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ACYLATED CYANIDIN 3,7,3′-TRIGLUCOSIDES IN FLOWERS OF × *LAELIOCATTLEYA* CV. MINI PURPLE AND ITS RELATIVES

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Key Word Index— × *Laeliocattleya* cv. Mini Purple; *Laelia pumila*; *Cattleya walkeriana*; Orchidaceae; red-purple flower colour; acylated cyanidin 3,7,3′-triglucosides; malonic acid; hydroxycinnamic acids.

Abstract—Ten acylated cyanidin 3,7,3′-triglucosides were isolated from the red-purple flowers of × *Laeliocattleya* cv. Mini Purple as major anthocyanins, along with a known pigment. The occurrence of these pigments was examined in the flowers of its parent species, *Laelia pumila* and *Cattleya walkeriana*, by HPLC. These ten pigments were observed in both parents, and FAB-mass measurements gave molecular ions $[M]^+$ at *m/z* 1459–1669, which were based on acylated cyanidin 3,7,3′-triglucosides with malonic acid, *p*-coumaric acid, caffeic acid, ferulic acid and glucosylated hydroxycinnamic acids. This was confirmed by the analysis of ^{1}H NMR spectra and results obtained from acid and alkaline hydrolysis. Four new acylated anthocyanin structures (**2**, **3**, **6** and **7**) were based on cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(acyl-I)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(acyl-II)-β-D-glucopyranosyl)-(acyl-III)-β-D-glucopyranoside], in which **3** was acylated with *p*-coumaric acid at acyl-I and -III, and caffeic acid at acyl-II, **6** was acylated with *p*-coumaric acid at acyl-I and caffeic acid at acyl-II and -III, **7** was acylated with three molecules of caffeic acid and the three acyl groups (acyl I–III) of **2** were composed of one molecule of caffeic acid and two molecules of ferulic acid.

INTRODUCTION

As part of our continuing work on flower colour variation due to acylated anthocyanins in orchids, six novel acylated cyanidin glycosides have been reported to be present in the red-purple flowers of *Dendrobium* cv. Pramot [1], × *Laeliocattleya* cv. Mini Purple [2] and *Blettila striata* [3]. In a further isolation study of acylated anthocyanins in the *Cattleya* alliance, we wish to report the occurrence of 10 acylated cyanidin 3,7,3′-triglucosides in the red-purple flowers of × *Laeliocattleya* cv. Mini Purple and its parents, *Laelia pumila* and *Cattleya walkeriana*, together with the structural elucidation of the main anthocyanins.

RESULTS AND DISCUSSION

In the course of an anthocyanin survey of the red-purple flowers of × *Laeliocattleya* cv. Mini Purple and its parent plants (*L. pumila* and *C. walkeriana*) by HPLC analysis, 16 anthocyanin peaks (**1**–**10** and **A**–**F**) were observed as major anthocyanins (Table 1). Ten main anthocyanins (**1**–**10**) including a known anthocyanin (**1**) reported previously [2] were obtained from the red-purple flowers of × *Laeliocattleya* cv. Mini Purple by the extraction with methanol-acetic acid-water. These pigments were purified using Diaion HP-20 column chromatography, PC and HPLC. Eight pigments (**1**–**3** and **6**–**10**) were successfully obtained as purified materials, but **4** and **5** were not completely purified.

The ten pigments (**1**–**10**) yielded only one deacylanthocyanin, cyanidin 3,7,3′-triglucoside, by alkaline hydrolysis. The mixed anthocyanins of these ten pigments gave cyanidin, glucose, *p*-coumaric acid, caffeic acid, ferulic acid and malonic acid by acid hydrolysis. Chromatographic and spectral properties of the ten pigments are shown in Table 2. Their components were analyzed by the FAB mass spectrometry and ^{1}H NMR spectra. Then, the molecular ratios between aglycone, sugar and acids were calculated (Table 3). Furthermore, structural determinations of the ten pigments were performed, and eight pigment structures (**1**–**3** and **6**–**10**) were successfully elucidated (see below). However, pigments **4** and **5** could not be determined because of their difficult separation, poor purification and low yields.

Pigment **1** was identified as the *Laeliocattleya* anthocyanin reported previously [2] (Tables 2–4), which is cyanidin 3-malonylglucoside-7-[6-*p*-coumarylglucoside]-3′-[6-(4-(6-*p*-coumarylglucosyl)-*p*-coumaryl)-glucoside]. Pigment **8**–**10** were identical with *Bletilla* anthocyanins **5**, **7** and **3** by analyses of HPLC and FAB-mass spectra [3]. The components produced by acid and alkaline hydrolysis also coincided with those of the *Bletilla* anthocyanins. The structure of **10** was determined to be cyanidin- 3-malonylglucoside-7-caffeylglucoside-3′-glucosylcaffeylglucosylcaffeylglucoside [3].

