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ACYLATED CYANIDIN GLYCOSIDES IN THE RED–PURPLE FLOWERS OF *PHALAENOPSIS*

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Key Word Index—*Phalaenopsis*; Orchidaceae; red–purple flower colour; acylated anthocyanins; cyanidin 3,7,3′-triglucoside; malonylglucoside; malonic and sinapic acids.

Abstract—Four acylated anthocyanins were isolated from the red–purple flowers of *Phalaenopsis* hybrid cultivars as major anthocyanins and their distribution was investigated in the flowers of five species; *P. equestris*, *P. intermedia*, *P. leucorrhoda*, *P. sanderiana* and *P. schilleriana* cultivar 'Pink Butterfly'. These four pigments were based on cyanidin 3,7,3′-triglucoside as their deacylanthocyanin, which was acylated with two molecules of hydroxycinnamic acid and/or one molecule of malonic acid. Two anthocyanins were characterized as cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7,3′-*O*-di-[6-*O*-(*trans*-sinapyl)-β-D-glucopyranoside] and its demalonyl derivative by spectral and chemical methods.

INTRODUCTION

Phalaenopsis plants are an economically important group of orchids which are native from Asia to Australasia with white, yellow, orange, red, red–purple and purple flowers. Griesbach isolated a sinapyl cyanidin 3,7,3′-triglucoside from the flowers of *Phalaenopsis schilleriana* [1], but its structure was not elucidated in detail. In the continuing work on flower colour variation due to acylated anthocyanins in orchids, we have already reported the occurrence of acylated cyanidin and peonidin glycosides in the flowers of *Dendrobium*, ×*Laeliocattleya*, *Bletilla* and *Cymbidium* cultivars [2–6]. In this paper we report the occurrence of new acylated cyanidin 3,7,3′-triglucosides in the red–purple flowers of *Phalaenopsis* and their structure elucidation.

RESULTS AND DISCUSSION

By HPLC analysis 20 anthocyanin peaks were observed in the MAW (methanol–acetic acid–water, 4:1:15) extracts from the red–purple flowers of five species and cultivars of *Phalaenopsis*. Among these peaks, four anthocyanins were isolated and identified to be **1** (9.2–33.3%), **2** (1.0–5.0%), **3** (26.0–64.4%) and **4** (1.0–5.0%) as major pigments (Table 1). These

Table 1. Distribution of anthocyanins in the flower extracts of *Phalaenopsis* species

Plant	Floral colour*	b/a†	Anthocyanin (as %)‡						
			A	**B**	**C**	**1**	**2**	**3**	**4**
Phalaenopsis equestris	Purple–violet 81C	−0.56	5.9	18.5	17.3	9.2	+	26.1	+
P. intermedia	Yellow white 158D	1.53	5.2	21.4	+	11.8	+	26.0	+
P. leucorrhoda	Yellow–white 158D	0.30	+	+	+	33.3	+	52.8	+
P. sanderiana	Purple–violet 81D	−0.62	+	+	+	27.3	+	64.4	+
P. schilleriana 'Pink Butterfly'	Purple violet 81D	−0.55	+	6.5	+	30.2	+	45.7	+

*R.H.S. colour chart.
†Hunter values (hue).
‡Percent of total absorbance of all detected anthocyanins at 530 nm in HPLC analysis, and +: under 5%. The anthocyanin no. was the same as in Fig. 1 and Table 2. R_t (min): **1**(15.9), **2**(17.5), **3**(17.9), **4**(18.1); unidentified anthocyanins **A**(12.1), **B**(13.2), **C**(15.2).

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anthocyanins (**1**–**4**) were isolated from the red–purple mixed flowers of *Phalaenopsis* hybrids with MAW solvent. These pigments were purified using a Diaion HP-20 column chromatography (C), paper C, HPLC and TLC. The R_f values. R_t (min) and spectral data of these four pigments are shown in Table 2.

