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ACYLATED CYANIDIN GLYCOSIDES IN THE PURPLE–RED FLOWERS OF *BLETILLA STRIATA*

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Key Word Index—*Bletilla striata*; Orchidaceae; purple–red flower colour; acylated anthocyanins; cyanidin 3,7,3′-triglucoside; malonic, *p*-coumaric and caffeic acids; glucosyloxycinnamic acids.

Abstract—Eight acylated anthocyanins were isolated from the purple–red flowers of *Bletilla striata* as major anthocyanins. These pigments were based on cyanidin 3,7,3′-triglucoside as their deacylanthocyanins, and were acylated with malonic, *p*-coumaric and caffeic acids and/or glucosylated hydroxycinnamic acids. Four structures were determined by spectral and chemical methods: cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-4-*O*-(β-D-glucopyranosyl)-*p*-coumaryl)-β-D-glucopyranosyl)-*p*-coumaryl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-*p*-coumaryl)-β-D-glucopyranoside]; the demalonyl derivative; cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-4-*O*-(β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-caffeyl)-β-D-glucopyranoside]; and the demalonyl derivative.

INTRODUCTION

Bletilla striata is an ornamental orchid which is mainly grown in Japan and south China. The flowers of these plants show rich mauve–pink and white. In the course of the investigation of flower colour variation due to acylated anthocyanins in orchid plants, we found two novel acylated cyanidin glycosides in *Dendrobium* [1] and × *Laeliocattleya* Mini Purple [2]. In this paper we report the isolation and structure elucidation of acylated cyanidin glycosides in the purple–red flowers of *B. striata*.

RESULTS AND DISCUSSION

In a survey of the flower parts of *B. striata* and its cultivars by HPLC analysis, eight anthocyanins (**1**–**8**) were observed (Table 1). These anthocyanins were isolated from the purple–red flowers of *B. striata* and analysed by standard procedures [1–4]. Their R_f values, R_t (min) and spectral data are shown in Table 2. Acid hydrolysis of all eight anthocyanis gave cyanidin, glucose and hydroxycinnamic acids. Among the components of acylating acids, caffeic acid was detected in the hydrolysis products of pigments **3**–**8**, and *p*-coumaric acid was detected in those of **1**, **2**, and **5**–**8** by acid and alkaline hydrolysis. Malonic acid was also observed in the products of **1**, **3**, **5** and **7** by alkaline hydrolysis, but were not detected in those of **2**, **4**, **6** and **8**. Alkaline hydrolysis yielded only one deacylanthocyanin, whose structure was elucidated to be cyanidin 3,7,3′-triglucoside by direct comparison with authentic samples of deacyl *Dendrobium* and × *Laeliocattleya* anthocyanins [1, 2]. Furthermore, this structure was confirmed by analysis of the FAB mass and ^{1}H NMR spectra (Tables 3 and 4).

The measurements of FAB mass and ^{1}H NMR spectra of these eight acylated anthocyanins led to the determination of the molecular ratios of chemical composition (aglycone, sugar and acid) as shown in Table 3. Among these eight pigments, the structures of four pigments (**1**–**4**) were successfully determined, but the other pigments (**5**–**8**) remain unknown because of the difficulty of purification and also the small amounts present.

Bletilla *anthocyanin* (**1**)

The FAB mass spectrum of **1** gave its molecular ion at 1621 m/z $[M]^+$, in good agreement with the mass calculated for $C_{75}H_{81}O_{40}$ (1621,430), cyanidin with five molecules of glucose, three of *p*-coumaric acid and one of malonic acid (Table 3). This result was confirmed by the ^{1}H NMR spectrum (Table 4). The proton signals of sugar parts were observed in the region of δ5.13–3.10. Signals of five anomeric protons appeared at δ4.95 (*d*, J = 6.8 Hz, Glc A), δ5.13 (*d*, J = 7.3 Hz, Glc B), δ4.99 (*d*, J = 7.0 Hz, Glc C), δ4.82 (*d*, J = 7.0 Hz, Glc D) and δ4.80 (*d*, J = 7.3 Hz, Glc E) and all observed vicinal

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Table 1. Distribution of anthocyanins in the flower extracts of *Bletilla striata* and its cultivars