Table 1. Distribution of main anthocyanins flower extracts of × *Laeliocattleya* cv. Mini Purple and its relatives

Species	Flower part	Floral† colour	b/a‡	Anthocyanin (as %)*														
				F	**10**	**9**	**8**	**7**	E	**6**	**5**	D	**4**	C	**3**	**2**	B	A	**1**
× *Laeliocattleya* cv.	Inner Perianth	PV81A	−0.57	+	+	3.7	4.2	12.6	+	12.2	8.6	3.0	15.2	+	7.0	9.1	+	+	7.0
Mini Purple	Outer perianth	PV81A	−0.60	+	+	3.5	4.2	10.8	+	11.9	6.9	4.5	14.6	+	8.1	10.0	+	+	8.3
	Labellum	PV80A	−0.11	+	3.8	4.8	5.4	6.9	+	8.9	5.0	3.1	11.7	+	7.2	7.4	+	+	8.0
Laelia pumila	I.P.	PV81A	−0.59	+	+	+	+	16.6	+	10.8	11.0	+	15.5	+	6.0	8.1	+	+	6.3
	O.P.	PV81A	−0.59	+	3.3	4.4	3.9	13.1	+	9.0	8.2	+	12.3	+	7.5	5.8	+	+	6.4
	L.	PV80A	−0.17	+	+	5.5	6.4	4.8	+	7.0	3.3	+	11.2	+	11.0	6.8	+	+	10.9
L. pumila 'Black Diamond'	I.P.	PV81A	−0.49	+	7.7	5.7	+	23.5	+	9.2	10.6	+	9.6	+	+	3.2	+	+	+
	O.P.	PV81A	−0.45	+	7.7	5.5	4.4	13.9	+	7.6	8.5	+	9.3	+	3.0	3.9	+	+	+
	L.	PV80A	−0.03	+	7.0	6.0	3.5	7.4	+	5.5	6.4	+	8.2	+	3.2	4.8	+	+	3.7
L. pumila var. *oculata*	I.P.	V84B	−0.76	+	9.2	7.0	6.2	16.5	+	10.4	6.9	+	8.8	+	3.1	+	+	+	+
'Imperitris'	O.P.	V84B	−0.70	+	8.2	7.3	5.2	15.2	+	12.6	5.8	+	6.5	+	+	+	+	+	+
	L.	PV81A	−0.47	3.6	4.8	7.0	5.8	10.3	+	8.6	6.3	+	9.3	+	+	4.7	+	+	3.2
L. pumila ssp. *praestans*§	I.P.	–	–	+	+	+	3.0	12.8	+	10.6	9.1	3.8	13.5	+	7.2	7.0	+	+	7.8
	O.P.	–	–	+	+	+	+	10.3	+	9.5	5.2	3.1	9.4	+	6.3	5.4	+	+	4.8
	L.	–	–	+	+	+	3.0	+	+	5.2	6.1	6.1	9.4	+	6.8	6.3	6.7	+	27.7
Cattleya walkeriana	I.P.	PV81B	−0.65	+	7.1	11.6	10.0	10.4	4.4	7.9	6.2	+	8.7	3.1	4.4	5.6	+	3.6	5.0
	O.P.	PV81B	−0.59	+	5.5	8.5	6.2	12.7	5.7	8.0	7.0	5.3	7.1	3.7	4.0	6.7	+	6.1	+
	L.	PV81A	−0.51	+	12.9	14.0	10.6	7.5	5.2	4.5	3.0	+	4.1	+	+	3.4	+	3.9	+
C. walkeriana var. *tipo*	I.P.	PV82B	−0.51	+	3.5	5.7	5.8	8.8	7.0	6.1	6.2	6.7	3.8	3.4	4.0	4.9	5.1	5.1	+
'Fett'	O.P.	PV82B	−0.51	+	7.2	11.8	8.2	12.2	+	8.5	7.2	4.2	5.1	5.2	3.2	5.9	+	5.2	+
	L.	PV80A	−0.49	+	7.3	8.9	8.2	12.1	+	8.3	9.0	3.7	6.4	4.8	3.9	5.5	+	4.0	+

*Percent of total absorbance of all detected anthocyanins at 530 nm by HPLC analysis; + < 3%. R_t (min); **1**(24.9), **2**(22.6), **3**(22.3), **4**(20.4), **5**(18.7), **6**(18.2), **7**(16.7), **8**(15.5), **9**(13.9) and **10**(12.4); Unidentified anthocyanins A(24.4), B(24.0), C(21.1), D(19.8), E(17.7), and F(11.3).

†R.H.S. colour chart.

‡Hunter values (hue).

§The Flowers of this subspecies were sent from Brazil as dried materials.

