Diacylated 8-*C*-Glucosylcyanidin 3-Glucoside from the Flowers of *Tricyrtis formosana*

Fumi TATSUZAWA,*[,a] Norio SAITO,[b] Kazumitsu MIYOSHI,[c] Koichi SHINODA,[d] Atsushi SHIGIHARA,[e] and Toshio HONDA[e]

[a] *Department of Agro-Environmental Science, Hokkaido Junior College, Takushoku University; Fukagawa, Hokkaido 074–8585, Japan:* [b] *Chemical Laboratory, Meiji-Gakuin University; Totsuka, Yokohama 244–8539, Japan:* [c] *Faculty of Bioresource Sciences, Akita Pref. University; Akita 010–0195, Japan:* [d] *National Agricultural Research Center for Hokkaido Region; Sapporo, Hokkaido 062–8555, Japan: and* [e] *Faculty of Pharmaceutical Sciences, Hoshi University; Shinagawa-ku, Tokyo 142–8501, Japan.*

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A new diacylated 8-*C*-glucosylanthocyanin was isolated from the purple flowers of *Tricyrtis formosana* 'Fujimusume' as one of the major anthocyanins along with four known pigments. The structure of this pigment was determined to be 8-*C*-(6-*O*-*trans*-sinapoyl)-β-glucopyranosylcyanidin 3-*O*-(6-*O*-malonyl-β-glucopyranoside) by chemical and spectroscopic methods. In addition, four known pigments, 8-*C*-glucosylcyanidin 3-malonylglucoside, cyanidin 3-glucoside, cyanidin 3-rutinoside and cyanidin 3-malonylglucoside, were identified as the major anthocyanins in the flowers.

Key words *Tricyrtis formosana*; Liliaceae; diacylated 8-*C*-glucosylanthocyanin; 8-*C*-glucosylcyanidin 3-*O*-glucoside; 8-*C*-(sinapyl)-glucosylcyanidin 3-*O*-(malonyl)-glucoside

Tricyrtis species are native in east Asia, and their cultivars are grown as ornamental plants. *T. formosana* 'Fujimusume' is a cultivar developed from the species of Taiwanese origin, and has purple perianth with darker red spots as well as with bluish edge in autumn. Recently, we reported the structure determination of a novel anthocyanin, 8-*C*-β-D-glucopyranosylcyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside), isolated from the flowers of this cultivar.[1)] In the present paper, we further report the finding of another novel diacylated 8-*C*-glycosylcyanidin glycoside in the flowers of *T. formosana* 'Fujimusume' along with three known common anthocyanins as well as 8-*C*-glucosylcyanidin 3-(6-malonylglucoside), and the effect of 8-*C*-glycosylation on producing the flower color is also discussed.

Results and Discussion

In a survey of *Tricyrtis formosana* 'Fujimusume' by HPLC analysis, six anthocyanins, the pigment (**1**) (content *ca.* 10.8% determined by HPLC analysis), 8-*C*-glucosylcyanidin 3-*O*-(malonyl)-glucoside (**2**) (*ca.* 9.8%), cyanidin 3-glucoside (**3**) (*ca.* 9.6%), cyanidin 3-rutinoside (**4**) (*ca.* 35.7%) and cyanidin 3-malonylglucoside (**5**) (*ca.* 29.5%) in addition to an unidentified anthocyanin were observed in the total flower extracts of *T. formosana* 'Fujimusume' with MAW (MeOH–HOAc–water, 4 : 1 : 5). These pigments isolated with 5% HOAc–water were successively purified using Diaion HP-20 column chromatography (CC), TLC and preparative HPLC. The four known anthocyanins were identified on the basis of TLC, HPLC and spectral data in comparison with those of authentic anthocyanins.[1,2)] The structure determination of a novel anthocyanin (**1**) was carried out as follows.