Plant	Flower*	Floral† colour	*h* a^{*}_{ab}‡	Anthocyanin (%)§ 4	3	8	7	6	5	2	1
B. striata	P	PV81B	−0.65	12.0	30.2	6.3	21.0	5.6	8.5	2.4	1.9
	L	P78A	0.61	1.8	6.3	—	8.4	4.2	14.9	4.1	31.1
B. striata cv. Marginata	P	PV80B	−0.72	6.5	25.9	5.2	26.3	5.7	13.1	4.7	2.3
	L	P78A	−0.65	3.8	10.0	1.9	12.6	5.2	16.4	4.1	24.5
B. striata cv. Kutibenis	P	P75C	−0.80	3.6	13.3	4.1	21.9	4.0	9.9	2.0	4.2
	L	P78A	−0.75	1.8	4.9	—	13.4	5.5	19.6	5.1	22.0
B. striata cv. Gebina	P	P75C	−0.70	5.2	9.9	4.8	16.7	3.6	4.3	4.8	4.9
	L	P75C	−0.73	6.9	9.4	2.3	14.7	6.6	11.4	4.0	8.0
B. striata	P	W155D	−0.59	4.3	16.2	5.4	10.0	2.0	3.6	1.9	1.0
cv. Albomarginata¶	L	W155D	−0.18	3.3	6.5	4.8	12.1	5.6	22.1	2.6	8.7

*P: perianth; L: labellum.

†R.H.S. colour chart.

‡Hunter values (hue).

§Percentage of total absorbance of all detected anthocyanins at 530 nm in HPLC analysis. Anthocyanin No. and HPLC condition are the same Table 2, and pigments are arranged in turn as R_f.

¶Slightly pigmented.

coupling constants of the five glucose moieties (A–E in Fig. 1) were 6.8–9.5 Hz. Therefore, all the glucose units must be β-D-glucopyranosides. The eight characteristic protons at δ4.25, 4.52 (Glc A), δ4.16, 4.70 (Glc B), δ4.00, 4.87 (Glc C) and δ4.13, 4.45 (Glc D) were assigned to be the C-6 methylenes of glucose units (A–D) by analysis of the ^{1}H–^{1}H COSY and negative nuclear Overhauser effect difference (DIFNOE) spectra [5, 6], indicating that the three *p*-coumaric acids and one malonic acid are each attached to these four OH-6 groups of glucose units (A–D), respectively.

Extensive analysis of the ^{1}H–^{1}H COSY spectrum of **1** made it possible to correlate most of the protons in the molecule, but unambiguous assignments of all protons of glucose units were unsuccessful due to heavily overlapping signals (Table 4). The application of the DIFNOE method made it possible to determine the linkages and/or the position of attachments of glucose, *p*-coumaric acid and malonic acid units in the molecule. Thus, the three glucoses (A, B and C) are attached to the OH-3, OH-7 and OH-3′ of cyanidin, respectively, as deduced from the observations of NOEs for anomeric protons of Glc A–C, as shown in Fig. 1 [5, 6]. Irradiation of H-1 of Glc D gave NOEs to H-3, 5 of *p*-coumaric acid-II, and also rather week NOEs to H-2, -6, -α and -β of *p*-coumaric acid-II. Thus, Glc D was determined to be attached to the OH-4 of *p*-coumaric acid-II by a glycosidic bond. Similar irradiation of H-1 of Glc E gave a DIFNOE spectrum in which strong NOEs were observed at H-3 and -5 signals of *p*-coumaric acid-III. Therefore, Glc E is determined to be attached to the OH-4 position of *p*-coumaric acid-III by a glycosidic bond. As irradiation of H-3 and -5 of *p*-coumaric acid-I gave no NOEs to these anomeric proton signals of Glc A–E, the OH-4 of *p*-coumaric acid-I is free from the glycosidic bond. By irradiation of H-2′ of cyanidin rather weak NOEs were observed at H-α and -β of *p*-coumaric acid-II as well as H-1, -2 and -4 of Glc C. This result indicated that Glc C was acylated with *p*-coumaric acid-II. Similarly, irradiation of H-8 of cyanidin gave the strong NOE at H-1 of Glc B and also rather weak NOEs at H-α and -β of *p*-coumaric acid-I, supporting the presence of the linkage between Glc B and *p*-coumaric acid-I. In order to determine the position of attachment of malonic acid, the demalonyl pigment of **1** was prepared by treatment with 1 N HCl–H_2O according to the previous procedure [1]. By the analysis of chemical and spectral properties this demalonylated pigment is identical to *Bletilla* anthocyanin **2** (shown in Tables 2–4). By analysis of the ^{1}H NMR and ^{1}H–^{1}H COSY spectra of demalonyl **1**, it was revealed that the proton chemical shifts of demalonyl **1** were in good agreement with those of **1** except for the proton signals of Glc A and malonic acid moieties (Table 4).