Acid hydrolysis gave cyanidin, glucose and hydroxycinnamic acids. Sinapic acid was detected in the hydrolysis products of **1**, **3** and **4**, and ferulic acid was detected in those of **2** and **4** by acid and alkaline hydrolysis. Alkaline hydrolysis of **1**–**4** yielded only one deacylanthocyanin, whose structure was identified to be cyanidin 3,7,3′-triglucoside by direct comparison with authentic samples prepared from *Dendrobium*, × *Laeliocattleya* and *Bletilla* anthocyanins [2–5]. The measurements of FAB mass and ^{1}H NMR spectra led to the determination of the molecular ratios of chemical composition (aglycone, sugar and acid) as shown in Table 3. Among these four pigments, the structures of two pigments (**1** and **3**) were completely determined, but the others were tentatively identified because of the difficulty of their purification and also the small amounts present.

Phalaenopsis *anthocyanin* **3**

The FAB mass measurement of **3** gave a molecular ion $[M]^+$ at 1271 *m/z*, in good agreement with the mass calculated for $C_{58}H_{63}O_{32}$ (requires 1271.330). The ^{1}H NMR spectrum of **3** was obtained at 500 MHz using DMSO-d_6 solvent containing 10% TFA-*d*. The ^{1}H NMR spectrum of **3** showed the presence of one molecule of cyanidin, three molecules of glucose, two molecules of sinapic acid and one molecule of malonic acid (Table 4). These proton signals were mainly assigned by ^{1}H-^{1}H COSY, and sugar and acyl linkages were confirmed by the negative difference nuclear Overhauser effect (DIFNOE) spectra (Fig. 1). The proton signals of the sugar parts were observed in the region of δ 3.12–5.22. The signals of three anomeric protons appeared at δ 5.14 (*d*, J = 7.7 Hz, Glc A), δ 5.22 (*d*, J = 7.1 Hz, Glc B) and δ 5.18 (*d*, J = 7.4 Hz, Glc C), and the assigned sugar protons have the coupling constants with J = 7.1–11.8 Hz indicating all these glucose units must be β-D-glucopyranose. Six methylene protons were assigned to H-6a and 6b of Glc A (δ 4.19 and 4.52), those of Glc B (δ 4.06 and 4.86) and those of Glc C (δ 4.18 and 4.72) by the DIFNOE experiments and also were correlated to each anomeric protons by analysis of the ^{1}H-^{1}H COSY spectrum. This result indicated that these three glucose units were acylated at the 6-OH groups with acids.

In order to determine the linkages and positions of the glucose and acyl units DIFNOE spectra of **3** were measured (Fig. 1). Observed DIFNOE between H-1 of Glc A and H-4 of cyanidin indicates that Glc A is attached to the 3-OH of cyanidin through a glucosidic bond. Glc B was determined to be attached to the 7-OH of cyanidin through a glycosidic bond, because of the presence of NOEs between H-6 and H-8 of

Table 2. Chromatographic and spectral properties of anthocyanins from flowers of *Phalaenopsis*

Anthocyanin	R_f values (× 100)				Spectral data in 0.1% HCl–MeOH			R_t (min)	FAB-MS $[M]^+$
	BAW	BuHCl	1% HCl	AHW	λ_{max} (nm)	E_{acyl}/E_{max} (%)	E_{440}/E_{max} (%)	$AlCl_3$		
1	41	11	16	47	534, 330, 295	154	28	0	15.9	1185
2	39	10	15	47	533, 330, 295	166	32	0	17.5	1241
3	34	10	15	54	535, 331, 295	121	29	0	17.9	1271
4	37	9	16	54	533, 330, 295	184	29	0	18.1	1211
Deacyl (**1**–**4**)	11	2	29	62	513, 280		39	0	3.7	773
Cy 3,7,3′-triglc.*	11	2	29	62	513, 280		36	0	3.7	773

*Deacylanthocyanin from *Dendrobium*, *Lc*. Mini Purple and *Bletilla striata* [1–4].

Table 3. The estimated molecular formulae of acylated anthocyanins from *Phalaenopsis* 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:	Sin:	Fer:	Mal	Cy:	Glc:	Sin:	Fer:	Mal
1	1185	$C_{55}H_{61}O_{29}$	1	3	2	0	0	1	3	2	0	0
2	1241	$C_{57}H_{61}O_{31}$	1	3	1	1	1	—	—	—	—	—
3	1271	$C_{58}H_{63}O_{32}$	1	3	2	0	1	1	3	2	0	1
4	1211	$C_{56}H_{59}O_{30}$	1	3	0	2	1	—	—	—	—	—
Deacyl anthocyanin	773	$C_{33}H_{41}O_{21}$	1	3	0	0	0	—	—	—	—	—

*$[M]^+$ and Mf = molecular ion mass values, and estimated molecular formulae as flavylium forms of anthocyanins isolated from *Phalaenopsis* based on FAB-mass data, respectively. Cy : Glc : Sin : Fer : Mal = molecular numbers of their components; Cy = cyanidin, Glc = glucose, Sin = sinapic acid, Fer = ferulic acid and Mal = malonic acid.

