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7-Polyacylated delphinidin 3,7-diglucosides from the blue flowers of *Leschenaultia* cv. Violet Lena

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Abstract

The triacyl anthocyanins, Leschenaultia blue anthocyanins **1** and **2** (LBAs **1** and **2**) were isolated from the blue flowers of *Leschenaultia* R. Br. cv. Violet Lena (Goodeniaceae), in which LBA **1** was present as a dominant pigment. The structure of LBA **1** was elucidated to be delphinidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(4-*O*-(6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranoside] by application of chemical and spectroscopic methods. Since LAB **2** was isolated in small amount, its structure was tentatively assigned as either delphinidin 3-(malonylglucoside)-7-[(glucosyl-*p*-coumaroyl)-(glucosylcaffeoyl)-glucoside] or delphinidin 3-(malonyl-glucoside)-7-[(glucosyl-caffeoyl)(glucosyl-*p*-coumaroyl)-glucoside]. This is the first report of the occurrence of 7-polyacylated anthocyanins in the family of Goodeniaceae, although others have been found in the families of the Ranunculaceae, Campanulaceae, and Compositae. Moreover, delphinidin 3-glycoside-7-di-(glucosylcaffeoyl)-glucoside has been reported only in the flowers of *Platycodon grandiflorum* (Campanulaceae). From a chemotaxonomical viewpoint, the Goodeniaceae may be closely related to the Campanulaceae.

Keywords: *Leschenaultia* R. Br. cv. Violet Lena; Goodeniaceae; Blue flower color; Acylated anthocyanins; Delphinidin 3-malonylglucoside-7-di-(glucosylcaffeoyl)-glucoside

1. Introduction

The *Leschenaultia* species, in the family of the Goodeniaceae, are native to Australia. There are 21 known species in Western Australia, where the aboriginal people have described the blue Leschenaultia (*Leschenaultia biloba*) flower as ''the floor of the sky''. In 1999, one (N.S.) of the authors came across the beautiful blue flowers of *L. biloba* in the parks and gardens of Western Australia during his sabbatical leave. Since then, he had been interested in the chemical structures of anthocyanin pigments of *L. biloba* flowers. In Japan, *Leschenaultia* R. Br. cv. Violet Lena (*L. biloba* cv. Sky Blue × *L. formosa* cv. Star Blaster) is grown as a popular greenhouse shrub and Japanese people admire its blue flower color. There has, however, been no report of anthocyanins in flowers of the genus *Leschenaultia*. In extending our acylated anthocyanin study (Honda and Saito, 2002), we thus decided to elucidate the structures of anthocyanin pigments of the blue flowers of *Leschenaultia* cv. Violet Lena, whose anthocyanins were anticipated to be heavily acylated with molecules of hydroxycinnamic acids and malonic acid.

In this paper, we report the structure elucidation of two acylated delphinidin 3,7-diglucosides (Leschenaultia blue anthocyanins **1** and **2**, abbreviated as LBAs **1** and **2**) in the blue flowers of *Leschenaultia* cv. Violet Lena.

2. Results and discussion

Two major and five minor anthocyanin peaks were observed in the 5% acetic acid extract from the blue flowers of *Leschenaultia* R. Br. cv. Violet Lena using high

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performance liquid chromatography (HPLC) with monitoring at 530 nm. The proportions were 57.5% (LBA **1**) and 12.3% (LBA **2**), based on a percentage of the total absorbance of the anthocyanin peaks. The two anthocyanins, LBAs **1** and **2**, were then extracted from the dried flowers with 5% aqueous acetic acid and purified using Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column chromatography (CC), preparative PC, HPLC and TLC, according to the procedures described previously (Tatsuzawa et al., 2004; Honda et al., 2005) to yield **1** and **2** in approximately 30 mg and 3 mg amounts, respectively. However, the other minor pigments could not be obtained in pure form due to their small amounts. The chromatographic and spectroscopic properties of LBAs **1** and **2** are summarized in Table 1.

Acid hydrolysis of LBAs **1** and **2** resulted in delphinidin, glucose, caffeic acid and malonic acid. In addition, *p*-coumaric acid was detected in the hydrolysate of LBA **2**. Alkaline hydrolysis of LBA **1** resulted in one deacylated anthocyanin, glycosylcaffeic acid and caffeic acid. On the other hand, similar treatment of LBA **2** resulted in only the same deacylated anthocyanin as that from LBA **1**; however, other compounds such as glycosyl hydroxycinnamic acids, caffeic acid, *p*-coumaric acid and malonic acid, could not be detected due to the small amounts of LBA **2** available.

