

Acylated Anthocyanins from Flowers of Cineraria, *Senecio cruentus*, Red Cultivar

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The structures of the two pigments in the red Cineraria, *Senecio cruentus*, flowers have been identified as malonylcaffeylecyanidin 3,3'-diglucoside (**2**) and as its demalonyl compound (**1**), new anthocyanins, by using chromatographic and spectroscopic techniques involving UV-VIS, IR, FAB-MS and NMR. In a neutral aqueous solution, the acylated anthocyanins retained their colorings for a long time.

Introduction

Cinerarin, the anthocyanin isolated from the blue flowers of garden cineraria, *S. cruentus* by Yoshitama *et al.* [1, 2], was identified as dicaFFEYlated delphinidin 3,7,3'-triglucoside on the basis of the chemical evidences and its extraordinary stability in a neutral aqueous solution was also pointed out. Goto *et al.* [3] established its complete structure as malonyltricaFFEYl delphinidin-tetra-glucoside mainly by means of NMR methods. On the other hand, Yoshitama *et al.* [4, 5] characterized the red pigment, named rubrocinerarin, from the cineraria red cultivar flowers, and tentatively determined its structure as cyanidin analogue of cinerarin with the same substituents.

Abbreviations: FAB-MS, fast atom bombardment-mass spectrometry; NMR, nuclear magnetic resonance; UV-VIS, ultraviolet and visible spectroscopy; IR, infrared spectroscopy; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; ODS, octadecylsilyl; Cy 3-Glc, cyanidin 3-glucoside; Cy 3,5-Glc, cyanidin 3,5-diglucoside; Ma-Glc, malonylglucose; TFA, trifluoroacetic acid; 2D-COSY, two-dimensional correlation spectroscopy; NOE, nuclear Overhauser effect; n.d., not detected; Fl, fluorescence; Ab, absorption.

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In the course of a survey of acylated anthocyanins in view of their utilization as natural food colorants and for chemotaxonomic interest, we have investigated the structures of the two major anthocyanins isolated from other cineraria red cultivar, and here describe their determination and stability in a neutral aqueous solution.

Materials and Methods

General procedures

TLC was carried out on microcrystalline cellulose plates (Avicel SF, Funakoshi) using solvent systems such as AHW (AcOH-conc. HCl-H₂O = 15:3:82, v/v), AAH (conc. HCl-AcOH-H₂O = 1:3:8), BAW (*n*-BuOH-AcOH-H₂O = 4:1:2), HCL (conc. HCl-H₂O = 3:97), BUH (*n*-BuOH-2N HCl = 1:1, upper phase), FOS (conc. HCl-AcOH-H₂O = 3:30:10), FOM (conc. HCl-HCOOH-H₂O = 2:5:3) for pigments, and EAA (EtOAc-AcOH-H₂O = 3:1:1), BEW (*n*-BuOH-EtOH-H₂O = 4:1:2.2), EFW (Et₂O-HCOOH-H₂O = 5:2:1), PFW (Phenol-HCOOH-H₂O = 75:1:25) for sugars and organic acids. The sample spots on chromatograms were detected with a UV-lamp for cinnamic acids, bromocresol green (BCG) spray reagent for aliphatic acids, and aniline hydrogenphthalate (AHP) spray reagent for sugars, respectively. HPLC was performed on an

L-6200 Intelligent pump system (Hitachi). Analytical HPLC was run on Inertsil ODS-2 (4.6 $\varnothing$ $\times$ 50 mm + 4.6 $\varnothing$ $\times$ 250 mm, GL Sciences Inc.) column at 35 $^{\circ}$ with a flow rate of 1 ml/min monitoring at 312 nm for UV-absorbing compounds and 530 nm for anthocyanins. Solvent systems employed were as follows: a linear gradient elution for 7 min from 25% to 32% solvent B, then for 40 min from 32% to 42% solvent B (1.5% H₃PO₄, 20% AcOH, 25% MeCN in H₂O) in solvent A (1.5% H₃PO₄ in H₂O). Preparative HPLC was done on Develosil ODS-5 (20 $\varnothing$ $\times$ 50 mm + 20 $\varnothing$ $\times$ 250 mm, Nomura Chemical) column with 7–10 ml/min by isocratic elution using mixture of solvent A (15% AcOH in H₂O) and solvent B (15% AcOH, 30% MeCN in H₂O), A:B = 3:1 at 520 nm. IR spectra were recorded on 270-30 (Hitachi) spectrometer in KBr, UV-VIS spectra on MPS-2000 (Shimadzu) spectrophotometer, FAB-MS spectra on JMX SX-102 (JEOL), and ¹H (400 MHz) and ¹³C (100 MHz) NMR on JNM GX-400 (JEOL) in DMSO-*d*₆:CF₃COOD = 9:1 with internal standard Me₄Si.