Table 2. Chromatographic and spectral properties of anthocyanins from flowers of × *Laeliocattleya* cv. Mini Purple

Anthocyanin	R_f values (× 100)				Spectral data on 0.1% HCl–MeOH					R_t (min)	FAB-MS $[M]^+$
	BAW	BuHCl	1% HCl	AHW	λ_{max} (nm)	$E_{u.v.}/E_{max}$(%)	E_{acyl}/E_{max}(%)	E_{440}/E_{max}(%)	$AlCl_3$		
1*	29	6	8	28	535, 305, 290	190	176	26	0	24.9	1459
2	22	10	10	25	537, 320, 290	146	134	23	0	22.6	1535
3	26	35	11	34	538, 310, 290	145	131	24	0	22.3	1475
4	20	27	10	25	536, 310, 290	128	120	26	0	20.4	1491
5	16	3	5	14	532, 315, 285	200	166	34	0	18.7	1521
6	17	13	8	18	538, 320, 290	109	105	29	0	18.2	1491
7	16	20	8	17	536, 325, 293	118	111	29	0	16.7	1507
8	9	4	14	38	538, 307, 290	178	147	24	0	15.5	1637
9	7	6	14	34	538, 309, 288	176	139	29	0	13.9	1653
10	7	4	10	24	537, 320, 288	183	140	36	0	12.4	1669
BA 5†	8	3	14	41	538, 307, 294	139	135	33	0	15.5	1637
BA 7†	7	6	11	35	538, 309, 288	205	168	32	0	13.9	1653
BA 3†	7	5	8	25	537, 320, 288	162	129	29	0	12.4	1669
Deacyl anthocyanin*	5	2	39	60	513, 280	97	19	36	0	3.7	773

For key to abbreviations, see Experimental.

* **1**: Cyanidin 3-malonylglucoside-3′-(*p*-coumarylglucoside)$_2$-7-*p*-coumarylglucoside. **Deacyl anthocyanin**: Cyanidin 3,7,3′-triglucoside [2].

† **BA 5, 7** and **3**: *Bletilla* anthocyanins **5, 7** and **3** [3].

Table 3. Estimated molecular formulae of acylated anthocyanins from × *Laeliocattleya* cv. Mini Purple and their molecular ratios of constituents based on FAB-MS and ^{1}H NMR data

	Based on FAB-MS*								Based on ^{1}H NMR†					
Anthocyanin	$[M]^+$	Mf	Cy:	Glc:	*p*-C:	Caf:	Fer:	Mal	Cy:	Glc:	*p*-C:	Caf:	Fer:	Mal
1	1459	$C_{69}H_{71}O_{35}$	1	4	3	0	0	1	1	4	3	0	0	1
2	1535	$C_{71}H_{75}O_{38}$	1	4	0	1	2	1	1	4	0	1	2	1
3	1475	$C_{69}H_{71}O_{36}$	1	4	2	1	0	1	1	4	2	1	0	1
6	1491	$C_{69}H_{71}O_{37}$	1	4	1	2	0	1	1	4	1	2	0	1
7	1507	$C_{69}H_{71}O_{38}$	1	4	0	3	0	1	1	4	0	3	0	1
4	1491	$C_{69}H_{71}O_{37}$	1	4	1	2	0	1	–	–	–	–	–	–
5	1521	$C_{70}H_{73}O_{38}$	1	4	0	2	1	1	–	–	–	–	–	–
8‡	1637	$C_{75}H_{81}O_{41}$	1	5	2	1	0	1	1	5	2	1	0	–
9‡	1653	$C_{75}H_{81}O_{42}$	1	5	1	2	0	1	1	5	1	2	0	–
10‡	1669	$C_{75}H_{81}O_{43}$	1	5	0	3	0	1	1	5	0	3	0	–

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

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

‡ Ref. [3]; **8** = *Bletilla* anthocyanin **5**, **9** = **BA7**, **10** = **BA 3**.

Table 4. ^{1}H NMR data of × *Laeliocattleya* anthocyanins [CF_3CO_2D–DMSO-d_6 (1:9) at 25°]