2.1. Deacylanthocyanin and glycosylcaffeic acid from LBA 1

The FAB mass spectrum of deacylanthocyanin (deacylated LBA **1**) gave a molecular ion $[M]^+$ at 627 *m/z* (calc. for $C_{27}H_{31}O_{17}$, 627.156 *m/z*), indicating that the deacylanthocyanin is composed of delphinidin with two molecules of glucose. The chromatographic and spectroscopic data of the deacylanthocyanin are shown in Table 1. The ^{1}H NMR spectrum (500 MHz in CF_3CO_2D–CD_3OD, 1:9) of deacylanthocyanin exhibited five aromatic protons being identified with those of delphinidin moieties (Toki et al., 1994), (Table 2). The anomeric protons of sugar moieties were assigned at δ 5.38 (*d*, $J = 7.9$ Hz, H-1 of Glu A) and δ 5.19 (*d*, $J = 7.4$ Hz, H-1 of Glu B), and both glucose moieties of Glu A and Glu B were determined to be in the β-glucopyranose form from their observed *J* values. By application of NOESY experiments, the long range NOEs between H-1 of Glu A and H-4 (δ 8.95) of delphinidin, and also between H-1 of Glu B and H-8 (δ 7.24) of delphinidin were observed (Fig. 1), suggesting that OH-3 and OH-7 of delphinidin are glycosylated with Glu A and Glu B, respectively. Therefore, the deacylanthocyanin was determined to be delphinidin 3-*O*-β-D-glucopyranoside-7-*O*-β-D-glucopyranoside.

The FAB mass spectrum of glycosylcaffeic acid gave a molecule ion $[M + 1]^+$ at 343 *m/z* (calc. for $C_{15}H_{19}O_9 = 343.095$) indicating that the glycosylcaffeic acid is composed of caffeic acid with a molecule of glucose. The structure of this glycosylcaffeic acid was elucidated to be 4-*O*-(β-D-glucopyranosyl)-*trans*-caffeic acid by the analysis of its ^{1}H NMR spectra (Section 4.4.2). Its linkage between glucose and caffeic acid moieties was confirmed by NOE experiments. Strong NOEs were observed between the H-1 signal (*d* 4.78, *d*, $J = 7.3$ Hz) of glucose and the H-5 resonance (*d* 7.11, *d*, $J = 8.2$ Hz) of caffeic acid by irradiation of H-1 (Glu) and H-5 (caffeic acid), respectively. Furthermore, this structure was confirmed by the analyses of TLC and HPLC with the authentic samples obtained from Cineraria and Evolvulus anthocyanins (Toki et al., 1994, 1995).

2.2. LBA 1

The molecular ion $[M]^+$ of LBA **1** was observed at *m/z* 1361 (calc. for $C_{60}H_{65}O_{36}$, 1361.325) using FABMS, indicating that LBA **1** is composed of delphinidin with four molecules of glucose, two molecules of caffeic acid and one molecule of malonic acid. The elemental components were confirmed by measuring its high-resolution; FABMS; calc. for $C_{60}H_{65}O_{36}$: 1361.3256. Found: 1361.3285. The structure of LBA **1** was further elucidated on the basis of analyses on its ^{1}H and ^{13}C NMR spectra [500 MHz for ^{1}H and 125.78 MHz for ^{13}C spectra in CF_3CO_2D–CD_3OD (1:9) or DCl–DMSO-d_6 (1:9)], including 2D COSY, 2D NOESY, HMQC and HMBC spectra.