Acid Hydrolysate of the Pigment (1) Acid hydrolysis of the pigment (**1**) gave an pigment (**6**) (*ca.* 1 mg), glucose (*ca.* 0.4 mg) as a sugar, and sinapic (*ca.* 0.4 mg) and malonic (*ca.* 0.2 mg) acids as its acyl components. By analysis of TLC using Forestal (HOAc–HCl–H_2O, 30 : 3 : 10) the pigment (**6**) showed an *Rf* value of 0.43 compared to that (0.39) of cyanidin (**8**), and was identical with that of 8-*C*-glucosylcyanidin.[1)]

Deacyl Pigment (8-*C*-Glucosylcyanidin 3-*O*-Glucoside (7)) Deacylation of the pigment (**1**) *via* alkaline hydrolysis afforded a deacylanthocyanin (**7**), and also sinapic and malonic acids. The FAB mass spectrum of deacylanthocyanin gave a molecular ion $[M]^+$ at 611 *m/z* in good agreement with the mass calculated for 8-*C*-glucosylcyanidin 3-*O*-glucoside ($C_{27}H_{31}O_{16}$) (Fig. 1). Its structure was confirmed by the analysis of its ^{1}H-NMR spectra as shown in Table 1. Twelve proton signals of 8-*C*-glucosylcyanidin, were assigned based on the analysis of the 2D COSY spectrum. Moreover, another anomeric proton of the sugar moiety appeared at δ 5.37 (d, *J*=8.0, H-1 of glucose B). Based on the consideration of the observed coupling constants, glucose B was determined to be a β-glucopyranoside form. The linkage between 3-OH of 8-*C*-glucosylcyanidin and H-1 of glucose B was confirmed by the analysis of its negative difference NOE (DIFNOE) spectra (Fig. 1). Therefore, the deacylanthocyanin (**7**) was determined to be 8-*C*-β-glucopyranosylcyanidin 3-*O*-β-glucopyranoside (Fig. 1).

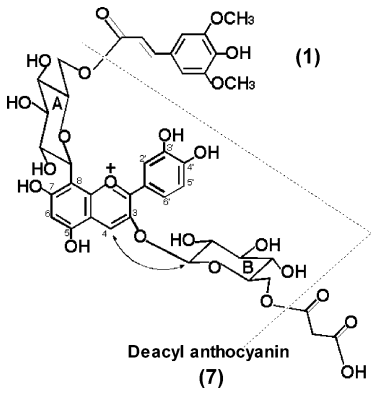

Fig. 1. Tricyrtis Anthocyanin (Pigment (**1**)) and Deacylanthocyanin (**7**)
NOE correlation is indicated by arrow.

* To whom correspondence should be addressed. e-mail: tatsuzawa@takushoku-hc.ac.jp

Table 1. ^{1}H-NMR Spectral Data of *Tricyrtis formosana* 'Fujimusume' Anthocyanin

	Pigment (**1**)	Deacylanthocyanin (**7**)
	δH	δH
Cyanidin		
4	8.91 s	8.97 s
6	6.92 s	6.90 s
8	—	—
2′	8.19 br d (2.2)	8.24 br d (2.1)
5′	7.15 d (8.6)	7.08 d (8.6)
6′	8.27 dd (2.2, 8.6)	8.28 dd (2.1, 8.6)
Glucose A[a,b]		
1	4.89 d (10.1)	4.83 d (9.5)
2	3.93 t (9.5)	3.83 m
3	3.41—3.46	3.41 m
4		3.50—3.70
5	3.70 m	
6a	4.33 dd (5.3, 12.0)	3.50—3.90
6b	4.63 br d (12.0)	3.70—3.90
Glucose B[a]		
1	5.40 d (7.9)	5.37 d (8.0)
2	3.61 t (8.5)	3.58 t (8.3)
3	3.41—3.46	3.41 t (9.5)
4	3.32 t (9.5)	3.31 t (9.5)
5	3.81 m	3.55 m
6a	4.18 dd (7.5, 10.7)	3.70—3.90
6b	4.52 br d (10.7)	
Malonic acid		
–CH_2–	3.41—3.46	
Sinapic acid[a]		
2.6	6.90 s	
α	6.47 d (15.6)	
β	7.54 d (15.6)	
–OCH_3	3.77 s	

500 MHz, in CF_3CO_2D–DMSO-d_6 (1 : 9), TMS as an internal standard, *J* Hz in parenthese. *a*) Assigned by ^{1}H–^{1}H COSY. *b*) Assigned by DIFNOE.