In the ^{1}H NMR spectrum of demalonyl **1** the upfield shift of methylene proton signals of Glc A were observed from δ4.25 and 4.52 to δ3.90–3.10 in comparison with those of **1**, indicating that the OH-6 of Glc A of demalonyl **1** was free from malonyl groups. Therefore, it was confirmed that malonic acid is bonded with Glc A at OH-6, and also the OH-6 of Glc D is bonded with *p*-coumaric acid III in **1**. Thus, **1** is cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-*p*-coumaryl)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-4-*O*-(β-D-glucopyranosyl)-*p*-coumaryl)-β-D-glucopyranosyl)-*p*-coumaryl)-β-D-glucopyranoside], which is a new anthocyanin [7, 8]. This pigment has three similar acylated side chains at the OH-3, -7 and -3′ of cyanidin to those of × *Laeliocattleya* anthocyanin [2].

Bletilla *anthocyanin* (2)

The FAB mass spectrum of **2** gave its molecular ion at 1535 *m/z*, in good agreement with the mass calculated for

Table 2. Chromatographic and spectral properties of anthocyanins from the purple-red flowers of *Bletilla striata*

Anthocyanin*	R_f values (×100)				Spectral data in 0.1% HCL–MeOH				R_t	FAB–MS
	BAW	BuH	1%HCl	AHW	λ_{max}(nm)	E_{UV}/E_{max} (%)	E_{acyl}/E_{max} (%)	E_{440}/E_{max} (%)	$AlCl_3$	(min)	$[M]^+$
1	30	6	16	44	537, 306, 290	156	141	27	0	17.7	1621
2	35	10	12	39	536, 306, 289	172	151	28	0	16.6	1535
3	22	6	10	28	537, 320, 288	162	129	29	0	12.4	1669
4	24	8	5	25	533, 320, 287	197	147	29	0	11.6	1583
5	27	4	18	45	538, 307, 294	139	135	33	0	15.5	1637
6	32	9	11	32	535, 307, 287	222	187	28	0	14.5	1551
7	22	7	14	39	538, 309, 288	205	168	32	0	13.9	1653
8	26	11	8	29	538, 309, 286	274	219	29	0	13.3	1567
Deacyl anthocyanin (**1**–**8**)	17	2	52	66	513, 280	77	23	42	0	3.7	773

*For pigment key, see Fig. 1 and Table 3. Deacyl *Bletilla* anthocyanin = cyanidin 3,7,3′-triglucoside.
For key to abbreviations, see Experimental.

$C_{72}H_{79}O_{37}$ (1535.430). This value is identical with the mass of the demalonylated pigment **1**, which was composed of cyanidin with five molecules of glucose and three molecules of *p*-coumaric acid, as shown in Table 3. Also, the R_f values, R_t (min) and spectral properties were in good agreement with those of the demalonylated pigment **1** (Table 2). The detailed structure was elucidated by analysis of the ^{1}HNMR spectra including ^{1}H–^{1}H COSY and DIFNOE spectral methods as described in the structure determination of **1**. The chemical shifts of proton of **2** are identical with those of demalonyl **1**, and also those of **1** except proton signals of Glc A and malonic acid units in which the methylene proton signals of **2** were shifted to the high-magnetic field from δ4.25, 4.52 (Glc A) to δ3.90–3.10. These results indicate that Glc A of **2** is free from malonic acid. The assigned protons of **2** are shown in Table 4. The structure of **2** was determined to be demalonyl **1**, which is a new anthocyanin in plants [7, 8].

