 78A	Cyanidin 3-sambubioside-5-glucoside	*p*-Coumaric acid, ferulic acid	1
Hesperis matronalis[d]	Purple 78C	Cyanidin 3-sambubioside-5-glucoside	*p*-Coumaric acid, caffeic acid, ferulic acid, sinapic acid	1 or 2
Matthiola incana[e]	Purple 76A–violet 84A	Cyanidin 3-sambubioside-5-glucoside	*p*-Coumaric acid, caffeic acid, ferulic acid, sinapic acid	1 or 2
Moricandia ramburii[f]	Purple 78B	Cyanidin 3-sophoroside-5-glucoside[m] and Peonidin 3-sophoroside-5-glucoside	*p*-Coumaric acid, ferulic acid, sinapic acid	2
Lobularia maritima[a,g]	Purple-violet 81A	Cyanidin 3-sambubioside-5-glucoside	*p*-Coumaric acid, caffeic acid, ferulic acid	2
Malcolmia maritima[h]	Purple-violet 81B	Cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside	*p*-Coumaric acid, sinapic acid	2
Iberis umbellata[i]	Purple-violet 81A and B	Cyanidin 3-sophoroside-5-glucoside	*p*-Coumaric acid, ferulic acid, sinapic acid	1, 2 or 3
Moricandia arvensis[j]	Purple-violet 82C	Cyanidin 3-sophoroside-5-glucoside	caffeic acid	3
Ionopsidium acaule	Violet 85D	Cyanidin 3-sambubioside-5-glucoside and Cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside	*p*-Coumaric acid, ferulic acid	3
Orychophragmus violaceus[k]	Violet-Blue 90C	Cyanidin 3-sambubioside-5-glucoside	*p*-Coumaric acid, caffeic acid, ferulic acid, sinapic acid	3
Heliophila colonopifolia[l]	Blue 99C	Cyanidin 3-sambubioside-5-glucoside[m] and Delphinidin 3-sambubioside-5-glucoside	*p*-Coumaric acid, ferulic acid	1

[a] Tatsuzawa et al. (2006).
[b] Ito et al. (2013).
[c] Tatsuzawa et al. (2012a) (no intermolecular copigmentation cultivar).
[d] Tatsuzawa (2012).
[e] Saito et al. (1995).
[f] Tatsuzawa et al. (2012c).
[g] Tatsuzawa et al. (2007).
[h] Tatsuzawa et al. (2008a).
[i] Saito et al. (2008).
[j] Tatsuzawa et al. (2013).
[k] Honda et al. (2005).
[l] Saito et al. (2011).
[m] Minor anthocyanin.

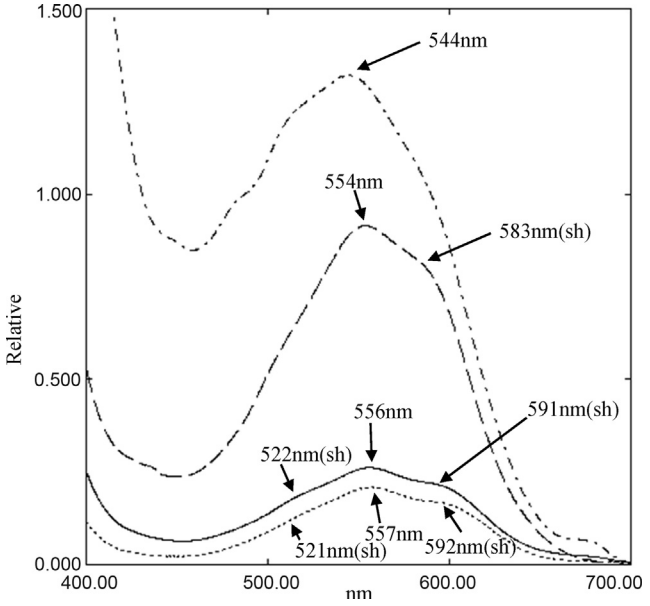

Fig. 3. Visible absorption spectra of fresh petals of Brassicaceae plants. —, *Ionopsidium acaule*: 3 (numbers of hydroxycinnamic acids); - - -, *Orychophragmus violaceus*: 3; – –, *Matthiola incana*: 2; and –·–, *Cheiranthus cheiri*: 1.

exhibited by forming the more stable intramolecular copigmentation between cyanidin and three molecules of hydroxycinnamic acids in their anthocyanin molecules (Honda and Saito, 2002).