The Structure of the Pigment (1) (Diacylated 8-*C*-Glucosylcyanidin 3-Glucoside) The FAB mass spectrum of the pigment (**1**) showed a molecular ion $[M]^+$ at *m/z* 903 ($C_{41}H_{43}O_{23}$). Its elemental components were confirmed by HR-FAB-MS indicating that pigment (**1**) is composed of 8-*C*-glucosylcyanidin with one molecule each of glucose, malonic acid and sinapic acid. The detailed chemical structure was elucidated by ^{1}H-NMR spectra including 2D COSY and DIFNOE spectra. In the ^{1}H-NMR [500 MHz proton FT-NMR in CF_3COOD–DMSO-d_6 (1 : 9)] spectrum of **1**, five aromatic proton signals of the anthocyanidin at δ 8.91 (s, H-4), δ 8.27 (dd, *J*=2.2, 8.6 Hz, H-6′), δ 8.19 (br d, *J*=2.2 Hz, H-2′), δ 7.15 (d, *J*=8.6 Hz, H-5′) and δ 6.92 (s, H-6), were assigned for the ring protons of the cyanidin nucleus. However, the H-8 signal was not observed in the spectrum suggesting the presence of a *C*-glycosyl-substituent at this position. The proton signals of the sugar moieties were observed in the region of δ 3.32—5.40. The signals of two anomeric protons of the sugars appeared at δ 5.40 (d, *J*=8.0 Hz, H-1 of glucose B) and δ 4.89 (d, *J*=10.1 Hz, H-1 of glucose A) and were identical with those of the deacyl anthocyanin described above. Based on the observed coupling constants, both glucose moieties were assumed to have a β-pyranose form. Two pairs of doublets (δ 6.47, 7.54) with large coupling constants (*J*=15.6 Hz) indicated the presence of the *trans*-olefinic protons of sinapic acid. In order to determine the linkages and positions of the glucose and acyl units DIFNOE spectra of the pigment (**1**) were measured. Observed DIFNOE between H-1 of Glc B and H-4 of cyanidin supported that Glc B is attached to the 3-OH of cyanidin through a glycosidic bond (Fig. 1). Since acid hydrolysis of the pigment (**1**) afforded 8-*C*-glucosylcyanidin (**6**),[1] C-1 of Glc A was confirmed to be linked directly with C-8 of cyanidin through *C*-glycosidic bond. Four methylene protons of Glc A and B were assigned to H-6a and H-6b of Glc A (δ 4.33, 4.63) and those of Glc B (δ 4.18, 4.52) by analysis of its 2D COSY spectrum. The down-field shifts of these methylene signals indicated that the malonyl and sinapyl moieties were attached to 6-OH of both glucose moieties. In order to determine the linkage of malonic acid in pigment (**1**), H_2O_2 degradation of the pigment (**1**) was carried out, and malonylglucose was detected in its product solution, indicating that malonic acid is attached to the 6-OH of Glc B.[3] Consequently, it was deduced that sinapic acid is bonding with 6-OH of Glc A. Thus, the pigment (**1**) was determined to be 8-*C*-[6-*O*-(sinapoyl)-β-glucopyranosyl]-cyanidin 3-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Fig. 1), which is a new naturally occurring *C*-glycosylanthocyanin.

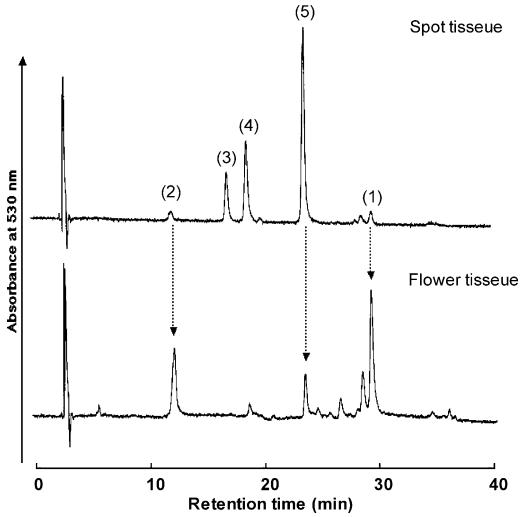

Fig. 2. HPLC Profiles for the Anthocyanins in the Dark Red Spot Tissues of *Tricyrtis formosana* 'Fujimusume' Flower (Upper), and in the Flower Tissue Removed the Dark Red Spot Tissues (Bottom)