Bletilla *anthocyanin* (3)

The FAB mass spectrum of **3** gave a molecular ion $[M]^+$ at 1669 *m/z*, in good agreement with the mass calculated for $C_{75}H_{81}O_{43}$ (1669.415), which was composed of cyanidin with five molecules of glucose, three molecules of caffeic acid and one molecule of malonic acid, as shown in Table 3. The detailed chemical structure was elucidated by ^{1}H NMR including ^{1}H–^{1}H COSY and DIFNOE spectral methods. Six proton signals of cyanidin moiety were observed as shown in Table 4. In caffeic acid moieties, three pairs of doublet signals (δ7.08, 5.79; δ7.43, 6.31; δ7.14, 6.27) with large coupling constants (J = 15.8, 15.8 and 15.0 Hz) indicated the presence of *trans* olefinic protons of caffeic acids (I–III). Also, three pairs of three aromatic protons were assigned to the ring protons of caffeic acids I–III, as shown in Table 4. The proton signals of five sugar moieties were assigned as shown in Table 4. These five glucose units were determined as β-D-glucopyranose forms. Eight characteristic proton signals corresponding to four methylene groups of glucoses were shifted to the lower magnetic field at δ4.37, 4.57 (Glc A), δ4.17, 4.78 (Glc B), δ4.04, 4.98 (Glc C) and δ4.14, 4.50 (Glc D), indicating that the four OH-6 of these glucose units are acylated with caffeic acids I–III and malonic acid, respectively.

In order to determine the attachments and positions of glucose and acyl units, DIFNOE spectra of **3** were measured as for the pigment **1** (Fig. 1). The observation of negative NOEs showed that Glc A is bonded at OH-3 of cyanidin, Glc B is attached at OH-7 of cyanidin, and Glc C is attached at OH-3′ of cyanidin through glycosidic bonds. These results were identical with those of pigments **1** and **2**. Irradiation of H-1 of Glc D gave a DIFNOE spectrum in which the NOE for a doublet signal of H-5 of caffeic acid-II was observed. Therefore, Glc D was attached at OH-4 of caffeic acid-II. Glc E was determined to be fixed at OH-4 of caffeic acid-III, because of the presence of NOEs between H-1 of Glc E and H-5 of caffeic acid-III. By irradiation at H-1 of Glc C rather

Table 3. The estimated molecular formulas of acylated anthocyanins from *Bletilla striata* and their molecular ratios of chemical composition based on FAB mass and ^{1}H NMR data

		Based on FAB–MS*						Based on ^{1}H NMR†			
Anthocyanin	$[M]^+$	Mf	Cy:	Glc:	*p*-C:	Caf:	Mal	Cy:	Glc:	*p*-C:	Caf
1	1621	$C_{75}H_{81}O_{40}$	1	5	3		1	1	5	3	
2	1535	$C_{72}H_{79}O_{37}$	1	5	3			1	5	3	
3	1669	$C_{75}H_{81}O_{43}$	1	5		3	1	1	5		3
4	1583	$C_{72}H_{79}O_{40}$	1	5		3		1	5		3
5	1637	$C_{75}H_{81}O_{41}$	1	5	2	1	1	1	5	2	1
6	1551	$C_{72}H_{79}O_{38}$	1	5	2	1		1	5	2	1
7	1653	$C_{75}H_{81}O_{42}$	1	5	1	2	1	1	5	1	2
8	1567	$C_{72}H_{79}O_{39}$	1	5	1	2		1	5	1	2
Deacyl anthocyanin	773	$C_{33}H_{41}O_{21}$	1	3				1	3		

*$[M]^+$ and Mf = molecular ion mass values, and estimated molecular formulae as flavylium forms of anthocyanins isolated from *B. striata* based on FAB mass data, respectively. Cy:Glc:*P*-C:Caf:Mal = molecular numbers of their components; Cy = cyanidin, Glc = glucose, *p*-C = *p*-coumaric acid, Caf = caffeic acid, Mal = malonic acid.

†Molecular numbers were based on the integrated intensities of proton signals such as cyanidin = H-4, glucose = H-1, *p*-coumaric acid and caffeic acid = olefinic proton (H-α). Each integrated intensity of proton signal was normalized in such a way that cyanidin H-4 is 1.

