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ACYLATED CYANIDIN GLYCOSIDES IN THE ORANGE-RED FLOWERS OF *SOPHRONITIS COCCINEA*

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Key Word Index—*Sophronitis coccinea*; Orchidaceae; orange-red flower colour; acylated anthocyanins; cyanidin 3,7,3′-triglucoside; malonylglucoside; malonic, caffeic, *p*-coumaric and ferulic acids.

Abstract—Five acylated anthocyanins were isolated from the orange-red flowers of *Sophronitis coccinea*. Their structures were based on cyanidin 3,3′,7-triglucoside, acylated variously with malonic, *p*-coumaric, caffeic and ferulic acids. Three anthocyanins were fully determined to be cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside] and its demalonyl derivative, and cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-feruloyl)-β-D-glucopyranoside]. Two other pigments were partly characterized as *p*-coumaroyl cyanidin 3-malonylglucoside-7,3′-diglucoside and feruloyl cyanidin 3,7,3′-triglucoside.

INTRODUCTION

Sophronitis plants with red, orange, pink and yellow colours are popular orchid ornamentals, and native to Brazil. Recently some species of *Sophronitis* have been hybridised with *Cattleya* with the purpose of producing the red or orange-red flower colour cultivars in the *Cattleya* alliance. There are two preliminary reports on the occurrence of anthocyanins in the flowers of *Sophronitis* [1, 2]. In continuing work on flower colour variation in orchids, we have already reported the occurrence of acylated cyanidin and peonidin glycosides in the flowers of *Dendrobium* "Pramot" [3], × *Laeliocattleya* "Mini Purple" [4, 5], *Bletilla striata* [6], *Cymbidium* hybrids [7] and *Phalaenopsis* hybrids [8]. In this paper we report the occurrence of new acylated cyanidin 3,7,3′-triglucosides in the orange-red flowers of *Sophronitis coccinea* and their structural elucidation.

RESULTS AND DISCUSSION

By HPLC analysis of the MAW (methanol-acetic acid-water, 4:1:15) extracts from the orange-red flowers of *Sophronitis coccinea*, we observed the presence of twenty anthocyanin peaks. Five anthocyanins (**1**-**5**) were isolated from this extracts, and purified using Diaion HP-20 column chromatography (C), paper C, HPLC and TLC. The relative concentrations were **1** (49.3%), **2** (16.4%), **3** (12.3%), **4** (6.7%) and **5** (6.3%). The Rf values, Rt (min) and spectral data of these five pigments are shown in Table 1.

Acid hydrolysis of these anthocyanins gave cyanidin, glucose and hydroxycinnamic acids. By alkaline hydrolysis these five pigments yielded only one deacylanthocyanin, identified as cyanidin 3,7,3′-triglucoside by direct comparison with an authentic sample from deacyl *Dendrobium*, × *Laeoliocattleya* and *Bletilla* anthocyanins [3–6]. Also as acyl moieties, caffeic acid was detected in the hydrolysis products of **1** and **2**, ferulic acid in **3** and **5**, and *p*-coumaric acid in **4**, and malonic acid in **1**, **3** and **4**, respectively, by acid and alkaline hydrolysis.

The measurements of FAB mass and ^{1}H NMR spectra of these five acylated anthocyanins led to the determination of the molecular ratios of chemical composition (aglycone, sugar and acid) as shown in Table 2. Among these five pigments, the detailed structures of the three pigments (**1**–**3**) were successfully determined by spectral and chemical methods, but the other two pigments (**4**, **5**) could not be determined because of the small amounts obtained.