The chemical shifts of 11 aromatic protons of delphinidin and caffeic acid moieties with their coupling constants

Table 1
Chromatographic and spectroscopic data for Leschenaultia blue anthocyanins of *Leschenaultia* cv. Violet Lena*

Anthocyanins**	R_f values (×100)				Spectral data in 0.1% HCl–MeOH				HPLC	FABMS***
	BAW	BuHCl	1% HCl	AHW	λ_{max} (nm)	E_{acyl}/E_{max} (%)	E_{440}/E_{max} (%)	$AlCl_3$	R_t (min)	$[M^+]$
LBA **1**	5	0	3	17	551, (318), 285	129	16	+	23.7	1361
LBA **2**	5	0	4	20	548, (308), 284	198	19	+	25.0	1345
Demalonyl LBA **1**	4	0	1	12	554, (317), 285	143	16	+	22.3	1275
Deacyl LBAa **1** and **2**	6	0	4	18	539, 278	–	21	+	7.7	627

LBA **2**: delphinidin 3-malonylglucoside-7-glucosylcaffeoylglucosyl-*p*-coumaroylglucoside or delphinidin 3-malonylglucoside-7-glucosyl-*p*-coumaroylglucosylcaffeoylglucoside; ***(calc. for $C_{60}H_{65}O_{35}$: 1345.331).

Demalonyl LBA **1**: delphinidin 3-glucoside-7-glucosylcaffeoylglucosylcaffeoylglucoside; ***(calc. for $C_{57}H_{63}O_{33}$: 1275.324).

Deacyl LBAa **1** and **2**: delphinidin 3,7-diglucoside; ***(calc. for $C_{27}H_{31}O_{17}$: 627.156).

* The composition of the solvents is given in Section 4.1.

** LBA **1**: delphinidin 3-malonylglucoside-7-glucosylcaffeoylglucosylcaffeoylglucoside; ***(calc. for $C_{60}H_{65}O_{36}$: 1361.325).

Table 2
NMR spectroscopic data of Leschenaultia blue anthocyanin 1

	LBA **1**[a]		Demalonyl LBA **1**[b]	Deacyl LBA **1**[a]
	^{13}C δ (ppm)	^{1}H δ (ppm)	^{1}H δ (ppm)	^{1}H δ (ppm)
Delphinidin				
2	163.1			
3	146.2			
4	133.0	8.52 *s*	8.65 *s*	8.95 *s*
5	155.8			
6	105.6	6.74 *d* (2.2)	7.28 *brs*	6.84 *brs*
7	166.9			
8	94.5	6.98 *d* (2.2)	7.38 *brs*	7.24 *brs*
9	157.3			
10	113.6			
1′	119.9			
2′	113.6	7.79 *s*	7.88 *s*	7.85 *s*
3′	147.8			
4′	147.3			
5′	147.8			
6′	113.6	7.79 *s*	7.88 *s*	7.85 *s*
Caffeic acid (I)				
1	129.8			
2	118.7	6.91 *d* (1.9)	7.18 *brs*	
3	147.7			
4	148.6			
5	116.0	6.66 *d* (8.6)	6.79 *d* (8.6)	
6	121.4	6.79 *dd* (1.9, 8.6)	6.95 *brd* (8.6)	
α	118.4	6.52 *d* (15.9)	6.53 *d* (15.9)	
β	144.2	7.58 *d* (15.9)	7.58 *d* (15.9)	
CO_2H	168.0			
Caffeic acid (II)				
1	130.2			
2	114.9	6.75 *d* (2.1)	7.02 *brs*	
3	147.9			
4	148.2			
5	116.5	6.53 *d* (8.2)	6.66 *d* (8.3)	
6	121.4	6.29 *dd* (2.1, 8.2)	6.90 *m*	
α	118.4	6.08 *d* (15.9)	6.27 *d* (16.2)	
β	144.2	6.93 *d* (15.9)	7.02 d (16.2)	
CO_2H	168.0			
Malonic acid				
$–CH_2–$	40–50	3.58–3.63		
COOH	169.1			
COOH	170.3			
Glucose A				
1	102.9	5.39 *d* (7.7)	5.33 *d* (8.0)	5.38 *d* (7.9)
2	74.5	3.78 *dd* (7.7, 9.0)	3.75 *m*	3.74 *dd* (7.9, 9.2)
3	77.7	3.62 *brt* (9.0)		3.52–3.58
4	71.1	3.58 *dd* (8.9, 9.0)	3.15–3.65	3.46 *brt* (9.6)
5	76.2	3.92 *m*		3.57–3.65
6a	65.7	4.52 *dd* (7.6, 10.9)		3.73 *dd* (6.2, 12.2)
6b		4.76 *brd* (10.9)	3.82 *d* (11.3)	3.97 *dd* (2.2, 12.2)
Glucose B				
1	101.8	5.24 *d* (8.0)	5.24 *d* (8.0)	5.19 *d* (7.4)
2	74.2	3.73 *dd* (8.0, 9.2)	3.53 *m*	3.54 *m*
3	77.6	3.59 *m*		3.52–3.55
4	74.3	3.37 *dd* (9.2, 9.5)	3.15–3.65	3.41 *brt* (9.5)
5	74.7	4.02 *ddd* (2.5, 9.5, 11.9)		3.58–3.65
6a	66.0	4.34 *dd* (9.5, 11.9)	4.25 *m*	3.72 *dd* (6.1, 12.2)
6b		4.73 *dd* (1.9, 11.9)	4.62 *d* (11.3)	3.94 *dd* (1.9, 12.2)
Glucose C				
1	101.8	4.91 *d* (7.9)	4.92 *m d* (8.0)	
2	73.9	3.60 *dd* (7.9, 8.7)	3.46 *m*	