Reference compounds

Cy 3-Glc and Cy 3,5-Glc were isolated from *Glycine soja* seeds and *Rosa hybrida* flowers, respectively. Six standard aglycones (anthocyanidins) were prepared by complete acid hydrolysis of corresponding anthocyanins isolated from suitable plant sources [6]. Ma-Glc was prepared from *Cinerarin* [3] by H₂O₂ oxidation. Other authentic sugars and organic acids were purchased commercially in Japan.

Plant materials

The seeds of *Senecio cruentus* cv. Starlet Red Shade were purchased from Sakata Nursery in Japan, and seeded in September 1990 in the garden of Minami-Kyushu University. The red flower petals were collected on April 1991 and dried at 50 $^{\circ}$ C overnight, and stored in silica-gel desiccator until experimental use.

Isolation of pigments

The dried petals (9.5 g) were steeped in 5% AcOH (21 $\times$ 2) overnight and filtered. The crude pigment filtrate contained two anthocyanins (**1** and **2**) at retention times (contents %) **1**: 18.36

(40%) and **2**: 21.00 (58%) min respectively by HPLC analysis. The red extract was applied on a HP-20 (Diaion) resin column (45 $\varnothing$ $\times$ 200 mm). The column was washed with 1% AcOH (2 l) and then eluted with 1% AcOH in 70% EtOH. The pigment fraction was separated on a Sephadex LH-20 column and finally purified by a preparative ODS-HPLC using AcOH solvent system. The major anthocyanin fractions were evaporated to dryness, dissolved in a small amount of TFA, and precipitated with excess Et₂O to give TFA salts of **1** and **2** as red powders. The anthocyanins **1** and **2** were obtained in amounts of *ca.* 11 and 26 mg, respectively.

Preparation of deacylated anthocyanin (**3**)

Ten mg of **2** were dissolved in 1 ml of 2 N NaOH and N₂ was bubbled into the solution and saturated for 5 min. Then the solution was kept standing for 30 min, acidified with 1.2 ml of 2 N HCl, and washed five times with 2 ml of Et₂O. The Et₂O washings were used for the analysis of the acylating acid. The aqueous layer was subjected to Amberlite XAD-2000 column (20 $\varnothing$ $\times$ 100 mm), washed with 1% AcOH and eluted with 1% AcOH in 70% EtOH and the eluate was evaporated *in vacuo* to dryness. The residue was powdered with TFA–Et₂O described above and 4 mg of deacylated anthocyanin TFA salt (**3**·TFA) was obtained as dark red powders.

Hydrolytic analyses

Complete and partial acid hydrolyses [7] and alkaline hydrolysis, and H₂O₂ oxidation [8] were performed in the usual manner.

Stability test

Pigments were weighed and dissolved in pH 7.0 buffer solution (McIlvaine buffer: 0.1 M citrate–0.5 M phosphate mixture) in concentration 5 $\times$ 10^{–5} M. Molecular weights of anthocyanins were calculated as mono TFA salts. The VIS spectra were measured immediately after dissolution, and the measurements were repeated for appropriate interval times at room temperature (*ca.* 25 $^{\circ}$ C). The relative residual color intensities were calculated based on the absorbance at visible maxima ($\lambda_{\text{vis-max}}$) which initial data was 100%.