Pigment **1** *and* **2**

The FAB mass measurement of **1** gave a molecular ion $[M]^+$ at 1021 *m/z* in good agreement with the mass

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Table 1. Chromatographic and spectral properties of anthocyanins from flowers of *Sophronitis*

Anthocyanin	Rf values (× 100)				Spectral data in 0.1% HCl-MeOH					Rt (min)	FAB-MS $[M]^+$
	BAW	BuHCl	1% HCl	AHW	λ_{max} (nm)	E_{UV}/E_{max}(%)	E_{acyl}/E_{max}(%)	E_{440}/E_{max}(%)	$AlCl_3$		
1	23	2	17	45	521,332,283	80	60	29	0	19.8	1021
2 (demalonyl 1)	21	2	19	41	521,332,283	91	70	33	0	16.3	935
3	33	4	25	48	524,331,284	89	79	28	0	23.0	1035
4	39	4	27	51	520,319,284	103	92	28	0	23.3	1005
5	23	2	15	42	523,332,283	82	68	27	0	20.4	949
Deacyl (1–5)	11	1	36	64	513,280			37	0	5.3	
Cy 3,7,3′-triglc.*	11	1	36	64	513,280			36	0	5.3	773

*Refs [4–6, 8].

Table 2. The estimated molecular formulae of acylated anthocyanins from *Sophronitis* and their molecular ratios of chemical composition based on FAB mass and ^1H NMR data.

Anthocyanin	Based on FAB-MS*								Based on ^1H NMR**					
	$[M]^+$	Mf	Cy:	Glc:	pC:	Caf:	Fer:	Mal	Cy:	Glc:	pC:	Caf:	Fer:	Mal
1	1021	$C_{45}H_{49}O_{27}$	1	3	0	1	0	1	1	3	0	1	0	1
2	935	$C_{42}H_{47}O_{24}$	1	3	0	1	0	0	1	3	0	1	0	0
3	1035	$C_{46}H_{51}O_{27}$	1	3	0	0	1	1	1	3	0	0	1	1
4	1005	$C_{45}H_{49}O_{26}$	1	3	1	0	0	1	1	3	1	0	0	1
5	949	$C_{43}H_{49}O_{24}$	1	3	0	0	1	0	—	—	—	—	—	—

Abbreviations: *$[M]^+$ and Mf = molecular ion mass values, and estimated molecular formulae as flavylium forms of anthocyanins isolated from *Sophronitis* based on FAB-mass data, respectively.Cy: Glc: pC: Caf: Fer: Mal = molecular numbers of their components; Cy = cyanidin, Glc = glucose, pC = *p*-coumaric acid, Caf = caffeic 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 hydroxycinnamic acid = olefinic proton (H-α). Each integrated intensity of proton signal was normalized in such a way that cyanidin H-4 is 1, respectively.

calculated for $C_{45}H_{49}O_{27}$ (1021.246), indicating the presence of one molecule of cyanidin, three of glucose, one of caffeic acid and one of malonic acid (Tables 2 and 3). The 500 MHz proton NMR spectra of **1** were measured in DMSO-d_6 solvent containing 10% TFA-*d*. The proton signals were mainly assigned by analysis of its ^{1}H-^{1}H COSY spectrum, and the sugar and acyl linkages were confirmed by the negative difference nuclear Overhauser effect (DIFNOE) spectra as described previously [9] (Fig. 1). The proton signals of the sugar moieties were observed in the region of δ 5.45–3.13. The signals of three anomeric protons appeared at δ 5.45 (*d*, $J = 8.0$ Hz, Glc A), δ 5.30 (*d*, $J = 7.5$ Hz, Glc B) and δ 4.88 (*d*, $J = 7.5$ Hz, Glc C), and the assigned sugar protons had the coupling constants with $J = 7.5$–12.0 Hz indicating all these glucose units to be β-D-glucopyranose forms. Four methylene protons, being shifted to a lower magnetic field, were

Table 3. ^{1}H NMR data of *Sophronitis* anthocyanins (CF_3CO_2D-DMSO-d_6, 1:9, at 25°C)