From the chemotaxonomical point of view, there are three typical glycoside patterns at the OH-3 position of anthocyanidins in the Brassicaceae, such as 3-sambubioside, 3-(3^X-glucosylsambubioside) and 3-sophoroside (Andersen and Jordheim, 2006; Tatsuzawa et al., 2008a) as summarized previously (Tatsuzawa et al., 2008b). Therefore, *I. acaule* is grouped into the both patterns of 3-sambubioside and 3-(3^X-glucosylsambubioside). Moreover, the distribution of cyanidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] has been reported in the 7 genera of this family, *Arabidopsis*, *Arabis*, *Aubrieta*, *Heliophila*, *Hesperis*, *Lunaria* and *Matthiola* (Ito et al., 2013; Nakabayashi et al., 2009; Saito et al., 1995, 2011; Tatsuzawa, 2012; Tatsuzawa et al., 2006, 2012a,c; Tatsuzawa, 2012). Therefore, *Ionopsidium* is the eighth genus producing cyanidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside].

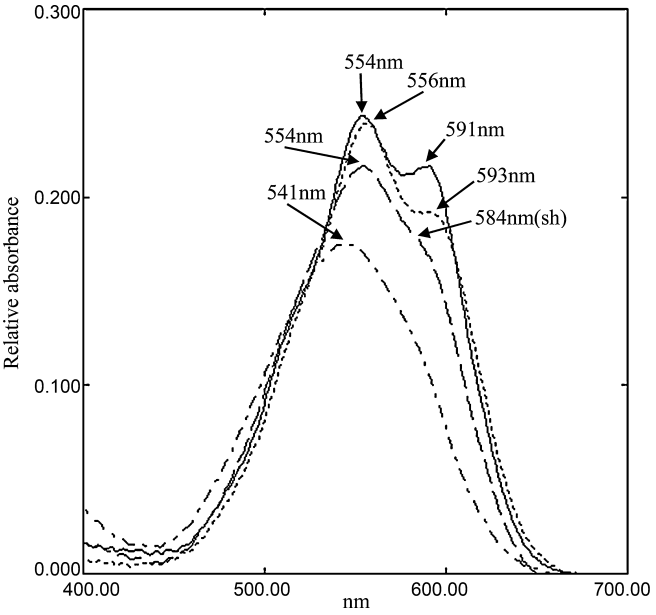

Fig. 4. Visible absorption spectra of typical four major floral anthocyanins in the Brassicaceae plants in a buffer solution pH 5.6. (phosphate-citrate buffer (MacIlvaine)). —, *Ionopsidium* anthocyanin **3**: Cy 3-[2-(2-(Fer)-Xyl)-6-(4-(6-(Fer)-Glc)-p-Cou)-Glc]-5-[6-(Mal)-Glc](1 mg/10 mL) -; - - -, *Orychophragmus* anthocyanin: Cy 3-[2-(2-(4-(6-(4-(Glc)-Caf)-Glc)-Caf)-Xyl)-6-(4-(Glc)-Sin)-Glc]-5-[6-(Mal)-Glc] (1 mg/10 mL); – –, *Matthiola* anthocyanin: Cy 3-[6-(Fer)-2-(2-(Sin)-Xyl)-Glc]-5-[6-(Mal)-Glc] (1 mg/10 mL); –·–, *Cheiranthus* anthocyanin: Cy 3-[2-(Xyl)-6-(*p*-Cou)-Glc]-5-Glc (1 mg/10 mL).