H	**1***	**2**	**3**	**6**	**7**
Cyanidin moiety					
4	8.41 *s*	8.60	8.48	8.50	8.60
6	6.71 *br s*	6.72	6.78	6.79	6.75
8	6.87 *br s*	6.84	6.93	6.98	6.91
2′	7.75 *br s*	7.89	7.72	7.77	7.72
5′	7.09 *d* (8.5)	7.15 *d* (9.4)	7.09 *br d* (9.4)	7.07 *d* (9.1)	7.07 *d* (8.6)
6′	8.51 *br d* (8.5)	8.59 *d* (9.4)	8.52 *br d* (9.4)	8.51 *br d* (9.1)	8.52 *d* (8.6)
p-Coumaryl, caffeyl or ferulyl moiety‡					
(I)					
2 or 2,6	6.63 *d* (8.1)	6.34 *s*	6.74 *d* (8.6)	6.79 *d* (8.3)	6.28 *s*
5 or 3,5	6.56 *d* (8.1)	6.68 *d* (8.2)	6.64 *d* (8.6)	6.63 *d* (8.3)	6.65 *d* (8.2)
6	–	6.29 *m*	–	–	6.15 *d* (8.2)
α	5.86 *d* (15.8)	5.82 *d* (15.8)	5.92 *d* (15.8)	5.93 *d* (15.5)	5.82 *d* (15.6)
β	7.05 *d* (15.8)	7.11 *d* (15.8)	7.12 *d* (15.8)	7.12 *d* (15.5)	7.09 *d* (15.6)
(II)					
2 or 2,6	6.95 *d* (8.1)	6.70	6.78	6.74	6.75
5 or 3,5	6.59 *d* (8.1)	7.29 *d* (8.6)	6.57 *d* (8.1)	6.65 *d* (9.1)	6.11 *d* (8.6)
6	–	6.68 *d* (8.6)	6.11 *d* (8.1)	6.25 *m*	6.25 *d* (8.6)
α	6.12 *d* (15.8)	6.23 *d* (15.8)	6.19 *d* (15.8)	6.17 *d* (15.9)	6.24 *d* (15.3)
β	7.23 *d* (15.8)	7.35 *d* (15.8)	7.33 *d* (15.8)	7.36 *d* (15.9)	7.30 *d* (15.3)
(III)					
2 or 2,6	7.15 *d* (8.1)	6.96	7.24 *d* (8.6)	6.98	6.97
5 or 3,5	6.59 *d* (8.1)	7.12 *d* (8.1)	6.68 *d* (8.6)	7.03 *m*	7.02 *m*
6	–	6.70 *d* (8.1)	–	6.80 *m*	6.81 *m*
α	6.22 *d* (15.8)	6.32 *d* (16.3)	6.23 *d* (15.8)	6.32 *d* (15.9)	6.16 *d* (15.8)
β	7.36 *d* (15.8)	7.44 *d* (16.3)	7.31 *d* (15.8)	7.53 *d* (15.9)	7.35 *d* (15.8)
$-OCH_3$		3.78(×2)§			
Glucose moiety†‡					

	(A)	(C)	(A)	(C)	(A)	(C)	(A)	(C)	(A)	(C)
1	4.96	4.96	5.16	5.05	4.95	5.00	4.98	5.06	5.06	5.03
2	3.67	3.44	3.65	3.48	3.71	3.51	3.69	3.49	3.71	3.48
3	3.48	3.34	3.80	~3.41	3.52	3.42	3.49	3.45–3.35	3.56	3.39
4	3.44	3.28	3.30	3.36	3.42	3.28	3.41	3.27	3.41	3.29
5	3.76	3.71	3.90	3.85	3.84	3.78	3.78	3.80	3.92	3.76
6a	4.30	4.12	4.30	4.05	4.41	4.12	4.39	4.06	4.39	4.03
6b	4.25	4.74	4.55	4.89	4.56	4.93	4.52	4.90	4.54	4.93

Table 4. (continued)

H	1*		2		3		6		7	
	(B)	(D)	(B)	(D)	(B)	(D)	(B)	(D)	(B)	(D)
1	5.13	4.82	5.16	4.90	5.20	4.75	5.21	4.76	5.17	4.77
2	3.45	3.37	3.48	3.39	3.51	3.51	3.50	3.45	3.50	3.44
3	3.35	3.34	3.45	3.35	3.43	3.45	3.46	{3.60	~ 3.50 ~ 3.39	{3.55 ~
4	3.22	3.32	3.30	3.30	3.35	3.39	3.38	{3.30	3.39	{3.39
5	3.76	3.71	3.78	3.78	3.81	3.79	3.80	3.75	3.79	3.79
6a	4.00	4.12	4.21	4.21	4.40	4.25	4.16	4.28	4.12	4.30
6b	4.88	4.42	4.74	4.43	4.83	4.44	4.79	4.39	4.83	4.39
Malonyl moiety										
$-CH_2-$	3.44		3.85 ~ 3.30		3.50 ~ 3.30		3.50 ~ 3.20		3.50 ~ 3.35	

*Ref. [2].

†Assigned by ^1H–^1H COSY.

‡Assigned by DIFNOE.

§Not possible to determine which hydroxycinnamic acid I ~ III were bonded with these methoxyl groups.

Coupling constants (J in Hz) in parentheses.