(*continued on next page*)

Table 2 (*continued*)

	LBA **1**[a]		Demalonyl LBA **1**[b]	Deacyl LBA **1**[a]
	^{13}C δ (ppm)	^{1}H δ (ppm)	^{1}H δ (ppm)	^{1}H δ (ppm)
3	77.4	3.47 *dd* (8.7, 9.2)		
4	71.1	3.48 *dd* (9.2, 9.5)	3.15–3.65	
5	75.9	3.57 *m*		
6a	66.0	3.94 *m*	3.95 *m*	
6b		4.76 *brd* (10.9)	4.62 *brd* (11.0)	
Glucose D				
1	102.2	4.53 *d* (7.7)	4.49 *d* (7.4)	
2	74.7	3.44 *dd* (7.7, 9.6)	3.32 *m*	
3	77.1	3.71 *dd* (7.7, 9.6)		
4	73.1	3.63 *m*	3.15–3.65	
5	75.9	3.46 *m*		
6a	61.9	3.72 *m*		
6b		3.94 *m*	3.98 *m*	

TMS as an internal standards.
[a] $CD_3OD/CF_3CO_2D = 9:1$.
[b] $CD_3OD/DMSO\text{-}d_6/CF_3CO_2D = 4:2:1$.

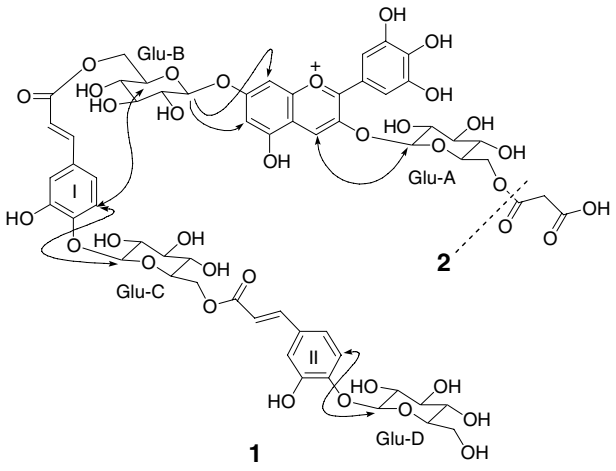

Fig. 1. **1**: Leschenaultia blue anthocyanin **1** (LBA **1**). **2**: Demalonyl LBA **1** Observed NOE's are indicated by arrows.