Anthocyanin Distribution in the Flower Tissues There are spot patterns like a bird's hototogisu in the flower and the leaf of *T. formosana* 'Fujimusume'. HPLC analysis revealed that the mauve flower tissues removed the dark red spot tissues from the flower contained mainly 8-*C*-sinapylglucosylcyanidin 3-*O*-malonylglucoside (**1**) and 8-*C*-glucosylcyanidin 3-*O*-malonylglucoside (**2**) as major pigments. On the other hand, the dark red spot tissues in the flowers contained cyanidin 3-*O*-rutinoside (**4**) and cyanidin 3-*O*-malonylglucoside (**5**) as main anthocyanins (Fig. 2). Therefore, the mauve or purple flower color might be considered not to depend on common cyanidin 3-*O*-glycosides, but 8-*C*-glucosylcyanidin 3-*O*-glycosides.

8-*C*-Glucosylcyanidin (**6**) exhibited its λ_{max} (0.1% HCl–MeOH) at 541 nm, in the UV–Vis spectrum, which was shifted to the longer wavelength region than that of cyanidin (**8**) (535 nm), and was very close to that of delphinidin (546 nm). Even in the UV–Vis spectrum of 3-*O*-glycosides

of the new anthocyanidin, 8-*C*-glucosylcyanidin 3-*O*-glucoside showed its λ_{max} (0.1% HCl–MeOH) at 532 nm, which was also shifted to the longer wavelength region than that of cyanidin 3-*O*-glucoside (527 nm). On the basis of these results, 8-*C*-glycosylation may be considered as another new bluing factor in producing flower color, such as the hydroxylation in the B-ring, acylation, metal complexation, and so on.[4)]

Experimental

General Procedures TLC was carried out on plastic sheets cellulose (Merck) using six mobile phases: BAW (*n*-BuOH–HOAc–H_2O, 4 : 1 : 2), BuHCl (*n*-BuOH–2 N HCl, 1 : 1), 1% HCl, AHW (HOAc–HCl–H_2O, 15 : 3 : 82) and Forestal (HOAc–HCl–H_2O, 30 : 3 : 10) for anthocyanins, and BAW, EAA (EtOAc–HOAc–H_2O, 3 : 1 : 1) and ETN (EtOH–NH_4OH–H_2O, 16 : 1 : 3) for organic acid and sugars.[5)] Analytical HPLC was performed on an LC-10A system (Shimadzu), using a Waters C18 (240×4.6 mm) column at 40 °C with a flow rate of 1 ml min^{-1} monitoring at 530 nm. The solvent was applied as a linear gradient elution for 40 min from 20 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). UV–Vis spectra were recorded on a MPS-2400 (Shimadzu) in 0.1% HCl–MeOH (from 200 to 700 nm). FAB mass spectra were recorded in positive mode using the magic bullet. NMR spectra were recorded at 500 MHz for ^1H spectra in DMSO-d_6–CF_3COOD (9 : 1). Chemical shifts are reported relative to TMS as an internal standard (δ) and coupling constants (*J*) are in Hz.

Plant Materials A *T. formosana* 'Fujimusume' plant was a gift from Mr. S. Kubota of Gensouen Nursery (Tokyo, Japan) and propagated by division in the garden of K. M. in Akita City, Japan. These were also grown in the green house of Hokkaido Junior College, Takushoku University. The fresh flowers collected from early September to early November on the day of their open were dried overnight at 37 °C and kept at −10 °C until used. To examine the distribution of anthocyanins in the flower, tissues were cut and separated into the two parts of the dark red spot tissues and the purple flower tissues free from the dark red spot tissues by the knife. The anthocyanins of both flower tissues were extracted with MAW.