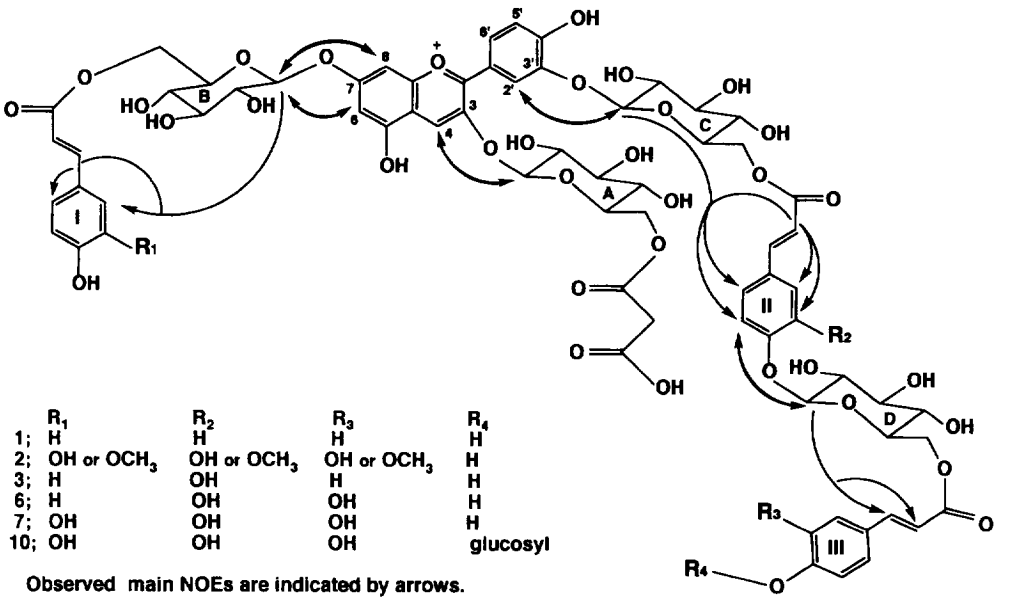

Fig. 1. Anthocyanins, from × *Laeliocattleya*.

Laeliocattleya anthocyanin **3**. The FAB-mass spectrum of **3** gave a $[M]^+$ at m/z 1475, corresponding to $C_{69}H_{71}O_{36}$ (*ca* 1475.370), which was composed of cyanidin with four molecules of glucose, two of *p*-coumaric acid and one each of caffeic and malonic acids (Table 3). This result was confirmed by ^1H NMR spectral measurement of **3**. The detailed structure of **3** was elucidated by analysis of ^1H NMR spectra, including ^1H-^1H COSY and DIFNOE (negative nuclear Overhauser effect difference) techniques [4, 5] (Table 4). Six proton signals of cyanidin and 11 ring proton signals of three hydroxycinnamic acids (acyl I, II and III in Fig. 1) were observed in the region at 8.48 ppm ~ 6.11 ppm (Table 3). Three pairs of doublet signals with large coupling constants (J = 15.8 Hz) indicated the presence of the *trans*-olefinic protons of *p*-coumaric acids (acyl I δ5.92, 7.12 and III δ6.23, 7.31) and caffeic acid (II δ6.19, 7.33). The signals of four anomeric protons of glucose units appeared at δ4.95 (d, J = 7.7 Hz, Glc A), δ5.20 (d, J = *ca* 7 Hz, Glc B), δ5.00 (d, J = *ca* 7 Hz, Glc C) and δ4.75 (d, J = 7.2 Hz, Glc D); all the observed vicinal coupling constants of these four glucose moieties (Glc A–D in Fig. 1) were *ca* 7.0–9.0 Hz. Therefore, all the glucose units must be of β-D-glucopyranoside. All the methylene proton signals (δ4.41, 4.56 Glc A; δ4.40, 4.83 Glc B; δ4.12, 4.93 Glc C; and δ4.25, 4.44 Glc D) of the four glucose units were shifted to a lower magnetic field, indicating all the OH-6 groups of these four glucose units to be acylated with four acids. Application of the DIFNOE method made it possible to determine the linkages and the attachment positions of

sugar and acid units in the molecule. Then, the three glucose units (A, B and C) were shown to be attached to the 3-OH, 7-OH and 3′-OH of cyanidin, respectively, by the observation of NOEs irradiating at the anomeric protons of Glc A–C (Fig. 1). Irradiation of the H-1 of Glc D gave a strong NOE to H-5 of caffeic acid (II) and also rather weak NOEs of H-α and -β of caffeic acid (II) and H-α and -β of *p*-coumaric acid (III). Therefore, Glc D was glycosylated at the 4-OH of caffeic acid (II) and also acylated with *p*-coumaric acid (III). Irradiation of the H-1 of Glc B gave rather weak NOEs to H-α and -β of *p*-coumaric acid (I), as well as a strong NOE with H-8 and -6 of cyanidin, indicating that Glc B was acylated with *p*-coumaric acid (I). In the same way, it was revealed that caffeic acid (II) was acylated with Glc C. By H_2O_2 degradation of **3**, malonylglucose was detected, indicating that malonic acid is attached to the 6-OH of Glc A [2]. Therefore, **3** was determined to be 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*-*p*-coumaryl)-β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranoside], which is a new anthocyanin [6, 7].