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

cyanidin and H-1 of Glc B. Similarly, Glc C was determined to be glycosylated at 3′-OH of cyanidin, because of the presence of NOEs between H-2′ of cyanidin and H-1 of Glc C. Moreover, irradiation at H-1 of Glc B gave a DIFNOE spectrum in which NOEs to protons of H-α and β of sinapic acid I were observed. Thus, sinapic acid I is attached to the 6-OH of Glc B. As irradiation at H-1 of Glc C gave NOEs to olefinic protons of sinapic acid II, and irradiation at H-2 and H-6 of sinapic acid II gave NOEs to H-1 of Glc C, sinapic acid II was attached to the 6-OH of 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 [7]. Therefore, **3** is cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7,3′-di-*O*-[6-*O*-(sinapyl)-β-D-glucopyranoside], which is a new anthocyanin [8, 9].

Phalaenopsis *anthocyanin* **1**

The FAB mass measurement of **1** gave a molecular ion $[M]^+$ at 1185 *m/z*, in good agreement with the mass calculated for $C_{55}H_{61}O_{29}$ (1185.330), indicating the presence of one molecule of cyanidin, three molecules of glucose and two molecules of sinapic acid. By hydrolysis of **3** with 1 N HCl at room temp. for 5 days [1], demalonyl pigment **3** (Rt. 15.9 min) was detected by analysis of HPLC, and this demalonyl pigment **3** is identical to **1**. Furthermore, glucose was produced by H_2O_2 degradation of **1**. Thus, the structure of **1** is cyanidin 3-*O*-[β-D-glucopyranoside]-7,3′-di-*O*-[6-*O*- (sinapyl)-β-D-glucopyranoside], which is a new anthocyanin [8, 9]. This was fully confirmed by NMR measurements on **1** (Table 4).

Phalaenopsis *anthocyanins* **2** *and* **4**

The FAB mass spectra of **2** and **4** gave their $[M]^+$ at 1241 *m/z* and 1211 *m/z* (Tables 2 and 3). These values indicate that **2** is composed of cyanidin with three molecules of glucose and one molecule each of ferulic, sinapic and malonic acid, and **4** is composed of cyanidin with two molecules of ferulic acid and one molecule of malonic acid. By alkaline hydrolysis, both anthocyanins gave a same cyanidin 3,7,3′-triglucoside, and also **2** yielded ferulic and sinapic acids and **4**

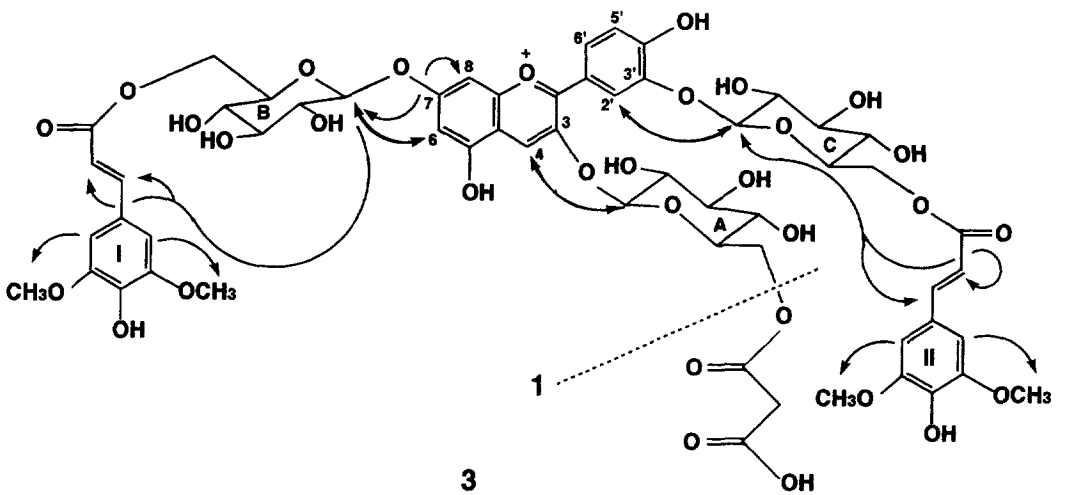

Fig. 1. *Phalaenopsis* anthocyanins. Observed NOEs are indicated by arrows.