Cyanidin-3-glucoside-3'-caffeylglucoside·TFA (**1**)

UV-VIS $\lambda_{\max}$ (0.01% HCl–MeOH) nm 526 (not shifted bathochromically with AlCl₃), 332, 283, $E_{\text{acid-max}}/E_{\text{vis-max}} = E_{332}/E_{526} = 76\%$, $E_{440}/E_{526} = 40\%$; IR $\nu_{\max}$ (KBr) cm⁻¹ 3404 (O–H), 1684 (C=O), 1644 (aromatic C=C), 1608 (aromatic C=C), 1074 (C–O); FAB-MS (positive mode with *m*-nitrobenzyl alcohol): m/z 773 [M = C₃₆H₃₇O₁₉]⁺, +NaI 795 [M + Na = C₃₆H₃₆O₁₉Na]⁺.

Cyanidin 3-malonylglucoside-3'-caffeylglucoside·TFA (**2**)

UV-VIS $\lambda_{\max}$ (0.01% HCl–MeOH) nm 528 (not shifted bathochromically with AlCl₃), 332, 282, $E_{\text{acid-max}}/E_{\text{vis-max}} = E_{332}/E_{528} = 73\%$, $E_{440}/E_{528} = 40\%$; IR $\nu_{\max}$ (KBr) cm⁻¹ 3404 (O–H), 1684 (C=O), 1644 (aromatic C=C), 1608 (aromatic C=C), 1064 (C–O); FAB-MS (positive mode with *m*-nitrobenzyl alcohol): m/z 859 [M = C₃₉H₃₉O₂₂]⁺, +NaI 881 [M + Na = C₃₉H₃₈O₂₂Na]⁺; high resolution FAB-MS: found m/z 859.1932, calcd for C₃₉H₃₉O₂₂⁺ = 859.1933, error –0.1 mmu.

Cyanidin 3, 3'-diglucoside·TFA (**3**)

UV-VIS $\lambda_{\max}$ (0.01% HCl–MeOH) nm 517 (not shifted bathochromically with AlCl₃) 278, $E_{440}/E_{517} = 37\%$; IR $\nu_{\max}$ (KBr) cm⁻¹ 3412 (O–H), 1680 (aromatic C=C), 1646 (aromatic C=C), 1074

(C–O); FAB-MS (positive mode with *m*-nitrobenzyl alcohol): m/z 611 [M = C₂₇H₃₁O₁₆]⁺, +NaI 633 [M + Na = C₂₇H₃₀O₁₆Na]⁺.

Results and Discussion

Petals of *S. cruentus* were extracted with 5% AcOH and the extract was purified by HP-20 and LH-20 resin columns and subsequent preparative ODS-HPLC using AcOH solvent system. Two major anthocyanins, **1** and **2**, were obtained as TFA salts of red powders (Table I).

On acidic hydrolysis, both **1** and **2** gave cyanidin (Cy) as aglycone (Table I), and D-glucose (Glc) as sugar component (Table II). In the UV-VIS spectra of **1** and **2**, the presence of the characteristic absorptions at 332 nm suggested that these were acylated with cinnamic acid(s) [6]. This was also confirmed by the presence of conjugated ester carbonyl bands at 1684 cm⁻¹ in the IR spectra. The number of the binding aromatic acid(s) were estimated to be 1–2 mols in **1** and **2** due to the values of $E_{\text{acid-max}}/E_{\text{vis-max}}$ (ca. 75%) [6]. On alkaline hydrolysis, the aromatic acids were identified to be caffeic acid (Cf) in **1**, and Cf and malonic acid (Ma) in **2** as depicted in Table II. Apart from organic acids, as shown in Table II the alkaline hydrolysis of **1** and **2** gave the same deacylated anthocyanin (**3**) which was determined to have the dimonogly-

Table I. Chromatographic properties of the red *S. cruentus* flower pigments **1**, **2** and related compounds.