Laeliocattleya anthocyanin **6**. The FAB-mass spectrum of **6** gave a $[M]^+$ at *m/z* 1491, corresponding to $C_{69}H_{71}O_{37}$ (*ca* 1491.365). Analysis of the ^1H NMR and ^1H-^1H COSY spectra indicated the presence of one molecule of cyanidin, four molecules of glucose, two molecules of caffeic acid and one each of *p*-coumaric and malonic acids. The signals of four anomeric protons appeared at δ4.98 (*d*, *J* = 7.1 Hz Glc A), δ5.21 (*d*, *J* = 7.0 Hz Glc B), δ5.06 (*d*, *J* = 7.1 Hz Glc C) and δ4.76 (*d*, *J* = 6.7 Hz Glc D); all the observed vicinal coupling constants of these four glucose moieties were 6.7–9.0 Hz, indicating them to be β-D-glucopyranosides. The eight characteristic protons of the four methylenes-CH_2- of glucoses (A–D) were shifted to a low magnetic field (δ4.39, 4.52 Glc A; δ4.16, 4.79 Glc B; δ4.06, 4.90 Glc C; δ4.28, 4.39 Glc D), indicating that the four hydroxyl groups (6-OH) of Glc A–D are acylated with four molecules of acid. Also, from analysis of the ^1H-^1H COSY spectrum of **6**, each anomeric protons of Glc A–D was finally correlated to each methylene proton of Glc A–D, respectively (Table 4). Application of the DIFNOE method made it possible to determine the linkages of glucose, hydroxycinnamic acid and malonic acid units in the pigment molecule. Irradiation of the anomeric protons of Glc A–D, revealed that OH-3, -7 and 3′ of cyanidin and also the OH-4 of caffeic acid-II were glycosylated by Glc A, B, C and D, respectively, because of the observation of NOEs as shown in Fig. 1. Irradiation of H-1 of Glc B gave rather weak NOEs with the H-α, -β, -2 and -6 of *p*-coumaric acid (I), as well as strong NOEs with the H-8 and -6 of cyanidin. Thus, *p*-coumaric acid (I) was shown to be attached to OH-6 of Glc B. Similarly, irradiation of H-1 of Glc C gave rather weak NOEs with H-2, -5 and -6 of caffeic acid (II), as well as a strong NOE with H-2′ of cyanidin. Therefore, caffeic acid (II) was attached to OH-6 of Glc C. By H_2O_2 degradation of **6**, malonylglucose was obtained. Consequently, malonic acid is attached to OH-6 of Glc A [2] and another caffeic acid (III) is linked to OH-6 of Glc D. The structure of **6** was thus determined to be 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*-caffeyl)-β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranoside], which is also a new anthocyanin.

Laeliocattleya anthocyanin **7**. The FAB-mass spectrum of **7** gave a $[M]^+$ *m/z* 1507, in good agreement with a formula of $C_{69}H_{71}O_{38}$ (1507.360), which was composed of cyanidin with four molecules of glucose, three molecules of caffeic acid and one molecule of malonic acid (Table 3). The detailed chemical structure was elucidated by ^1H NMR measurement, including ^1H-^1H COSY and DIFNOE methods. Six proton signals of the cyanidin moiety and nine proton signals of three caffeic acid moieties (acyl I, II and III) were observed (Table 4). In these caffeic acid moieties, three olefinic proton pairs of doublet signals were assigned to each caffeic acid moiety at δ5.82, 7.09 (I), δ6.24, 7.30 (II) and δ6.16, 7.35 (III), large coupling constants (*J* = 15.6, 15.3 and 15.8 Hz) indicating the olefinic configuration of these three acyl groups to be *trans*. Concerning the proton signals of the four sugar parts (Glc A–D), four anomeric protons were observed at δ5.06 (*d*, *J* = 7.8 Hz Glc A), δ5.17 (*m*, Glc B), δ5.03 (*d*, *J* = 7.1 Hz Glc C) and δ4.77 (*d*, *J* = 7.5 Hz Glc D); the observed vicinal coupling constants of all four glucose moieties were 7.1–9.0 Hz, supporting a β-D-glucopyranoside structure. The eight characteristic protons at δ4.39, 4.54 (Glc A), δ4.12, 4.83 (Glc B), δ4.03, 4.93 (Glc C) and δ4.30, 4.39 (Glc D) were assigned to the C-6 methylenes of glucose units (A–D) and were correlated with each anomeric proton of Glc A–D from analysis of the ^1H-^1H COSY spectrum (Table 4). Therefore, all the four 6-OH groups of glucose units (A–D) were acylated with three molecules of caffeic acid and one molecule of malonic acid, respectively.

Measurements of DIFNOE spectra of **7** revealed the linkages and attachments of the four molecules of glucose and four molecules of acyl groups in this pigment. Irradiations of the H-1 of Glc A, H-1 of Glc B, H-1 of Glc C and H-1 of Glc D caused negative NOEs at the H-4, H-8 and H-2′ of the cyanidin nucleus and the H-5 of caffeic acid (II), respectively. Therefore, Glc A, B, C and D were attached to the OH-3, OH-7 and OH-3′ of cyanidin, and the OH-4 of caffeic acid (II), respectively. Furthermore, irradiations of the H-1 of Glc B, C and D gave rather week NOEs to the H-α and -β of caffeic acids (I), (II) and (III), respectively, indicating that Glc B was acylated with caffeic acid (I), Glc C with caffeic acid (II) and Glc D with caffeic acid (III). The position of attachment of malonic acid on Glc A through an ester bond was shown from H_2O_2 degradation of **7** which gave malonylglucose.