Table 4. ^{1}H NMR (500 MHz) data of *Phalaenopsis* anthocyanins (CF_3CO_2D-DMSO-d_6, 1 : 9, at 30°)

H	**3**	**1**
Cyanidin		
4	8.62 *s*	8.69 *s*
6	6.69 *d* (2.0)	6.69 *d* (2.0)
8	6.98 *d* (2.0)	7.03 *d* (2.0)
2′	7.97 *br s*	7.99 *br s*
5′	7.02 *d* (8.3)	7.07 *d* (8.8)
6′	8.49 *dd* (8.3, 2.0)	8.51 *dd* (8.9, 2.0)
Glucose*,†		
(A) 3-G		
1	5.14	5.10
2	3.55	}
3	3.47	
4	3.31	} 3.29–3.85
5	3.81	
6a	4.18	
6b	4.52	}
(B) 7-G		
1	5.22	5.22
2	3.43	3.49
3	} 3.27–3.57	} 3.20–3.85
4		
5	3.81	3.79
6a	4.06	4.19
6b	4.86	4.89
(C) 3′-G		
1	5.18	5.20
2	3.47	3.45
3	3.45	} 3.20–3.85
4	3.29	
5	3.82	3.80
6a	4.19	4.08
6b	4.72	4.73
Sinapic acid*,†		
(I)		
2,6	6.43 *s*	6.43 *s*
α	6.17 *d* (15.7)	6.18 *d* (15.7)
β	7.25 *d* (15.7)	7.25 *d* (15.7)
-OCH_3	3.64 *s*	3.66 *s*
(II)		
2,6	6.67 *s*	6.64 *s*
α	6.44 *d* (15.9)	6.43 *d* (16.0)
β	7.44 *d* (15.9)	7.43 *d* (16.0)
-OCH_3	3.66 *s*	3.67 *s*
Malonic acid		
-CH_2-	3.42–3.49	—

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

yielded ferulic acid as hydroxycinnamic acids, respectively (Table 3). The further structure determination study of these pigments was, however, not possible due to the small amounts available for analysis. Based on the above findings, *Phalaenopsis* anthocyanins **2** and **4** are considered to be similar tri-acyl 3,7,3′-triglucosides of cyanidin to that of **3**. Therefore, these structures are assumed to be cyanidin 3,7,3′-triglucosides whose 7,3′-glucosides are acylated with hydroxycinnamic acids, and also whose 3-glucoside is acylated with malonic acid.

The occurrence of sinapyl cyanidin 3,7,3′-triglucoside was firstly reported in the flowers of *P. schilleriana* by Griesbach [1]. However, its detailed structure was not determined. In this study, it is clear that all *Phalaenopsis* anthocyanins are glycosylated at the 3, 7 and 3′ hydroxyls of cyanidin and, particularly, the glucosyl residues of the 7-OH and 3′-OH are acylated with hydroxycinnamic acids, and also that of the 3-OH was acylated with malonic acid in the cases of **2**–**4**. From a chemotaxonomic point of view, it is of note that the glycosylation pattern of *Phalaenopsis* anthocyanins is identical with those of *Bletilla* and × *Laeliocattleya* anthocyanins, but the acylation pattern of *Phalaenopsis* anthocyanins differs from those of *Bletilla* and × *Laeliocattleya* in that the 3′-glucosyl residue in cyanidin, of the *Phalaenopsis* anthocyanins is acylated with only one molecule of hydroxycinnamic acid, but *Bletilla* and × *Laeliocattleya* anthocyanins are more complex than this [3–5].

EXPERIMENTAL

Plant material. Phalaenopsis species and cultivars were grown in the greenhouses of Dogashima Orchid Center (Shizuoka, Japan) and also of Tokyo University of Agriculture (Tokyo, Japan). The fresh red-purple flowers were collected in spring of 1994 and 1995.