H	1	2	3
Cyanidin			
4	8.89 *s*	8.99 *s*	8.85 *s*
6	6.83 *brs*	6.82 *brs*	6.82 *brs*
8	7.50 *brs*	7.51 *brs*	7.50 *d* (2.0)
2′	8.18 *brs*	8.19 *brs*	8.10 *d* (2.0)
5′	7.07 *d* (9.5)	7.08 *d* (8.5)	7.01 *d* (8.0)
6′	8.63 *brd* (9.5)	8.63 *brd* (8.5)	8.57 *brd* (8.0)
Hydroxycinnamyic acid*, **			
(I)			
2	6.81 *brs*	6.81 *brs*	6.82 *brs*
5	6.56 *d* (8.5)	6.55 *d* (8.5)	6.55 *d* (7.5)
6	6.68 *brd* (8.5)	6.71 *brd* (8.5)	6.66 *brd* (7.5)
α	6.20 *d* (15.5)	6.20 *d* (16.0)	6.38 *d* (15.5)
β	7.38 *d* (15.5)	7.37 *d* (16.0)	7.39 *d* (15.5)
-OCH_3	—	—	3.83 *s*
Glucose *, **			
(A) 3-G			
1	5.45 *d* (8.0)	5.52 *d* (7.5)	5.48 *d* (8.0)
2	3.53	3.27	3.53
3	3.43	3.10 ~ 3.92	3.27 ~ 3.40
4	3.30		
5	3.87		3.88
6a	4.18		4.14
6b	4.50 *d* (10.5)		4.49
(B) 7-Gt			
1	5.30 *d* (7.5)	5.30 *d* (7.5)	5.32 *d* (7.5)
2	3.40	3.37	3.43
3	3.36	3.10 ~ 3.92	3.20 ~ 3.80
4	3.52		
5	3.81		3.85
6a	4.20	4.20	4.18
6b	4.58 *d* (11.5)	4.60 *d* (12.0)	4.59
(C) 3′-G			
1	4.88 *d* (7.5)	4.88 *d* (7.5)	4.87 *d* (7.5)
2	3.38	3.40	3.39
3	3.28	3.10 ~ 3.92	3.27 ~ 3.36
4	3.13		3.11
5	3.52		3.55
6a	3.55		3.56
6b	3.90		3.91
Malonic acid			
-CH_2-	3.43	—	3.43

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

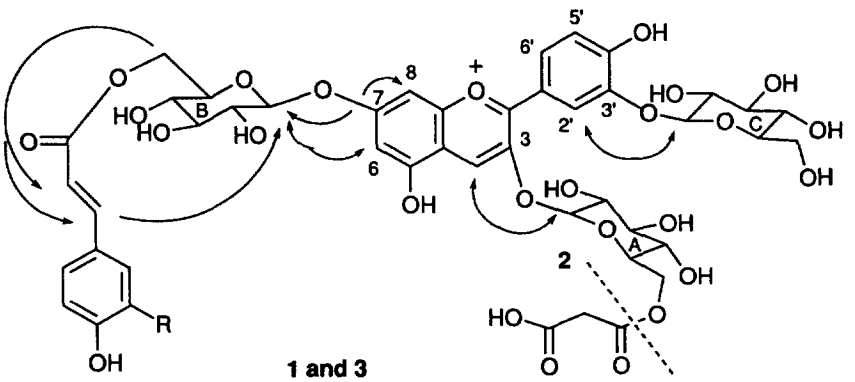

Fig. 1. Anthocyanins from *Sophronitis coccinea*. R 1: OH 2: OH 3: OCH_3. Observed NOEs are indicated by arrows.

assigned to H-6a and 6b of Glc A (δ 4.18 and 4.50) and those of Glc B (δ 4.20 and 4.58) and also were correlated to their anomeric protons by analysis of the ^{1}H-^{1}H COSY spectrum. This result indicated that these two glucose units were acylated with acids at their OH-6 groups.

In order to determine the linkages and/or positions of the attachments between the glucose and acyl units in this pigment molecule, DIFNOE spectra of **1** were measured (Fig. 1). Observed DIFNOE spectra between H-1 of Glc A and H-4 of cyanidin indicates that Glc A is attached to OH-3 of cyanidin through a glycosidic bond by irradiations of H-1 of Glc A and H-4 of cyanidin. Glc B was determined to be attached to the OH-7 of cyanidin through a glycosidic bond, because of the presence of NOEs between H-6 and H-8 of cyanidin and H-1 of Glc B. Similarly, Glc C was determined to be glycosylated at OH-3′ of cyanidin, because of the presence of NOEs between H-2′ of cyanidin and H-1 of Glc C. Moreover, irradiation at H-6b of Glc B gave a DIFNOE spectrum in which NOEs to protons of H-α and β of caffeic acid were observed. Thus, caffeic acid is confirmed to be attached to OH-6 of Glc B. By H_2O_2 degradation of **1** malonylglucose was detected, indicating that malonic acid is attached to 6-OH of Glc A [10]. Therefore, **1** is cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucopyranoside], which is a new anthocyanin [11, 12].