Pigment	AHW	AAH	TLC [$R_f \times 100$]				FOS	FOM	Color	Identity
			BAW	HCL	BUH					
1	33	44	54	4	63	–	–	–	red	–
2	34	46	52	4	63	–	–	–	red	–
Deacylated anthocyanin										
of 1	40	50	46	17	10	–	–	–	red	–
of 2 (= 3)	40	50	46	17	10	–	–	–	red	–
Aglycone										
of 1	7	13	–	–	–	43	35	–	magenta	Cy
of 2	7	13	–	–	–	43	35	–	magenta	Cy
Authentic sample										
Cy 3-Glc	22	31	62	6	22	–	–	–	magenta	–
Cy 3,5-Glc	33	44	43	12	8	–	–	–	magenta	–
Pelargonidin	12	21	–	–	–	62	56	–	red	–
Cyanidin	6	13	–	–	–	42	35	–	magenta	–
Peonidin	8	15	–	–	–	58	48	–	magenta	–
Delphinidin	4	6	–	–	–	23	19	–	purple	–
Petunidin	5	9	–	–	–	37	30	–	purple	–
Malvidin	6	14	–	–	–	55	46	–	purple	–

Abbreviations: see footnote.

Table II. Chromatographic properties of sugar, H₂O₂ oxidation products, and acylating acids of the red *S. cruentus* flower pigments **1**, **2** and related compounds.

Sample	EAA	TLC [$R_f \times 100$]				PFW	Colored by		Identity
		BAW	BEW	EFW			AHP	BCG	
Sugar									
of 1	31	46	31	29	50	brown	n.d.	Glc	
of 2	31	46	31	29	50	brown	n.d.	Glc	
H ₂ O ₂ oxidation product									
of 1	31	46	31	29	50	brown	n.d.	Glc	
of 2	49	54	31	41	56	brown	yellow	Ma-Glc	
of 3	31	46	31	29	50	brown	n.d.	Glc	
Acylating acid									
of 1	94	99	88	86	87	blue Fl	n.d.	Cf	
of 2	94	99	88	86	87	blue Fl	n.d.	Cf	
	81	85	66	82	63	n.d.	yellow	Ma	
Authentic sample									
D-Glucose	31	46	31	29	50	brown	n.d.	—	
D-Galactose	28	44	33	28	55	brown	n.d.	—	
D-Arabinose	38	52	36	39	62	red	n.d.	—	
D-Xylose	44	53	40	42	55	red	n.d.	—	
Ma-Glc	49	54	31	41	56	brown	yellow	—	
<i>p</i> -Coumaric acid	98	94	96	94	85	purple Ab	n.d.	—	
Caffeic acid	94	98	88	86	87	blue Fl	n.d.	—	
Ferulic acid	98	99	94	94	91	blue Fl	n.d.	—	
Sinapic acid	96	99	91	93	89	blue Fl	n.d.	—	
Malonic acid	81	85	66	82	63	n.d.	yellow	—	
Succinic acid	90	96	82	86	73	n.d.	yellow	—	

Abbreviations: see footnote.

cosidic substitution pattern by partial acid hydrolysis [7] because other than itself, **3** gave three hydrolyzed pigments involving Cy 3-Glc and Cy. As shown in Table II, H₂O₂ oxidation of **3** gave Glc showing the presence of one Glc at 3-OH of Cy [8]. Other Glc was determined to be at the position 3'-OH on B-ring based on the following evidences. The visible maximum wavelength ($\lambda_{\text{vis-max}} = 517$ nm) of **3** occurred the hypsochromic shift from those of common Cy-diglycosides (*e.g.* $\lambda_{\text{vis-max}} = 526$ nm of Cy 3,5-Glc) and the $\lambda_{\text{vis-max}}$ of **3** as well as those of **1** and **2** didn't shift bathochromically on addition of AlCl₃ solution, and the large value of $E_{440}/E_{\text{vis-max}} = 37\%$ (*cf.* 13% of Cy 3,5-Glc) showed it to have 3,3'-diglycosidic substitution pattern [5]. On FAB-MS measurement, **3** gave a molecular ion m/z 611 and Na⁺ adduct at 633 hence its molecular formula as the flavylum ion was deduced to be C₂₇H₃₁O₁₆⁺ which is composed of Cy and two molecules of Glc.