Isolation of Phalaenopsis *anthocyanins.* Fresh red–purple flowers (2 kg) of *P.* cultivars were extracted with MAW (20 l, methanol–acetic acid–water, 4 : 1 : 15). The extract was concd to 500 ml. The concd extract was purified by Diaion HP-20 CC, PC, TLC and HPLC by the previous procedures [2–6]. Solvents used were 15% HOAc, BAW (*n*-BuOH–HOAc–H_2O, 4 : 1 : 2), 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∅ × 150 mm) column at 40° with a flow rate of 4 ml min^{-1} monitoring at 530 nm for anthocyanins. Solvent systems used were as follows: 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 volume of 5% HOAc–EtOH followed by addition of excess Et_2O, and then drying to give pigment powders, pigment **1**, 10 mg; **2**, 3 mg; **3**, 30 mg; **4**, 3 mg.

Standard analysis. 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 BAW, EAA (EtOAc–HOAc–H_2O, 3 : 1 : 1), ETN (EtOH–NH_4OH–H_2O, 16 : 1 : 3) and EFW (EtOAc–HCOOH–H_2O, 5 : 2 : 1) for organic acids and sugars. The processes of acid hydrolysis, alkaline deacylation, H_2O_2 oxidation and

partial acid hydrolysis of anthocyanins were performed according to the standard procedures [10].

After H_2O_2 degradation of pigment **1** and **3**, prep. TLC of the products carried out using solvent BAW and the sugar component part of TLC was eluted with 50% MeOH and evapd to dryness. The residues were analysed by TLC. R_f values ($\times 100$) of the product-**1** (glucose) were 21 (BAW), 33 (EAA), 83 (ETN) and 52 (EFW), and the product-**3** (malonylglucose) were 25 (BAW), 47 (EAA), 73 (ETN) and 53 (EFW).

Authentic glucose and malonylglucose were used and prepared from commercial glucose (Tokyo Kasei Co., Ltd) and anthocyanins of *C. ternatea* [7], respectively.

Distribution of anthocyanins in the flowers of Phalaenopsis *species.* A fresh flower of each plant was extracted with small volume of MAW, and their analytical HPLC were performed on a Waters C_{18} (4.6∅ × 250 mm) column at 40° with a flow rate of 1 ml min^{-1} monitoring at 530 nm for anthocyanins. A solvent system used as follows: a linear gradient elution for 30 min from 40 to 85% solvent B in solvent A.

FAB mass and NMR measurements. FAB mass spectra were recorded in the positive mode with a magic bullet. NMR spectra were recorded at a frequency of 500 MHz for ^1H spectra in DMSO–d_6–CF_3COOD (9 : 1). Chemical shifts are reported relative to a TMS int. standard (δ) and coupling constants are reported in Hz.

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REFERENCES

1. Griesbach, R. J., *Lindleyana*, 1990, **5**, 231.
2. Saito, N., Toki, K., Uesato, K., Shigihara, A. and Honda, T., *Phytochemistry*, 1994, **37**, 245.
3. Tatsuzawa, F., Saito, N., Yokoi, M., Shigihara, A. and Honda, T., *Phytochemistry*, 1994, **37**, 1179.
4. Saito, N., Ku, M., Tatsuzawa, F., Lu, T. S., Yokoi, M., Shigihara, A. and Honda, T., *Phytochemistry*, 1995, **40**, 1523.
5. Tatsuzawa, F., Saito, N., Yokoi, M., Shigihara, A. and Honda, T., *Phytochemistry*, 1996, **41**, 635.
6. Tatsuzawa, F., Saito, N. and Yokoi, M., *Lindleyana*, 1996, **11**(4), 214.
7. Terahara, N., Saito, N., Honda, T., Toki, K. and Osajima, Y., *Phytochemistry*, 1990, **29**, 949.
8. Harborne, J. B. and Grayer, R. J., in *The Flavonoids, Advances in Research Since* 1986, ed. J. B. Harborne. Chapman and Hall, London, 1988, p. 1.
9. Strack, D. and Wray, V., in *The Flavonoids, Advances in Research since* 1986, ed. J. B. Harborne. Chapman and Hall, London, 1994, p. 1.
10. Harborne, J. B., *Phytochemical Methods*, 2nd edn. Chapman and Hall, London, 1984.