were assigned by the analysis of its 2D COSY spectrum as shown in Table 2. The ^{1}H NMR spectrum exhibited four olefinic proton signals of caffeic acid with large coupling constants ($J = 15.9$ Hz and 15.9 Hz). These two caffeic acids were determined to be in the *trans* configuration. The chemical shifts of the sugar moieties were observed in the region of δ 5.39 – 3.37, where the four anomeric protons resonated at δ 5.39 (*d*, $J = 7.7$ Hz, Glu A-H1), δ 5.24 (*d*, $J = 8.0$ Hz, Glu B-H1), δ 4.91 (*d*, $J = 7.9$ Hz, Glu C-H1), and δ 4.53 (*d*, $J = 7.7$ Hz, Glu D-H1), respectively. Based on the observed coupling constants (Table 2), these four sugars were assumed to be in the β-pyranose forms. By analysis of the 2D COSY spectrum, six characteristic proton signals, shifted to lower magnetic field, were assigned as methylene protons of Glu A (δ 4.52 and 4.76, H-6a and -6b), Glu B (δ 4.34 and 4.73, H-6a and -6b) and Glu C (δ 3.94 and 4.76, H-6a and -6b), respectively. These results indicated that the three OH-6 groups of Glu A, B and C were acylated with two molecules of caffeic acid and one molecule of malonic acid, respectively. By analysis of the NOESY spectrum, long range NOEs between H-1 of Glu A and H-4 (δ 8.52) of delphinidin, H-1 of Glu B and H-8 (δ 6.98) of delphinidin, H-1 of Glu C and H-5 (δ 6.66) of caffeic acid I, and H-1 of Glu D and H-5 (δ 6.53) of caffeic acid II were observed (Fig. 1). These data indicated that the OH-3 and OH-7 groups of delphinidin were glycosylated with Glu A and Glu B, respectively, while the OH-4s of caffeic acid I and II were glycosylated with Glu C and Glu D, respectively. Furthermore, rather weak long range NOEs were observed between H-5 (δ 4.02) of Glu B and H-5 (δ 6.66) of caffeic acid I, and also between H-5 of Glu B and H-6 (δ 6.79) of caffeic acid I. These results supported the view that the OH-6 of Glu B was esterified with the COOH group of caffeic acid I (Fig.1). Unfortunately, the linkages between Glu A and malonic acid, as well as between Glu C and caffeic acid II, could not be confirmed by IDNOE, NOESY and HMBC experiments. In order to determine the bonding position of malonic acid in LBA **1**, demalonyl LBA **1** was prepared according to the procedure described previously (Saito et al., 1994). The structure determination of demalonyl LBA **1** is described in the following Section 2.3. The results revealed that OH-6 of Glu A was not acylated in demalonyl LBA **1**, but it was acylated with malonic acid in LBA **1**. Then, OH-6 of Glu C is acylated with caffeic acid II, and Glu D is terminal of this long substituent group in the molecule. Consequently, the structure of LBA **1** was elucidated to be delphinidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(4-*O*-(6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranoside], which is a new anthocyanin in plants.

2.3. Demalonyl LBA *1*

Hydrolysis of LBA **1** (ca. 15 mg) with 1 N HCl at room temperature for 18 days, followed by separation with HPLC

gave its demalonylanthocyanin (demalonyl LBA **1**, ca. 7 mg). The chromatographic and spectroscopic data of demalonyl LBA **1** are shown in Table 1. The FAB mass spectrum of demalonyl LBA **1** exhibited a molecular ion at 1275 *m*/*z* in agreement with the mass $C_{57}H_{63}O_{33}$ (1275.324). The elemental components were also confirmed by measuring its high-resolution FABMS; calc. for $C_{57}H_{63}O_{33}$: 1275.3252. Found: 1275.3264. The ^{1}H NMR spectrum of demalonyl LBA **1** showed the presence of one molecule of delphinidin, two molecules of caffeic acid and four molecules of glucose (Table 2). By analyses of its ^{1}H NMR spectrum, as well as its 2D COSY and NOESY spectra, it was revealed that the proton chemical shifts of demalonyl LBA **1** were almost in agreement with those of LBA **1** except for the proton signals of Glu A and malonic acid moieties (Table 2). In particular, upfield shifts of methylene protons (δ 3.82, 3.65–3.15 H-6b and 6a) of Glu A in demalonyl LBA **1** were observed in comparison to those (δ 4.52, 4.76 H-6a and 6b) of LBA **1**. Therefore, the OH-6 of Glu A in demalonyl LBA **1** was free of malonic acid. Other proton signals of demalonyl LBA **1** were assigned in the process described in LBA **1** (Table 2). Therefore, demalonyl LBA **1** was determined to be delphinidin 3-*O*-[β-D-glucopyranoside]-7-*O*-[6-*O*-(4-*O*-(6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranoside], which has not been described before.