Thus, **7** 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*-caffeyl)-β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranoside], which is another new anthocyanin [6, 7].

Laeliocattleya anthocyanin **2**. The FAB-mass spectrum of **2** gave a $[M]^+$ at *m/z* 1535, corresponding to the

formula $C_{71}H_{75}O_{38}$ (1535.391). The proton chemical shifts of the ^{1}H NMR spectrum of **2** were assigned as shown in Tables 3 and 4. They indicated the presence of one molecule of cyanidin, four molecules of glucose, two molecules of ferulic acid and one each of caffeic and malonic acids. By analysis of the ^{1}H-^{1}H COSY spectrum, four anomeric protons were assigned at δ5.16 (*d*, J = 7.2 Hz Glc A), δ5.16 (*d*, J = 7.2 Hz Glc B), δ5.05 (*d*, J = 8.1 Hz Glc C) and δ4.90 (*d*, J = 6.8 Hz Glc D); all the observed vicinal coupling constants of these four glucose units were 6.8–9.0 Hz. Therefore, these glucose units are of the β-D-glucopyranose form. Four characteristic protons, shifted to a lower magnetic field at δ4.30, 4.55 (Glc A), δ4.21, 4.74 (Glc B), δ4.05, 4.89 (Glc C) and δ4.21, 4.43 (Glc D), were assigned to the four methylene protons of Glc A–D and correlated with each of the anomeric protons of Glc A–D, respectively. Thus, all glucose units in this pigment were acylated with acids at their OH-6 groups, like those of **3**, **6** and **7**. By H_2O_2 degradation of **2**, malonylglucose was obtained. Consequently, malonic acid is confirmed to be attached to OH-6 of Glc A. A DIFNOE experiment on **2** could not be performed because of its low yield. Therefore, the linkages and/or the position of attachments of the three hydroxycinnamic acid units (I–III) were not elucidated precisely in this pigment. Thus, **2** was tentatively assigned as cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-hydroxycinnamyl)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-hydroxycinnamyl)-β-D-glucopyranosyl)-hydroxycinnamyl)-β-D-glucopyranoside], in which the three hydroxycinnamic acid units were composed of two molecules of ferulic acid and one molecule of caffeic acid.

Anthocyanins **4**, **5**. Though both compounds were still mixed with other pigments, FAB-mass spectra of **4** and **5** gave their $[M]^+$ as *m/z* 1491 and 1521 (Tables 2 and 3). These values are in good agreement with the mass calculated from their theoretical molecules, respectively, which are composed of cyanidin with four molecules of glucose, three molecules of hydroxycinnamic acids and one molecule of malonic acid. By alkaline hydrolysis, these anthocyanins gave cyanidin 3,7,3′-triglucoside and several organic acids, such as *p*-coumaric, caffeic, ferulic and malonic acid, and glucosylhydroxycinnamic acids (Table 3). Based on the above findings, *Laeliocattleya* anthocyanins **4** and **5** are considered to have similar tetra-acyl 3,7,3′-triglucoside structures to those of **1**–**3**, **6** and **7**, and both structures are thought to be cyanidin 3,7,3′-triglucosides whose 7,3′-glucosides are acylated with varied kinds and lengths of hydroxycinnamic acid-glucose side-chains, and also whose 3-glucoside is acylated with malonic acid.

To date, there are four reports on the occurrence of polyacylated cyanidin 3,7,3′-triglucosides in the flowers of Orchid cultivars [1–3, 8]. *Laeliocattleya* and *Bletilla* anthocyanins [2, 3] and *Phalaenopsis* anthocyanin [8] are acylated with hydroxycinnamic and/or malonic acids. On the other hand, *Dendrobium* anthocyanin [1] is acylated with *p*-hydroxybenzoic and malonic acids. These pigments show similar acylated structures which have three characteristic side-chains at 7-, 3- and 3′-OH of cyanidin, except *Phalaenopsis* anthocyanin which is acylated only at the 3-glucoside of cyanidin.

Several anthocyanins of *Laeliocattleya* and *Bletilla* exhibit the same chromatographic and spectral data. As shown in Tables 2 and 3, *Laeliocattleya* anthocyanins (*LA* **8**, **9** and **10**) showed similar retention times on HPLC and FAB mass data to those of *Bletilla* ones (BA **5**, **7** and **3**), respectively. Therefore, this result indicated an affinity among *B. striata*, *C. walkeriana* and *L. pumila* from a chemotaxonomical point of view.

