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ACYLATED CYANIDIN 3,7,3'-TRIGLUCOSIDES WITH *p*-HYDROXYBENZOIC ACID FROM THE FLOWERS OF *DENDROBIUM*

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Abstract – Two new acylated anthocyanins were isolated from the purple flowers of *Dendrobium* ×*superbiens*, along with four known pigments. These pigments were all based on cyanidin 3,7,3'-triglucoside, and acylated variously with malonic, sinapic, and *p*-hydroxybenzoic acids. One of new anthocyanins was fully determined to be cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*p*-hydroxybenzoyl)-β-D-glucopyranoside]-3'-*O*-[6-*O*-(4-(β-D-glucopyranosyl)-*p*-hydroxybenzoyl)-β-D-glucopyranoside] by chemical and spectral methods. Another new pigment was provisionally determined to be cyanidin 3-malonylglucoside-7-glucosyl-*p*-hydroxybenzoylglucoside-3'-*p*-hydroxybenzoylglucoside.

In our continuing work on flower color variation due to acylated anthocyanins in orchids, we have already reported the occurrence of acylated cyanidin, peonidin, and delphinidin glycosides in the flowers of *Dendrobium*,[1-3] ×*Laeliocattleya*, *Cattleya*, *Laelia*,[4,5] *Bletilla*,[6] *Cymbidium*,[7] *Phalaenopsis*,[8] *Sophronitis*,[9] and *Vanda*.[10] In this paper, we wish to report the structure determination of two new acylated cyanidin 3,7,3'-triglucosides isolated from the purple flowers of *Dendrobium* ×*superbiens*. Dry flowers (*ca*. 100 g) of *Dendrobium* ×*superbiens* and hybrids mixture [11] were immersed in 5% HOAc for 24 h at room temperature and extracted. By the analysis of HPLC, [12] more than 30 anthocyanin peaks were observed in the extract from the flowers of *D.* ×*superbiens* (Figure 1). Anthocyanins of four peaks (C-F) were

identified to be cyanidin 3-malonylglucoside-7,3'-di(glucosyl-*p*-hydroxybenzoylglucoside) for D,[13] cyanidin 3-malonylglucoside-7,3'-disinapoylglucoside for F,[14] and their demalonyl derivatives for C[15] and E[16] described previously[3] (Figure 1). In this study, we investigated the isolation and structure determination of two major remaining peaks **A** and **B** from this plant as follows: The concentrated extract

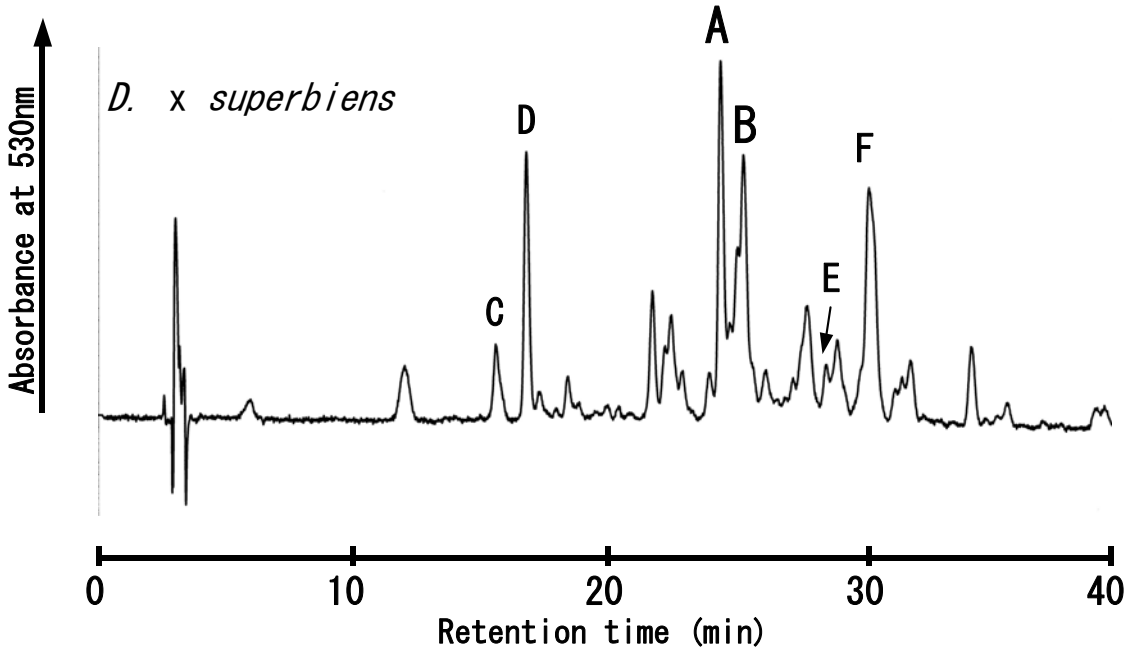

Figure 1. HPLC analysis of the pigments in the flowers of *Dendrobium* × *superbiens*.
C: cyanidin 3-glucoside-7,3'-di(glucosyl-*p*-hydroxybenzoylglucoside),
D: cyanidin 3-malonylglucoside-7,3'-di(glucosyl-*p*-hydroxybenzoylglucoside),
E: cyanidin 3-glucoside-7,3'-disinapoylglucoside,
F: cyanidin 3-malonylglucoside-7,3'-disinapoylglucoside.

was purified by DIAION HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column chromatography with 5% HOAc and 5% HOAc-MeOH, and then by paper chromatography with BAW (*n*-BuOH/HOAc/H_2O, 4:1:2, v/v/v) and 5% HOAc according to the process described previously.[1-3] The fractions containing two anthocyanins were further purified by preparative HPLC,[12] and precipitated with Et_2O to afford pure pigments; pigment **A** (*ca*. 5 mg)[17] and pigment **B** (*ca*. 2 mg).[18]

By acid hydrolysis, pigments **A** and **B** gave cyanidin, glucose, *p*-hydroxybenzoic acid and malonic acid. The alkaline hydrolysis of pigments **A** and **B** yielded cyanidin 3,7,3'-triglucoside,[19] 4-glucosyloxybenzoic acid,[20] *p*-hydroxybenzoic acid and malonic acid.