2.4. LBA 2

The chromatographic and spectroscopic properties of LBA **2** are shown in Table 1. As mentioned before, LBA **2** gave delphinidin, glucose, caffeic acid, *p*-coumaric acid, and malonic acid by acid hydrolysis. Furthermore, delphinidin 3,7-diglucoside was obtained from the alkaline hydrolysate as its deacyl anthocyanin. The FAB mass spectrum of LBA **2** gave its molecular ion $[M]^+$ at 1345 *m*/*z* (calcd for $C_{60}H_{65}O_{35}$ 1345.330) and at 1259 *m*/*z* (M^+-malonyl), indicating that LBA **2** is composed of delphinidin with four molecules of glucose and one molecule each of caffeic acid, *p*-coumaric acid and malonic acid. Because of the small amounts obtained, no further structural studies of LBA **2** could be carried out. However, since both LBA **1** and **2** coexist in the flower petals, it is very likely that the substitution pattern of LBA **2** would be similar to that of LBA **1**. Based on this assumption and the above chromatographic and spectroscopic data, a possible structure of LBA **2** would be either delphinidin 3-(malonylglucoside)-7-[(glucosyl-*p*-coumaroyl)(glucosylcaffeoyl)-glucoside] or delphinidin 3-(malonylglucoside)-7-[(glucosylcaffeoyl)(glucosyl-*p*-coumaroyl)-glucoside], either of which is a new anthocyanin in plants.

3. Concluding remarks

To date, the distribution of 7-polyacylated anthocyanins, which are acylated with more than two molecules of aromatic acids at 7-glycosyl residues, has been reported only in the three families of the Ranunculaceae, Campanulaceae, and Compositae (Honda and Saito, 2002). From a chemotaxonomical point of view, since Leschenaultia blue anthocyanins LBAs **1** and **2** were found to be 7-polyacylated anthocyanins, the family of Goodeniaceae, to which Leschenaultia belongs, should be added to the above three families. Furthermore, the structure of LBA **1** is similar to platyconin isolated from the blue-violet flowers of *Platycodon grandiflorum* in the family of Campanulaceae (Saito et al., 1971; Goto et al., 1983). The difference in the structures is only at the residue of 3-malonylglucoside for LBA **1** and of 3-rutinoside for platyconin. These results may be chemotaxonomically important as they suggest that the Goodeniaceae are closely related to the Campanulaceae. According to DNA sequence studies, this is apparently also the case (Angiosperm Phylogeny Group, 1998).

The flowers of *Leschenaultia* cv. Violet Lena exhibited a blue color (Blue 99C by RHS Colour Chart) with chromaticity value $b^*/a^* = -1.95$. As shown in Fig. 2, the visible absorption curve of intact flower petals gave λ_{max} at 542, 579, and 630 nm and was similar to that of *Platycodon grandiflorum* having λ_{max} at 538, 573, and 615 nm (Saito, 1967). This indicates that the blue flower color of *L.* cv. Violet Lena is very likely due to the formation of intramolecular co-pigmentation between delphinidin and hydroxycinnamic acids (Honda and Saito, 2002). However, the visible absorption spectra of LBA **1** showed λ_{max} at 537–540, 573–576, and 613–616 nm in the phosphate–citrate buffer (MacIlvains) at pH 6 and 7 (Fig. 2) which were not the same as the λ_{max} observed for fresh flower petals (Fig. 2). The difference in the λ_{max} between *L.* cv. Violet Lena and LBA **1** in buffer solutions strongly indicates that an additional bluing factor (e.g. intermolecular co-pigmentation) may be involved in the blue Leschenaultia flowers as is the case for the blue flower of *Ceanothus papillosus* (Bloor, 1997).

4. Experimentals

4.1. General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using eight 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, and BAW (4:1:5), 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 acids with UV light and aniline hydrogen phthalate spray reagent (Harborne, 1984).

Analytical HPLC was performed on a LC 10A system (Shimadzu), using a Waters C18 (4.6 $\phi \times 250$ mm) column at 40 °C with a flow rate of 1 mL/min and monitoring at 530 nm. The eluant was applied as a linear gradient elution for 40 min from 20% to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in