Table 4. ^{1}H NMR data for *Bletilla striata* anthocyanins using DMSO-d_6 with CF_3COOD

H	**1**	**2** (demalonyl **1**)	**3**	**4** (demalonyl **3**)	Deacyl pigment
Cyanidin*					
4	8.36 (*s*)	8.47 (*s*)	8.51 (*s*)	8.61 (*s*)	9.04 (*s*)
6	6.68 (*br s*)	6.67 (*br s*)	6.67 (*br s*)	6.66 (*br s*)	6.85 (*br s*)
8	6.91 (*br s*)	6.91 (*br s*)	6.90 (*br s*)	6.91 (*br s*)	7.23 (*br s*)
2′	7.84 (*br s*)	7.88 (*br s*)	7.83 (*br s*)	7.87 (*br s*)	8.27 (*d* 2.0)
5′	7.14 (*d*, 7.8)	7.10 (*d*, 6.8)	7.12 (*d*, 8.6)	7.13 (*d*, 8.9)	7.16 (*d*, 9.2)
6′	8.56 (*br d*, 7.8)	8.57 (*br d*, 6.8)	8.60 (*br d*, 8.6)	8.60 (*br d*, 8.9)	8.69 (*dd*, 2.0, 9.2)
p-Coumaric or caffeic acid*†					
(I)					
2,6 or 2	6.69 (*d*, 7.8)	6.72 (*d*, 7.8)	7.00 (*br s*)	7.12 (*br s*)	
3,5 or 5	6.55 (*d*, 7.8)	6.56 (*d*, 7.8)	6.63 (*d*, 8.1)	6.63 (*d*, 8.2)	
6	—	—	6.23 (*d*, 8.1)	6.25 (*d*, 8.2)	
α	5.89 (*d*, 16.6)	5.88 (*d*, 16.1)	5.79 (*d*, 15.8)	5.80 (*d*, 15.7)	
β	7.07 (*d*, 16.6)	7.07 (*d*, 16.1)	7.08 (*d*, 15.8)	7.09 (*d*, 15.7)	
(II)					
2,6 or 2	7.06 (*d*, 7.8)	7.10 (*d*, 6.8)	6.86 (*br s*)	6.88 (*br s*)	
3,5 or 5	6.62 (*d*, 7.8)	6.68 (*d*, 6.8)	6.59 (*d*, 8.6)	6.67 (*d*, 8.2)	
6	—	—	6.33 (*d*, 8.6)	6.32 (*d*, 8.2)	
α	6.30 (*d*, 16.1)	6.31 (*d*, 16.1)	6.31 (*d*, 15.8)	6.31 (*d*, 15.7)	
β	7.46 (*d*, 16.1)	7.47 (*d*, 16.1)	7.43 (*d*, 15.8)	7.42 (*d*, 15.7)	
(III)					
2,6 or 2	7.26 (*d*, 8.3)	7.37 (*d*, 7.8)	6.34 (*br s*)	6.31 (*br s*)	
3,5 or 5	6.82 (*d*, 8.3)	6.84 (*d*, 7.8)	6.84 (*d*, 8.5)	6.90 (*d*, 8.2)	
6	—	—	6.66 (*d*, 8.5)	6. 80 (*d*, 8.2)	
α	6.25 (*d*, 16.6)	6.35 (*d*, 16.1)	6.27 (*d*, 15.0)	6.39 (*d*, 15.7)	
β	7.26 (*d*, 16.6)	7.32 (*d*, 16.1)	7.14 (*d*, 15.0)	7.32 (*d*, 15.7)	
Glucose*†					
(A)					
1	4.95 (*d*, 6.8)	4.96 (*d*, 7.8)	5.06 (*d*, 8.1)	5.06 (*d*, 7.5)	5.45 (*d*, 7.7)
2	3.59	3.60	3.63	3.63	3.77
3	3.49		3.54		
4	3.35		3.37		
5	3.79	3.90–3.10	3.90	3.90–3.20	3.90–3.10
6a	4.25		4.37		
6b	4.52		4.57		