The above estimated structure of **3** was verified by ¹H NMR spectra which could be assigned with the aid of 2D-COSY as shown in Table III. In ¹H

NMR spectra of **3**, six characteristic proton peaks (H-4, 6, 8, 2', 5', 6') in the downfield region showed those of the aglycone moiety, Cy. In the sugar region, the anomeric protons of two sugars (A-1 and B-1) in downfield with large *J* values (7 ~ 8 Hz) showed the sugar moieties to have β forms and ring protons with large *J* values (7 ~ 8 Hz) showed the sugar moieties to have pyranoside forms. To determine the position of the glycosidic linkages, 1D-difference NOE (DIFNOE) was applied. Since on irradiation at A-1 and B-1, negative NOEs were observed at the H-4 and H-2', respectively, A-Glc and B-Glc showed to be attached at 3-OH and 3'-OH on Cy, respectively. Similarly, the observation of NOE for A-1 by irradiation at H-4 confirmed the presence of A-Glc at the 3-position unambiguously. Thus the chemical structure of **3** was identified as 3,3'-di-O-β-D-glucopyranosylcyanidin (Cy 3,3'-Glc) as shown in Fig. 1.

Molecular weights of the flavylum ions **1** and **2** were determined as 773 and 859 corresponding to C₃₆H₃₇O₁₉⁺ and C₃₉H₃₉O₂₂⁺, respectively, based on FAB-MS data, showing **1** to be composed with

Table III. ^1H NMR data of the red *S. cruentus* flower pigments **1**, **2** and their deacylated derivative **3** in $\text{DMSO}-d_6:\text{CF}_3\text{COOD} = 9:1$ (δ ppm).

	1	2	3
Aglycone moiety			
4	8.74 s	8.66 s	8.97 s
6	6.88 s	6.88 d (1)	7.05 br. s
8	6.60 s	6.63 d (2)	6.72 d (2)
2'	8.09 d (2)	8.10 d (2)	8.17 d (2)
5'	7.10 d (9)	7.10 d (9)	7.09 d (9)
6'	8.47 br. d (9)	8.44 dd (2, 9)	8.51 dd (2, 9)
Caffeyl moiety			
2''	5.90 d (16)	5.89 d (16)	
3''	7.12 d (16)	7.12 d (16)	
5''	6.54 br. s	6.50 d (1)	
8''	6.59 d (8)	6.43 br. d (8)	
9''	6.43 br. d (8)	6.58 d (8)	
Malonyl moiety			
2'''		3.38 d (5)	
A-Glucosyl moiety			
A1	5.15 d (8)	5.18 d (8)	5.34 d (8)
A2	3.44 m	3.50 t (8)	3.42 t (8)
A3	3.25 t (9)	3.43 m	3.22 m
A4	3.39 t (9)	3.24 t (9)	3.30 m
A5	3.50 t (8)	3.79 br. t (10)	3.40 m
A6A	3.75 d (11)	4.48 d (11)	3.80 d (11)
A6B	3.54 m	4.11 m	3.57 m
B-Glucosyl moiety			
B1	5.25 d (7)	5.25 d (7)	4.99 d (7)
B2	3.44 m	3.43 m	3.30 m
B3	3.48 t (8)	3.43 m	3.22 m
B4	3.27 t (8)	3.27 t (9)	3.30 m
B5	3.88 m	3.87 br. t (9)	3.50 m
B6A	4.58 d (11)	4.60 d (11)	3.72 d (10)
B6B	4.16 m	4.14 m	3.50 m

J [Hz] in parentheses.

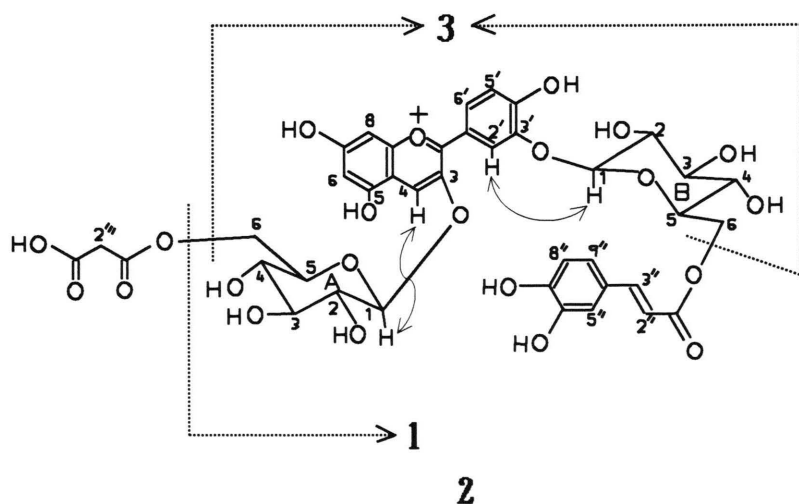


Fig. 1. Structures of the red *S. cruentus* flower pigments **1** and **2**. The bidirectional arrows indicate NOE correlations.