The FAB mass measurement of **2** gave a molecular ion $[M]^+$ at 935 *m/z* in good agreement with the mass calculated for $C_{42}H_{47}O_{24}$ (935.246), indicating the presence of one molecule of cyanidin, three molecules of glucose and one molecule of caffeic acid. The demalonyl pigment of **1** was prepared by the treatment of **1** with 1N HCl-H_2O according to previous procedure [3, 8, 13]. The Rf values, Rt and spectral data of the demalonyl pigment **1** were good identical with those of **2** (Table 1). Furthermore, by analysis of ^{1}H NMR and ^{1}H-^{1}H COSY spectra of **2** the proton chemical shifts of **2** were in good agreement with those of **1** without the proton signals of malonic acid and Glc A moieties (Table 3). In particular, the methylene proton signals of Glc A were shifted in the upper magnetic field than those of **1**, indicating that the OH-6 of Glc A was free from malonic acid. Therefore, the structure of **2** was determined to be demalonyl **1**, namely, cyanidin 3,3′-di-*O*-[β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside], which is a new anthocyanin [11, 12].

Pigment **3**, **4** *and* **5**

The FAB mass spectra of **3**, **4** and **5** gave their molecular ions 1035 *m/z* (**3**), 1005 *m/z* (**4**) and 949 *m/z* (**5**) as shown in Tables 1 and 2. These values are in good agreement with the mass calculated for their theoretical molecules (Table 2), respectively, which are composed of cyanidin 3,7,3′-triglucoside with each of *p*-coumaric or ferulic acid. The pigments **3** and **4** have malonic acid as an additional acid. Their Rf values, Rt (min) and spectral properties are summarized in Table 1. By H_2O_2 degradation of **3** and **4**, malonylglucose was detected, however similar treatment of **5** afforded glucose without any acyl residue, indicating that the 3-glucose residues of **3** and **4** are substituted with malonic acid and that of **5** is free from malonic acid. On alkaline hydrolysis **3**–**5** gave cyanidin 3,7,3′-triglucoside as their deacylanthocyanin, where ferulic acid was obtained in **3** and **5**, and *p*-coumaric acid was detected in **4**. Based on these findings, the molecular ratios of cyanidin: glucose:acyls (acids) in **3**, **4** and **5** were determined by the analysis of FAB mass data as shown in Table 2. Furthermore, the structure of **3** was confirmed by analysis of their ^{1}H NMR spectra including ^{1}H-^{1}H COSY spectra. The ^{1}H NMR spectrum of **3** was superimposed on that of **1** except for the signals of methoxyl group in ferulic acid moieties (Table 3). Also, four methylene proton signals of Glc A and B were shifted to the lower magnetic field (δ 4.14, 4.49 and δ 4.18, 4.59). Thus, **3** is cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-feruloyl)-β-D-glucopyranoside]-3′-*O*-[β-D-glucopyranoside], which is a new pigment [11, 12].

In the ^{1}H NMR spectrum of **4**, the chemical shifts of cyanidin and *p*-coumaric acid moieties were determined, but other proton chemical shifts except three anomeric and four methylene protons were not detected due to the heavy overlapping of proton signals. Also the linkages between glucose units and a *p*-coumaric acid unit could not be determined because of a small amount of **4** obtained. Thus, **4** was tentatively assigned as *trans-p*-coumaroyl cyanidin 3-malonylglucoside-7,3′-diglucoside. The further structure study of **5** was not carried out because of its low yield. Consequently the structure of **5** was assumed to be feruloyl cyanidin 3,7,3′-triglucoside.