The distribution of acylated cyanidin glycosides has been reported from plants of its thirteen genera (Table 2). Among them, the distribution of acylated cyanidin 3-sambubioside-5-glucosides as the main anthocyanins has been reported in the eight genera (Table 2). Particularly, these glycosides are acylated with from one to three molecules of hydroxycinnamic acids in their glycosyl residues, such as cyanidin 3-[2-(2-(acyl-1)-xylosyl)-6-(acyl-2)-glucoside]-5-[6-(acyl-3)-glucoside] pattern, in which the acyl-1 was none, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid or glucosyl-caffeoyl-glucosyl-caffeic acid, the acyl-2 was *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, glucosyl-*p*-coumaric acid, glucosyl-ferulic acid or glucosyl-sinapic acid, and the last acyl-3 was none or malonic acid. In this study, the pigments **1**–**3** and **A** from the flowers of *I. acaule*, the pattern of acyl groups was determined such as the acyl-1 was none or ferulic acid, the acyl-2 was *p*-coumaric acid, glucosyl-*p*-coumaric acid or feruloyl-glucosyl-*p*-coumaric acid, and the acyl-3 was malonic acid. Therefore, it was very interesting that a long-linear side chain such as feruloyl-glucosyl-*p*-coumaric acid was found at the 6-OH of Glc A residue of *I. acaule*, similar to the case of *Iberis* anthocyanins 7–9, whose long-liner side chains of glucose and hydroxycinnamic acids were acylated at the 6-position of inner glucose moiety in their 3-sophorose residue (Saito et al., 2008).

3. Experimental

3.1. General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using nine mobile phases: BAW (*n*-BuOH/HOAc/H_2O, 4:1:2, v/v/v), BuHCl (*n*-BuOH/2 N HCl, 1:1, v/v, upper layer), AHW (HOAc/HCl/H_2O, 15:3:82, v/v/v), and 1% HCl and Forestal (HOAc/HCl/H_2O, 30:3:10, v/v/v) for anthocyanins, and BAW, APW (EtOAc/pyridine/H_2O, 15:7:5, v/v/v), EAA (EtOAc/HCOOH/H_2O, 5:2:1, v/v/v), EFW (Et_2O/HCOOH/H_2O, 5:2:1, v/v/v) and 15% HOAc-H_2O for sugars and organic acid with UV light and aniline hydrogen phthalate spray regent (Harborne, 1984).

Analytical HPLC was performed by LC 10A system (Shimadzu), using a Waters C18 (4.6 ϕ × 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 a MPS-2450 (Shimadzu) in 0.1% HCl-MeOH (from 200 to 700 nm) and in pH 5.6 buffer solution (MacIlvaine). FAB mass spectra were obtained in the positive ion mode using the magic bullet (5:1 mixture of dithiothreitol and dithioerythritol) as a matrix. NMR spectra ware recorded at 500 MHz for ^{1}H spectra in DMSO-d_6-CF_3COOD (9:1), including 2D COSY and 2D NOESY spectra for pigments **1** and **2**, and at 500 MHz for ^{1}H and 125.78 MHz for ^{13}C spectra in DMSO-d_6-CF_3COOD (9:1), including 2D COSY, 2D NOESY, HMQC and HMBC spectra for pigments **3** and **4**. Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants (J) are in Hz.

3.2. Plant materials

Seeds of pale-violet flowers of *I. acaule* were purchased from Takii Co. Ltd (Kyoto). Seeds were sown in August, 2009 and plants were grown in a greenhouse of Iwate University. Flowers with a pale violet color [Violet 85D by Royal Horticultural Society (R.H.S) Color Chart and $b^*(-4.79)/a^*(7.70) = -0.62$, $L^* = 79.42$ by a SE 2000 Spectro Color Meter (Nppon Denshoku Industries Co., Ltd.)] were collected in winter to spring in 2010. Spectral absorption of flowers were directly measured on intact petals using a recording spectrophotometer operating as a double-beam instrument (Type: MPS-2450) (Saito, 1967; Yokoi and Saito, 1973). The petals (3 mm in its diameter and four petals per a flower) were trimmed from flowers (about 10,000 flowers) by hand and dried by air for 1 day at 45 °C. Then they were kept at −20 °C until used.