Table 4. *Continued*

H	1	2 (demalonyl 1)	3	4 (demalonyl 3)	Deacyl pigment
(B)					
1	5.13 (*d*, 7.3)	5.12 (*d*, 7.0)	5.11 (*d*, 7.3)	5.12 (*d*, 6.7)	5.25 (*d*, 7.7)
2	3.45	3.46	3.49	3.52	3.38
3	3.32	3.33	3.37	3.37	
4	3.33	3.25	3.31	3.30	
5	3.72	3.78	3.79	3.75	3.90–3.10
6a	4.16	4.20	4.17	4.19	
6b	4.70	4.68	4.78	4.75	
(C)					
1	4.99 (*d*, 7.0)	5.00 (*d*, 6.4)	4.99 (*d*, 7.2)	5.02 (*d*, 7.3)	4.98 (*d*, 7.7)
2	3.45	3.40	3.43	3.45	3.43
3	3.29	3.33	3.35	3.39	
4	3.40	3.47	3.31	3.25	
5	3.77	3.80	3.75	3.78	3.90–3.10
6a	4.00	4.04	4.04	4.05	
6b	4.87	4.86	4.98	4.91	
(D)					
1	4.82 (*d*, 7.0)	4.86 (*br s*)	4.75 (*d*, 8.1)	4.80 (*d*, 7.5)	
2	3.33	3.31	3.45	3.37	
3	3.25	3.29	3.38	3.48	
4	3.35	3.24	3.35	3.39	
5	3.73	3.75	3.75	3.78	
6a	4.13	4.15	4.14	4.16	
6b	4.45	4.50	4.50	4.56	
(E)					
1	4.80 (*d*, 7.3)	4.80 (*d*, 7.8)	4.68 (*d*, 7.3)	4.67 (*d*, 7.5)	
2	3.25	3.24	3.34	3.35	
3					
4					
5	3.80–3.25	3.90–3.10	3.90–3.20	3.90–3.20	
6a					
6b					
Malonic acid					
$-CH_2-$	3.50–3.25		3.60–3.30		

*Assigned by ^1H–^1H COSY.
†Assigned by DIFNOE.
Coupling constants (*J* in Hz) in parentheses.

weak NOEs were observed at H-α,-β,-2,-5 and -6 of caffeic acid-II as well as a strong NOE at H-2′ of cyanidin, indicating Glc C to be bonded with caffeic acid-II. Furthermore, irradiation at H-1 of Glc B gave a DIFNOE spectrum in which rather weak NOEs were found at H-α,-β and -6 of caffeic acid-I. Thus caffeic acid-I was presumed to be attached to OH-6 of Glc B. In order to determine the attachment of malonic acid in **3**, the demalonylated pigment of **3** was prepared by the same process of demalonyl **1** described before. This demalonyl **3** was found to be identical with *Bletilla* anthocyanin **4** on analysis by HPLC and from the spectral properties (Tables 2 and 3). The structure of this demalonyl **3** was elucidated by analysis of the ^1H NMR spectra including ^1H–^1H COSY and DIFNOE spectra (Table 4). By analysis of the COSY spectrum of demalonyl **3** the proton signals of methylene (Glc A) were clearly shifted to the up-magnetic field from δ4.37, 4.57 (Glc A of **3**) to δ3.9–3.2 of demalonyl **3**, indicating that the malonyl group was free from the OH-6 of Glc A in demalonyl **3**. From this result for demalonyl **3**, it was revealed that Glc A of pigment **3** is bonded with malonic acid at OH-6. Therefore, caffeic acid-III is bonded with Glc D at OH-6 of Glc D in **3**. Thus, *Bletilla* anthocyanin **3** was determined to be cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-caffeyl)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-4-*O*-(β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranoside], which is a new anthocyanin in plants.

Bletilla *anthocyanin* (4)

The FAB mass spectrum of **4** gave its molecular ion $[M]^+$ at 1583 *m/z*, in good agreement with the mass