Cy, two Glc and Cf, and **2** with Cy, two Glc, Cf and Ma. The molecular formula of **2** was further supported by its high resolution FAB-MS exhibiting the molecular mass at 859.1932. Hence the chemical structures of **1** and **2** were estimated as caffeoyl-Cy 3,3'-Glc and malonylcaffeoyl-Cy 3,3'-Glc, respectively.

Of assignable peaks in the ^1H NMR spectra of **1** and **2**, six proton peaks in the downfield region showed the aglycone moiety to be Cy. In the sugar region, the anomeric protons of two sugars (A-1 and B-1 for **1** and **2**, respectively) in downfield with large J values (7–8 Hz) demonstrated the sugar moieties to have β forms. Methylene protons (B-6 for **1** and A-6 and B-6 for **2**, respectively) appeared as the shifted signals in the downfield area (δ 4.1–4.6 ppm) indicating the sugar parts to be the A-6-OH of **1** and A-6-OH and B-6-OH of **2** to be acylated. The other sugar ring protons gave overlapped signals in the upfield (3.3–3.9 ppm) region but some peaks were clarified by the analysis of COSY spectra. The assignable ring protons with large vicinal couplings (J = 7–10 Hz, Table III) showed that both A- and B-Glc moieties were pyranosyl configurations. Therefore, the sugar moieties were confirmed to be 3-(6-acylated- β -D-glucopyranosyl)-3'- β -D-glucopyranosyl for **1** and 3,3'-di-(6-acylated- β -D-glucopyranosyl) for **2**, respectively.

In the Cf moieties of **1** and **2**, as H-2'' and H-3'' in both olefinic parts had large coupling constants (J = 16 Hz), the α,β -unsaturated carbonyl moieties had *trans* (*E*) configuration, and the aromatic protons H-8''5, H-9'' and H-5'' appeared as a ABM type splittings suggesting the presence of a 1,3,4-substituted benzene rings (Table III). For the malonyl groups of **2**, the signals of the methylene protons (H-2''') appeared as doublet type peaks in the sugar proton region (3.38 ppm). Linkage positions

of acyl residues on aglycone were confirmed by H_2O_2 degradations of **1** and **2** [7]. Since **1** and **2** gave Glc and malonylglucose (Ma-Glc), respectively (Table II), Cf was linked at 3'-Glc in **1** and **2**, and Ma was linked at 3-Glc in **2**.

Thus the chemical structures of red *S. cruentus* flower anthocyanins **1** and **2** were identified as 3-O- β -D-glucopyranosyl-3'-O-(6-O-(*E*)-caffeoyl- β -D-glucopyranosyl)cyanidin and as 3-O-(6-O-malonyl- β -D-glucopyranosyl)-3'-O-(6-O-(*E*)-caffeoyl- β -D-glucopyranosyl)cyanidin, respectively (Fig. 1). It is interesting that cinerarin and rubro-cinerarin have 3,3',7-trisubstitution pattern but these pigments (**1** and **2**) have 3,3'-type substituents despite the isolation from same cineraria species.

The color stabilities of **1** and **2** in neutral aqueous solution were almost the same and superior to their deacylated derivative **3**. This indicates that the caffeic acid in **1** and **2** contributes to the stability but malonic acid in **2** does not. In general, when dissolved in weakly acidic or neutral aqueous solutions, colorations of polyacylated anthocyanins with two or more cinnamic acids such as cinerarin and Heavenly Blue Anthocyanin are stabilized by sandwich-type intramolecular stacking mechanism between the aglycone chromophore and cinnamic acids [9–11]. However, **1** and **2** showed high stability in spite of anthocyanins monoacylated with caffeic acid. From the fact, **1** and **2** are considered to be stabilized by cooperative mechanisms with self-association in addition to the half-side intramolecular stacking [12]. They are expected to be potential natural food and other colorants.

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