Isolation of Anthocyanins Dried flowers (80 g) of *T. formosana* 'Fujimusume' were immersed overnight in MAW (1 l) at room temperature. The extract was concentrated to 100 ml and purified by successive chromatography on Diaion HP-20 CC, TLC (BAW, 4 : 1 : 2) and preparative HPLC by previous procedures.[1)] Preparative HPLC was run on a Waters C18 (150×19 mm) column at 40 °C with a flow rate of 4 ml min^{-1} monitoring at 530 nm for anthocyanins. The solvent systems used were as follows: a linear gradient elution for 25 min from 25 to 70% solvent B in solvent A. Fractions (*ca.* 50 ml) were transferred Diaion HP-20 column. Anthocyanins were eluted with 5% HOAc–MeOH, and concentrated to give a residue, which was dissolved in a small volume of 5% HOAc–EtOH, followed by addition of excess Et_2O, and then dried to give pigment powders, **1** (5 mg), **2** (10 mg), **3** (1 mg), **4** (2 mg) and **5** (2 mg). Pigment (**6**) (2 mg) was obtained from the acid hydrolysates of both pigments (**1**) and (**2**) by TLC, and **7** (2 mg) was obtained from the alkaline hydrolysates of pigments (**1**) and (**2**) as purified pigment products by TLC.

Analysis of Anthocyanins For quantitative analysis of the spot and flower tissues (fr.wt. *ca.* 25 mg), anthocyanins were extracted with MAW (1 ml) and analysed by HPLC. Characterization of pigments were carried out by measuring UV–Vis, FAB-MS and NMR spectra and also by using TLC and HPLC. Furthermore, the components of the pigments (**1**) and (**7**) were analyzed by TLC after acid and alkaline hydrolyses and H_2O_2 degradation.[5)] Acid hydrolysis is carried out with 2 M HCl at 100 °C for 2 h. Alkaline hydrolysis is carried out in deflated syringe with 2 M NaOH at room temperature for 15 min, and acidified with 2 M HCl. H_2O_2 degradation is carried out with 50% H_2O_2 at room temperature for 1 h was quenched with MnO_2, and followed by alkaline hydrolysis with NH_3 gas for 1 h at room temperature. Pigment (**6**) was obtained from the hydrolysates of the pigments (**1**) and (**2**) by acid hydrolysis, and also (**7**) was obtained from those of the pigments (**1**) and (**2**) by alkaline hydrolysis.[5)] After H_2O_2 degradation of the pigment (**1**), preparative TLC of its products was carried out using solvent BAW, and its malonylglucose fraction was eluted with 50% MeOH from the TLC spot and evaporated to dryness. The components of sugar, acid and acylated sugar, obtained from the pigment (**1**) by acid and alkaline hydrolyses, and H_2O_2 degradation, were confirmed to be glucose [0.13 (BAW), 0.20 (EAA), 0.51 (ETN)], malonic acid [0.64 (BAW), 0.71 (EAA), 0.11 (ETN)], sinapic acid [0.88 (BAW), 0.93 (EAA), 0.60 (ETN)], malonylglucose [0.17 (BAW), 0.28 (EAA), 0.30 (ETN)], respectively, by co-TLC analysis with authentic samples. The authentic samples used were commercial glucose, malonic acid and sinapic acid (Wako Chemicals), and also malonylglucose obtained from H_2O_2 degradation product of *Phalaenopsis* anthocyanin 3.[3)]

Pigment (**1**) (8-C-[6-*O*-(Sinapoyl)-β-glucopyranosyl]-cyanidin 3-*O*-[6-*O*-(Malonyl)-β-glucopyranoside]): UV–Vis (0.1% HCl–MeOH): λ_{max} 287, 330, 531 nm, E_{440}/E_{max} (%)=24, E_{acyl}/E_{max} (%)=70, $AlCl_3$ shift +, TLC: *Rf*-values BAW 0.32, BuHCl 0.11, 1% HCl 0.21, AHW 0.67, HPLC: t_R (min) 28.2. HR-FAB mass Calcd for $C_{41}H_{43}O_{23}$: 903.2194. Found: 903.2195.

Deacylanthocyanin (**7**) (8-*C*-Glucosylcyanidin 3-*O*-Glucoside): UV–Vis (0.1% HCl–MeOH): λ_{max} 282, 532 nm, E_{440}/E_{max} (%)=27, $AlCl_3$ shift +, TLC: *Rf*-values BAW 0.14, BuHCl 0.03, 1% HCl 0.25, AHW 0.49, HPLC: t_R (min) 5.7. HR-FAB mass Calcd for $C_{27}H_{31}O_{16}$: 611.1612. Found: 611.1639.

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