The occurrence of acylated anthocyanins (**1**–**10**) in × *Laeliocattleya* cv. Mini Purple indicates a typical intergeneric hybrid distribution of anthocyanins between *L. pumila* and *C. walkeriana*. Pigments **1**–**4** and **6** were the main ones in the labellum of *L. pumila*. On the other hand, **7**–**10** were observed in the labellum of *C. walkeriana* as dominant anthocyanins. The mode of transmission of these characteristic anthocyanins from parental species to progenies was recognized by this analysis (Table 2).

The relative colour stabilities of the polyacylated anthocyanins (**1**–**3**, **6** and **7**) were very stable in neutral solution in comparison with the deacylated pigment [9]. The polyacylated side-chains in these pigments were effective in maintaining colour stability; these pigments also might have an intramolecular stacking structure in the flowers [9–12].

EXPERIMENTAL

Plant material. Fresh flowers of the × *Laeliocattleya* cv. Mini Purple were obtained from Taguchi Nursery, Kawanishi Orchids and ALF Ltd., Shizuoka, Japan. Flowers of *L. pumila*, *L. pumila* ssp. *praestans*, *L. pumila* 'Black Diamond' and *L. pumila* var. *oculata* 'Imperitris' were gifts from the cultivations of Mr Y. Yamauchi (Omori Meter Co. Ltd., Tokyo, Japan), Mr T. Yamamoto (ALF Ltd., Shizuoka, Japan) and Mr S. Nakashima and Mr N. Tanaka (Orquidário São Bernardo, São Paulo, Brazil). Flowers of *C. walkeriana* and *C. walkeriana* var. *tipo* 'Fett' were gifts, from the cultivations of Mr J. Miura (Miura Mericlone Co. Ltd., Kanagawa, Japan) and Mr T. Yamamoto. These flowers were dried overnight at 37° and kept at −20°.

Isolation of anthocyanins. Dried red-purple petals (*ca* 200 g) of × *Laeliocattleya* cv. Mini Purple were immersed in MAW (2 l, MeOH–HOAc–H_2O, 9:1:10), kept at 4° for 1 week and extracted. The extract (*ca* 1.8 l) was concd to 100 ml. The red-purple concd extract was purified by Diaion HP-20 gel CC, PC and HPLC as described previously [1–3]. Solvents used were 15% HOAc, BAW 1 (*n*-BuOH–HOAc–H_2O, 8:1:5), 5% HOAc–MeOH and MAW for CC and PC. Prep. HPLC was run on a Waters C18 (19ϕ × 150 mm) column at 40° with a flow rate of 4 ml min^{-1} monitoring at 530 nm for anthocyanins. Solvents systems used for HPLC were as follows: a linear gradient elution for 30 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). Pigment frs

were evapd *in vacuo* to dryness. The residues were dissolved in a small vol. of 5% HOAc–MeOH followed by addition of excess Et_2O and then dried. Yields: **1**, *ca* 20 mg [2], **2**, *ca* 10 mg, **3**, *ca* 25 mg, **4**, *ca* 30 mg, **5**, *ca* 10 mg, **6**, *ca* 25 mg, **7**, *ca* 30 mg, **8**, *ca* 5 mg, **9**, *ca* 5 mg and **10**, *ca* 5 mg.

Anthocyanin analysis. Pigment identifications were carried out by standard procedures involving H_2O_2 oxidation, deacylation with alkali, demalonylation and hydrolysis with acid [13, 14]. Solvents used were BAW (*n*-BuOH–HOAc–H_2O, 4:1:2), 1% HCl, BuHCl (*n*-BuOH–2N HCl, 1:1) and AHW (HOAc–HCl–H_2O, 15:3:82) for anthocyanins, and EFW (EtOAc–HCO_2H–H_2O, 5:2:1), BAW and EAH (EtOAc–HOAc–H_2O, 3:1:1), for organic acids and sugars.

Distribution of anthocyanins in flower parts. Dried and fresh inner and outer perianths and labellums (*ca* 0.02 g) from flowers of *L. pumila*, *C. walkeriana* and related species were extracted with MAW. TLC and HPLC were carried out as described in ref. [13]. Quantitative analysis was performed by HPLC using a Waters C18 ($4.6\phi \times 250$ mm) column at 40° with a flow rate of 1 ml min^{-1} monitoring at 530 nm for anthocyanins. Solvent systems used were as follows: a linear gradient elution for 30 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).

FAB mass and NMR measurements. FAB MS were recorded in the positive mode using a magic bullet and in the negative mode in glycerol. NMR spectra were recorded at 400 MHz for ^1H NMR spectra in DMSO–d_6–CF_3CO_2D (9:1). Chemical shifts are reported relative to a TMS int. standard (δ) and coupling constants are reported in Hz.

Colour stability in neutral solution. Each anthocyanin (*ca* 1 mg) was dissolved in a Pi buffer (4 ml, pH 6.9) and kept at room temp. (*ca* 20–25°). Then, A at 540 nm were measured at intervals over 20 days.

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