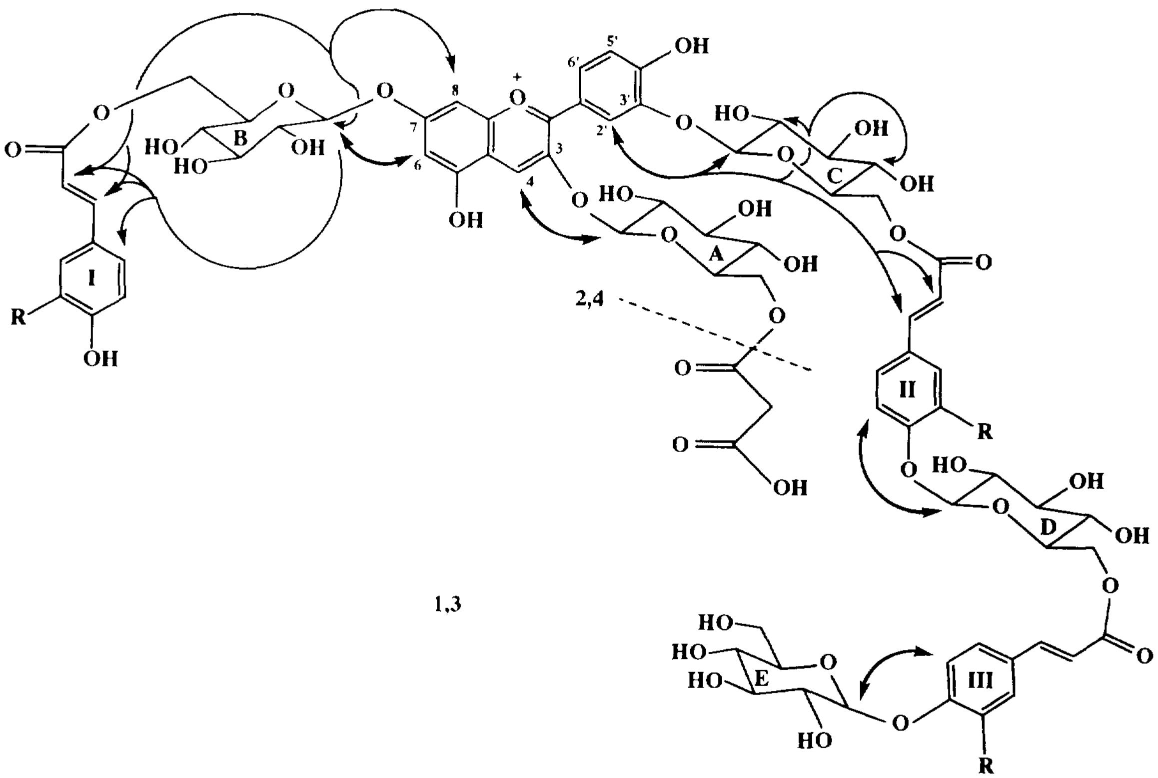

Fig. 1. *Bletilla* anthocyanins. **1**, **2**: R = H; **3**, **4**: R = OH. Observed NOEs are indicated by arrows.

calculated for $C_{72}H_{79}O_{40}$ (1583.415). This value is identical with the mass of the demalonylated pigment **3**, which was composed of cyanidin with five molecules of glucose and three molecules of caffeic acid as shown in Table 3. The R_f values, R_t (min) and spectral data are shown in Table 2. The detailed structure was determined by analysis of the ^{1}H NMR spectra including COSY and DIF-NOE spectra. The proton chemical shifts of **4** were identical with those of demalonyl pigment **3**, and also in good agreement with those of **3** except those of Glc A and malonic acid parts (Table 4). Since the methylene proton signals of Glc A of **4** were clearly shifted to the up-magnetic field from δ4.37, 4.57 (pigment **3**) to δ3.90–3.20 (**4**), Glc A of **4** is supposed to be free from malonic acid. Thus, the structure of **4** was determined to be demalonyl pigment **3**, which is a new anthocyanin in plants.

Other Bletilla *anthocyanins*

The FAB mass spectra of *Bletilla* anthocyanins **5–8** gave their molecular ions (1551–1653 *m/z*) as shown in Tables 2 and 3. These values are in good agreement with the mass calculated for their theoretical molecules, respectively, which are composed of cyanidin with five molecules of glucose, each of two molecules of hydroxycinnamic acids and also one (or none) of a molecule of malonic acid. Their R_f values, R_t (min) and spectral properties are summarized in Table 2. By alkaline hydrolysis these anthocyanins gave the same deacylanthocyanin, cyanidin 3,7,3′-triglucoside, and also several organic acids such as caffeic acid, *p*-coumaric acid, malonic acid and glucosyl hydroxycinnamic acids, respectively. Based on the above finding, the structures of *Bletilla* anthocyanins **5–8** are estimated to be cyanidin 3,7,3′-triglucoside whose 7,3′-glucosides are acylated with various hydroxycinnamic acid–glucose side chains, and also (except **6** and **8**) whose 3-glucoside is acylated with malonic acid. The molecular ratio of cyanidin : glucose : acyls (acids) in *Bletilla* anthocyanin **5–8** was determined by the result of FAB mass data as shown in Table 3 and confirmed by analysis of their ^{1}H NMR spectra.