3.3. Isolation and purification of anthocyanins

Dried flowers (*ca.* 50 g) of *I. acaule* were immersed in 5% HOAc (5 l) at room temperature for 5 h and extracted. Five anthocyanin pigments was isolated and purified by Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column (90 × 150 mm) chromatography, paper chromatography and preparative HPLC from the extract as described previously (Tatsuzawa et al., 2006, 2007). The purified five anthocyanins were obtained from the flowers as follows; pigment **1** (*ca.* 3 mg), pigment **2** (*ca.* 3 mg), pigment **3** (*ca.* 15 mg), pigment **4** (*ca.* 9 mg) and pigment **A** (*ca.* 2 mg).

3.4. Chemical and spectroscopic analyses of purified anthocyanins

The identification of anthocyanins was carried out by standard procedures (Harborne, 1984).

3.4.1. Acid hydrolyses

Acid hydrolyses of pigments **1**–**4** (*ca.* 0.5 mg, each) were carried out with 2 N HCl (1 ml) at 100 °C for 2 h, and resulted in cyanidin, glucose, xylose, *p*-coumaric acid, ferulic acid and malonic acid. These compounds were confirmed by direct comparison of TLC and/or HPLC with the authentic samples (Honda et al., 2005).

Partial acid hydrolysis of pigment **3** (*ca.* 0.5 mg) was carried out with 2 N HCl (0.5 ml) and hydrolyzed by heating in a water bath (*ca.* 90 °C) for 10 min. Two intermediary pigment products were detected in the hydrolysates as the main products by HPLC analysis, and isolated from the hydrolysates. These pigment products were identified by comparison with cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-xylosyl)-6-*O*-(4-*O*-glucosyl-*trans*-*p*-coumaroyl)-glucoside]-5-*O*-glucoside and cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-glucoside]-5-*O*-glucoside which were obtained from *Lobularia* purple-violet flowers described previously (Tatsuzawa et al., 2007). The data of two intermediary pigment products are shown in Sections 3.4.1.1 and 3.4.1.2.

*3.4.1.1. Cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-xylosyl)-6-*O*-(4-*O*-glucosyl-*trans*-p-coumaroyl)-glucoside]-5-*O*-glucoside.* UV–vis (in 0.1% HCl–MeOH): λ_{max} 532, 318sh, 298sh, 279 nm, E_{318}/E_{532}(%) = 95, E_{440}/E_{532} (%) = 13, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.43, BuHCl 0.19, 1% HCl 0.30, AHW 0.61, HPLC: R_t (min) 25.5.

*3.4.1.2. Cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-feruloyl)-xylosyl)-6-*O*-(trans-p-coumaroyl)-glucoside]-5-*O*-glucoside.* UV–vis (in 0.1% HCl–MeOH): λ_{max} 531, 320, 298, 281 nm, E_{320}/E_{531} (%) = 123, E_{440}/E_{531} (%) = 12, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.67, BuHCl 0.48, 1% HCl 0.20, AHW 0.51, HPLC: R_t (min) 31.9.

3.4.2. Alkaline hydrolyses

Alkaline hydrolyses of pigments **1**–**4** (*ca.* 0.5 mg, each) were carried out with 2 N NaOH solution (1 ml) using a degassed syringe stirring for 15 min. Then, cyanidin 3-sambubioside-5-glucoside, 4-*O*-glucosyl-*p*-coumaric acid, ferulic acid and malonic acid were obtained from **1** to **3** as their hydrolysates and cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside, 4-*O*-glucosyl-*p*-coumaric acid, ferulic acid and malonic acid were obtained from **4** as its hydrolysate. These compounds were confirmed by direct comparison of TLC, UV–vis and/or HPLC with the authentic samples which were obtained from anthocyanins of *Malcolmia* and *Lobularia* by alkaline hydrolysis (Tatsuzawa et al., 2007, 2008a).

*3.4.2.1. Deacyl anthocyanin of pigments **1**–**3** (cyanidin 3-sambubioside-5-glucoside).* UV–vis (in 0.1% HCl–MeOH): λ_{max} 527, 278 nm, E_{440}/E_{527} (%) = 13, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.28, BuHCl 0.04, 1% HCl 0.24, AHW 0.50; HPLC: R_t (min) 13.1.

*3.4.2.2. Deacyl anthocyanin of pigment **4**, cyanidin 3-(3^X-glucosylsambubioside-5-glucoside).* UV–vis (in 0.1% HCl–MeOH): λ_{max} 526, 278 nm, E_{440}/E_{526} (%) = 14, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.18, BuHCl 0.03, 1% HCl 0.34, AHW 0.53; HPLC: R_t (min) 13.7.