Six papers have so far been published on the occurrence of anthocyanidin 3-malonylglucosides in the flowers of *Dendrobium*, *Laelia*, *Cattleya*, *Bletilla*, *Cymbidium*, and *Phalaenopsis* [3–8, 14]. Thus, this is the seventh report of occurrence of malonylated anthocyanin in orchid flowers. Our previous studies showed that the orchids with red-purple flowers, such as *Cattleya*, *Bletilla* contained di- or tri-(hydroxycinnamoyl) cyanidin 3,7,3′-triglucosides as their major pigments [3–6, 8]. However, *Sophronitis coccinea* contained mono-(hydroxycinnamoyl) cyanidin 3,7,3′-triglucosides with carotenoid pigments [2]. A simple acylation in *Sophronitis* flowers might be thought to play an important role in producing orange-red flowers [16]. According to Manuel *et al.*, this colour of flowers attracts hummingbirds as a pollinator [15, 17] as contrasted with the red-purple flowered orchids with di- or tri-aromatic acid-acylation which are known to attract insects for pollination [18].

EXPERIMENTAL

Plant material

The flowers of *Sophronitis coccinea* were obtained from the cultivators of Utsunomiya Ranyukai (Utsunomiya Orchid Club, Tochigi, Japan), Mr. M. Abou (Gifu, Japan) and Mr. T. Yamamoto (α-Orchid Ltd., Shizuoka, Japan). The fresh orange-red flowers were collected in spring of 1994 and 1995.

Isolation of Sophronitis anthocyanins

Dried orange-red flowers (100 g) of *S. coccinea* were extracted with MAW (1 l, MeOH-HOAc-H_2O, 4:1:15). The extract was purified by Diaion HP-20, CC, PC, TLC and HPLC by the previous procedures [3, 4, 5, 6, 7, 8]. 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} (19ϕ × 150 mm) column at 40°C 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 25 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**, 30 mg; **2**, 10 mg; **3**, 8 mg; **4**, 5 mg; **5**, 3 mg.

Standard analysis

Characterization of pigments were carried out using PC, TLC and UV-VIS spectrometry. Solvents used were BAW, BuH (n-BuOH-2N 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 [19].

After H_2O_2 degradation of pigment **1**–**5**, prep. TLC of the products was carried out using solvent BAW and the sugar component part of TLC was eluted with 50% MeOH and evapd to dryness. The residues were analyzed by TLC. Rf values (× 100) of the product-**2** and **5** (glucose) were 21 (BAW), 33 (EAA), 83 (ETN), 52 (EFW), and the product-**1**, **3** and **4** (malonylglucose) were 25 (BAW), 47 (EAA), 73 (ETN), 53 (EFW).

Authentic glucose was purchased from (Tokyo Kasei Co, Ltd.) and malonylglucose was prepared from anthocyanins of *C. ternatea* by H_2O_2 degradation [9].

Analytical HPLC was performed on a Waters C_{18} (4.6ϕ × 250 mm) column at 40°C with a flow rate of 1 ml min^{-1} monitoring at 530 nm for anthocyanins. A solvent system used was as follows: a linear gradient elution for 40 min from 25 to 85% solvent B in solvent A.

FAB mass and NMR measurements

FAB mass spectra were recorded in positive mode using the magic bullet and in negative mode in glycerol. NMR spectra were recorded at 500 MHz for ^{1}H 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.

^{1}H NMR spectral data

Pigment **4**: cyanidin δ 8.87 (1H, *s*, H-4), 6.82 (1H, *brs*, H-6), 7.51 (1H, *brs*, H-8), 8.17 (1H, *brs*, H-2′), 7.08 (1H, *d*, J = 7.5, H-5′), 8.64 (1H, *brd*, J = 7.5, H-6′), *p*-coumaric acid 7.18 (2H, *d*, J = 8.5, H-2,6), 6.54 (2H, *d*, J = 8.5, H-3,5), 6.28 (1H, *d*, J = 15.0, H-α), 7.42 (1H, *d*, J = 15.0, H-β), Sugars 5.47 (1H, *d*, J = 7.3, Glc A-1), 5.31 (1H, *d*, J = 7.0, Glc B-1), 4.86 (1H, *d*, J = 7.6, Glc C-1), 4.20 (1H, *m*, Glc A-6a), 4.40 (1H, *brd*, J = 11.0, Glc A-6b), 4.23 (1H, *m*, Glc B-6a), 4.59 (1H, *brd*, J = 11.5, Glc B-6b) [14].

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