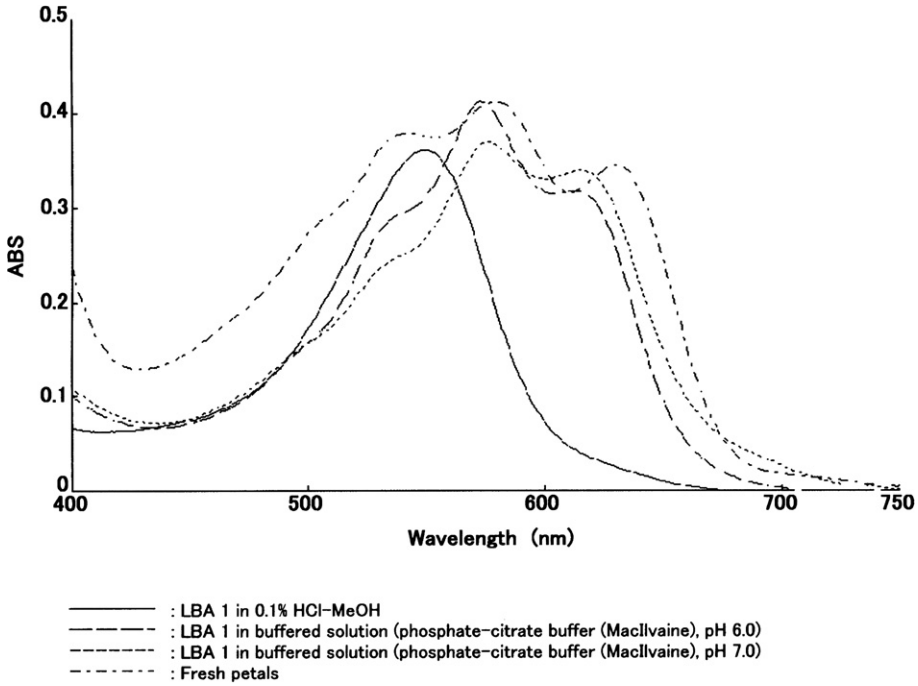

Fig. 2. Visible absorption spectra of LBA 1 and blue petal-tissue of *Leschenaultia*.

H_2O). UV–Vis spectra were recorded on a MPS-2400 (Shimadzu) in 0.1% HCl–MeOH (from 200 to 700 nm). Spectral absorption of the blue flower was directly measured on the intact petals using a recording spectrophotometer operated as a double-beam instrument (Type MPS-2400) (Saito, 1967; Yokoi and Saito, 1973).

High resolution FAB mass (FABMS) spectra were obtained in the positive ion mode using the magic bullet (5:1 mixture of dithiothreitol and dithioerythritol) as a matrix. NMR spectra were determined at 500 MHz for ^1H spectra and at 125.78 MHz for ^{13}C spectra in CF_3COOD–CD_3OD (1:9), DCl–DMSO-d_6 (1:9) and DCl–CD_3OD (1:9). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants are in Hz.

4.2. Plant materials

The plants of *Leschenaultia* cv. Violet Lena were purchased from Daiichi-engei (Tokyo), and grown in greenhouses in Hokkaido, Japan (Department of Agro-Environmental Science, Hokkaido Junior College, Takushoku University, Fukagawa, Hokkaido). Flowers were collected during winter seasons in Japan. The flowers exhibited a blue color (Blue 99C by Royal Horticultural Society Color Chart and its chromaticity value b^* (−41.7)/a^* (21.3) = −1.95 by Minolta CM-2002); they were dried at 40 °C overnight and stored at −10 °C in the refrigerator until needed.

4.3. Isolation of anthocyanins

Dried flowers (ca. 100 g) of *Leschenaultia* cv. Violet Lena were immersed in 5% HOAc–H_2O (2 L) at room temperature overnight. The extract was passed through a Diaion HP-20 resin (Mitsubishi Chemical's Ion Exchange Resins) column (90 $\phi \times 150$ mm), on which acylated anthocyanins were adsorbed. Then, the column was thoroughly washed with H_2O (2000 mL) and eluted with 5% HOAc–MeOH (500 mL) to recover the anthocyanins. After concentration, the eluates were separated and purified with paper chromatography (PC) using BAW. The separated pigments were further purified with prep. HPLC. Prep. HPLC was performed on a Waters C18 (19 $\phi \times 150$ mm) column at 40 °C with a flow rate of 4 mL/min and monitoring at 530 nm for anthocyanins. The solvent used was as follows: a linear gradient elution for 15 min from 50% to 60% solvent B in solvent A. Fractions were transferred to a Diaion HP-20 column, on which pigments were adsorbed. Anthocyanin pigments were eluted with 5% HOAc–MeOH followed by addition of excess Et_2O, and then dried. The entire process was repeated for a second 100 g dried flowers to yield a total of dark blue-violet color powders, LBA **1** (ca. 30 mg) and LBA **2** (ca. 3 mg).