*3.4.2.3. 4-*O*-Glucosyl-*p*-coumaric acid.* TLC: (R_f-values) BAW 0.76, EAA 0.82, EFW 0.79, HPLC: R_t (min) 8.0.

3.4.3. Pigment **1**

Dark red powder; for UV–vis (in 0.1% HCl–MeOH): λ_{max} 530, 315sh, 294, 283 nm, E_{315}/E_{530} (%) = 127, E_{440}/E_{530} (%) = 10, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.41, BuHCl 0.12, 1% HCl 0.10, AHW 0.37, HPLC: R_t (min) 34.9, ^{1}H NMR spectrum, see Table 1; HR-FABMS calc. for $C_{60}H_{65}O_{33}$: 1313.3408. Found: 1313.3376.

3.4.4. Pigment **2**

Dark red powder; for UV–vis (in 0.1% HCl–MeOH): λ_{max} 530, 315sh, 295, 283 nm, E_{315}/E_{530} (%) = 122, E_{440}/E_{530} (%) = 12, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.74, BuHCl 0.33, 1% HCl 0.27, AHW 0.64, HPLC: R_t (min) 27.0, ^{1}H NMR spectrum, see Table 1; HR-FABMS calc. for $C_{60}H_{65}O_{33}$: 1313.3408. Found: 1313.3422.

3.4.5. Pigment **3**

Dark red powder; for UV–vis (in 0.1% HCl-MeOH): λ_{max} 533, 315sh, 296, 284sh nm, E_{315}/E_{533} (%) = 146, E_{440}/E_{533} (%) = 11, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.74, BuHCl 0.38, 1% HCl 0.10, AHW 0.40, HPLC: R_t (min) 37.1, ^{1}H and ^{13}C NMR spectra, see Table 1; HR-FABMS calc. for $C_{70}H_{73}O_{36}$: 1489.3882. Found: 1489.3885.

3.4.6. Pigment **4**

Dark red powder; for UV–vis (in 0.1% HCl–MeOH): λ_{max} 532, 317sh, 295, 285sh nm, E_{317}/E_{532} (%) = 159, E_{440}/E_{532} (%) = 14, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.62, BuHCl 0.20, 1% HCl 0.36, AHW 0.60, HPLC: R_t (min) 37.1, ^{1}H and ^{13}C NMR spectra, see Table 1; HR-FABMS calc. for $C_{76}H_{83}O_{41}$: 1651.4410. Found: 1651.4465.

3.4.7. Pigment **A**

For the identification of pigment **A** isolated from the flowers of *I. acaule*, we used a purified anthocyanin, cyanidin 3-[2-(xylosyl)-6-(*trans*-*p*-coumaryl)-glucoside]-5-[6-(malonyl)-glucoside], obtained from the flowers of *Lunnaria annua* as a comparative standard (Tatsuzawa et al., 2006), and analyzed by the methods of TLC, HPLC, UV–vis and HR-FAB mass spectra. As the results, we were able to determine the structure **A** to be cyanidin 3-*O*-[2-*O*-(xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-glucoside]-5-*O*-[6-*O*-(malonyl)-glucoside] as follows.

*3.4.7.1. Cyanidin 3-*O*-[2-*O*-(xylosyl)-6-*O*-(*trans*-p-coumaroyl)-glucoside]-5-*O*-[6-*O*-(malonyl)-glucoside] (= pigment **A**).* UV–vis (in 0.1% HCl–MeOH): λ_{max}530, 311, 295, 281 nm, E_{311}/E_{530} (%) = 77, E_{440}/E_{530} (%) = 14, $AlCl_3$ shift + TLC: (R_f-values) BAW 0.68, BuHCl 0.36, 1% HCl 0.12, AHW 0.39; HPLC: R_t (min) 32.0; FAB-MS *m/z* 975 $[M]^+$ (calc. for $C_{44}H_{47}O_{25}$); HR-FAB MS calc. 975.2406. Found 975.2391.

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