To date, there are two reports on the occurrence of polyacylated cyanidin 3,7,3′-triglucosides in the flowers of orchid cultivars [1, 2, 7–9]. *Dendrobium* anthocyanin is acylated with *p*-hydroxybenzoic acid and malonic acid [1], and also × *Laeliocattleya* anthocyanin is acylated with *p*-coumaric acid and malonic acid [2]. The latter has a similar acylating pattern to those of *Bletilla* anthocyanins, which are mainly acylated with three molecules of hydroxycinnamic acids and also one molecule of malonic acid. The aliphatic acylating acid oxalic acid is linked to the 3-glucose in cyanidin in temperate members of the Orchidaceae [8–10], whereas malonic acid replaces it as acylating acid in tropical members of *Dendrobium* [1] and × *Laeliocattleya* [2].

The relative colour stabilities of **1–4** were compared in neutral solution with the deacylated pigment, cyanidin 3,7,3′-triglucoside. The polyacyl side chains of these

Bletilla anthocyanins were effective in maintaining colour stability, and also these pigments might have an intramolecular stacking structure in the flowers [11–13].

EXPERIMENTAL

Plant material. The fresh red–purple flowers of *B. striata* were collected from the plants growing in the experimental garden of Hoshi University, the experimental farm of Chiba University and the farms of Mr F. Tsukakoshi (Kawamoto, Saitama, Japan) and Mr M. Tatsuzawa (Mikkabi, Shizuoka, Japan). The flowers of four cultivars of these plants were also collected from the farm of Mr F. Tsukakoshi. These flowers were collected in May–June 1992, 1993 and 1994.

Isolation of Bletilla *anthocyanins.* Fresh red–purple flowers (1 kg) of *B. striata* were extracted with MAW (10 l, MeOH–HOAc–H_2O 9:1:10). The extract was concd to 500 ml. The concd extract was purified by Diaion HP-20 CC, PC, TLC and HPLC by previous procedures [1, 2]. Solvents used were 15% HOAc, BAW (*n*-BuOH–HOAc–H_2O, 4:1:5), 5% HOAc–MeOH and MAW for CC, PC and TLC. HPLC was performed on LC-6A system (Shimadzu). Prep. HPLC was run on a Waters C_{18} (19 $\phi \times 150$ mm) column at 40° with flow rate 4 ml min^{-1} and monitoring at 530 nm for anthocyanins. Solvent system used: a linear gradient elution for 40 min from 40 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). The evapn residues were dissolved in a small vol. of 5% HOAc–EtOH followed by addition of excess of Et_2O, and then drying to give pigment powder: pigment **1**, 25 mg; **2**, 25 mg; **3**, 15 mg; **4**, 15 mg; **5**, 20 mg; **6**, 10 mg; **7**, 10 mg; **8**, 5 mg.

Analysis of anthocyanins. Characterization of pigments was carried out using PC, TLC and UV–VIS spectrometry. Solvents used were BAW, BuH (*n*-BuOH–2 N HCl, 1:1), 1% HCl and AHW (HOAc–HCl –H_2O, 15:3:82) for anthocyanins, and EtOAc–HCOOH –H_2O (5:2:1), *n*-BuOH–HOAc–H_2O (4:1:2) and EtOAc –HOAc–H_2O (3:1:1) for organic acids and sugars. Acid hydrolysis, alkaline deacylation, H_2O_2 oxidation and partial acid hydrolysis of anthocyanins were performed according to standard procedures [3, 4].

Distribution of anthocyanins in the flowers of B. striata *and four cultivars.* Fresh perianths and labellums of each plant were extracted with MAW, and analyt. HPLC was performed on a Waters C_{18} (4.6 $\phi \times 250$ mm) column at 40° with flow rate 1 ml min^{-1} and monitoring at 530 nm for anthocyanins. Solvent system used: linear gradient elution for 30 min from 40 to 85% solvent B in solvent A.

FAB–MS and NMR measurements. FAB mass spectra were recorded on JEOL JMS SX-102A; positive mode in magic bullet and negative mode in glycerol. NMR spectra were recorded at 400 MHz for ^1H spectra by JEOL JNM GX-400 in DMSO-d_6–CF_3COOD (9:1). Chemical shifts are reported relative to TMS int. standard (δ) and coupling constants are reported in Hz.

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