4.4. Analyses of anthocyanins

The identification of anthocyanins was carried out by standard procedures involving deacylation with acid, and hydrolysis with alkaline and acid (Harborne, 1984; Terahara et al., 1990; Saito and Harborne, 1992). The data of TLC (R_f values), HPLC (R_t-min), UV–VIS (λ_{max}), and FABMS spectra are shown in Table 1 and are also described in Sections 4.4.1–4.4.3.

Leschenaultia blue anthocyanin 1 (**1**). Dark blue-violet powders; for UV–VIS, see Table 1; for TLC, see Table 1; for ^1H and ^{13}C NMR spectra, see Table 2; HR-FABMS calc. for $C_{60}H_{65}O_{36}$: 1361.3256. Found: 1361.3285.

Leschenaultia blue anthocyanin 2 (**2**). Dark blue-violet powders; UV–VIS: λ_{max} 548, (307), 284 nm, E_{acyl}/E_{max} (%) = 198, E_{440}/E_{max} (%) = 19, $+AlCl_3$ = +shift, TLC: R_f-values BAW 0.05, BuHCl 0.00, 1% HCl 0.04, AHW 0.20, HPLC: R_t (min) 25.0; FABMS calc. for $C_{60}H_{65}O_{35}$: 1345.331. Found *m*/*z* 1345.5.

4.4.1. Deacylanthocyanin, caffeic acid, and 4-O-glucosyl-caffeic acid

Acid hydrolysis of LBAs **1** and **2** (ca. 1 mg each) was carried out with 2 N HCl (10 mL) at 100 °C for 1 h, with the hydrolysates analyzed using of TLC and HPLC relative to authentic standard compounds. LBA **1** (ca. 15 mg) was dissolved in 2 N NaOH (10 ml) under a degassed syringe and allowed to stand for 15 min. The solution was next acidified with 2 N HCl and evaporated in vacuo to dryness. The residue was dissolved in 1% HCl–MeOH and applied on TLC (BAW) to yields deacylanthocyanin LBA1 (ca. 5 mg), caffeic acid (ca. 1 mg), and 4-*O*-glucosylcaffeic acid (ca. 3 mg). However, malonic acid could not be obtained because of its small amount. Caffeic acid was identified by the analyses of TLC and HPLC in comparison with authentic caffeic acid. In addition, alkaline hydrolysis of LBA **2** (ca. 1 mg) was carried out by a similar process to that for LBA **1** and only delphinidin 3,7-diglucoside was detected in its hydrolysate due to the small amount of LBA **2** available. The characteristic properties of deacylanthocyanin are shown in Table 1.

4.4.2. 4-O-Glucopyranosyl-trans-caffeic acid

UV: λ_{max} 316, 288 (MeOH), TLC: R_f-values BAW 0.52, BuHCl 0.46, 1% HCl 0.33 and 0.72, AHW 0.55 and 0.99, HPLC: R_t (min) 8.3, FABMS *m*/*z* 343 $[M + 1]^+$ (calc. for $C_{15}H_{18}O_9$, 342.095), ^1H NMR (500 MHz, DCl–DMSO-d_6 (1:9), standard TMS): δ caffeic acid: 7.14 (*d*, $J = 1.9$ Hz, H-2), 7.11 (*d*, $J = 8.2$ Hz, H-5), 7.08 (*dd*, $J = 1.9$ and 8.2 Hz, H-6), 7.46 (*d*, $J = 15.9$ Hz, H-α), 6.32 (*d*. $J = 15.9$ Hz, H-β). Glucose: 4.78 (*d*, $J = 7.3$ Hz, H-1), 3.29 (*dd*, $J = 7.3$ and 8.9 Hz, H-2), 3.17, (*t*, $J = 8.9$ Hz, H-3), 3.45–3.27 (H-4 and H-5), 3.47 (*m*, H-6a), 3.72 (*d*, $J = 11.6$ Hz, H-6b).

4.4.3. Demalonyl LBA *1*

LBA **1** (ca. 15 mg) was dissolved in 1 N HCl–H_2O solution (20 ml) and allowed to stand at room temperature for 18 days. At this point, demalonylated LBA **1** was formed in its solution. Demalonylated LBA **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 concentrated residues were 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 LBA **1** powder (ca. 7 mg).

For UV–VIS, see Table 1; for TLC, see Table 1; for ^1H spectrum, see Table 2; HR-FABMS calc. for $C_{57}H_{63}O_{33}$: 1275.3252. Found: 1275.3264.

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