The FAB MS spectra of pigments **A** and **B** gave the same molecular ion $[M]^+$ at 1261 *m/z*, in agreement with their mass calculated for $C_{56}H_{61}O_{33}$.

Pigment **A**: The elemental components of pigment **A** were confirmed by High Resolution-FABMS (calcd for $C_{56}H_{61}O_{33}$: 1261.3095. Found: 1261.3086). The result indicated that this pigment is composed of cyanidin with one molecule of malonic acid, two molecules of *p*-hydroxybenzoic acid and four molecules of glucose. The detailed structure of pigment **A** was elucidated by its ^{1}H NMR spectral measurement including ^{1}H-^{1}H COSY, NOEDIF and NOESY spectra in CF_3COOD-DMSO-d_6 (1:9), and the chemical shifts were assigned as shown in Table 1. The proton signals of cyanidin and two *p*-hydroxybenzoic acids were assigned by 2D COSY and NOEDIF spectra. The proton signals of the sugar moieties were observed in the region of δ 5.74 – 3.05 ppm. The chemical shifts of four anomeric protons were observed at δ 5.74 (*d*, *J*=7.7 Hz, Glc A), 5.41 (*d*, *J*=7.6 Hz, Glc B), 4.19 (*d*, *J*=7.6 Hz, Glc C), and 4.21 (*d*, *J*=7.6 Hz, Glc D), therefore, all glucose units must be β-glucopyranose form. Six methylene protons –CH_2- in glucose moieties which were acylated with two *p*-hydroxybenzoic acids (I and II) and malonic acid, were shifted to lower magnetic field (δ 3.98 – 4.90). These protons were assigned to the Glc A (δ 4.21 and 4.60, H-6a and 6b), Glc B (δ 4.21 and 4.90, H-6a and 6b), and Glc C (δ 3.98 and 4.54, H-6a and 6b) by the NOEDIF experiment and also by the analysis of its 2D COSY spectrum.

Table 1 ^{1}H NMR spectral data of pigment A of *Dendrobium* × *superbiens*.*

Cyanidin			Glucose A			Glucose C		
4	8.83	s	1	5.74	d (7.7)	1	4.19	d (7.6)
6	6.70	br s	2	3.78	m	2	3.26	m
8	7.71	br s	3	3.46	m	3	3.05	t (8.9)
2'	8.15	d (2.2)	4	3.33	m	4	3.31	m
5'	7.18	d (9.0)	5	3.87	m	5	3.36	m
6'	8.93	dd (2.2, 9.0)	6a	4.21	m	6a	3.98	m
			6b	4.60	d (11.6)	6b	4.54	d (9.8)
p-Hydroxybenzoic acid I								
			Glucose B			Glucose D		
2 and 6	7.45	d (8.9)						
3 and 5	6.58	d (8.9)	1	5.41	d (7.6)	1	4.21	d (7.6)
			2	3.49	m	2	3.29	m
p-Hydroxybenzoic acid II			3	3.44	m	3	3.20 – 3.90	
			4	3.40	m	4		
2 and 6	7.90	d (8.8)	5	4.01	m	5		
3 and 5	6.81	d (8.8)	6a	4.21	m	6a		
			6b	4.90	d (10.4)	6b		
Malonic acid								
CH_2–	3.38 – 3.43							

* ^{1}H NMR (500 MHz) (CF_3CO_2D–DMSO–d_6, 1:9), at 25°C, an internal standard of TMS; Coupling constants (*J* in Hz) in parentheses.

In order to determine the attachments and/or positions of the glucose and acyl units in pigment **A**, NOEDIF spectra were measured. Observed NOEDIF between H-1 of Glc A and H-4 of cyanidin indicates

that Glc A is attached to OH-3 of cyanidin through a glucosidic bond. 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. Similarly, Glc B was deduced to be attached to OH-7 of cyanidin through a glucosidic bond due to the presence of NOEs between H-6 and –8 of cyanidin and H-1 of Glc B. Irradiation at H-1 of Glc B gave a NOEDIF spectrum in which rather weak NOEs at protons of H-2, -3, -5 and -6 of *p*-hydroxybenzoic acid I were observed. Thus, *p*-hydroxybenzoic acid I is attached to OH-6 of Glc B. As both irradiations at H-6b of Glc C and H-1 of Glc D exhibited NOEs at H-2, -3, -5 and -6 of *p*-hydroxybenzoic acid II, Glc C was confirmed to be acylated with *p*-hydroxybenzoic acid II at OH-6 of Glc C and also *p*-hydroxybenzoic acid II was glucosylated with Glc D at OH-4 of *p*-hydroxybenzoic acid II. On H_2O_2 degradation of pigment **A**, malonylglucose was determined in its product,[1] supporting that Glc A was acylated with malonic acid. Thus, the structure of pigment **A** was determined to be cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*p*-hydroxybenzoyl)-β-D-glucopyranoside]-3'-*O*-[6-*O*-(4-(β-D-glucopyranosyl)-*p*-hydroxybenzoyl)-β-D-glucopyranoside], which is a new anthocyanin.[21,22]

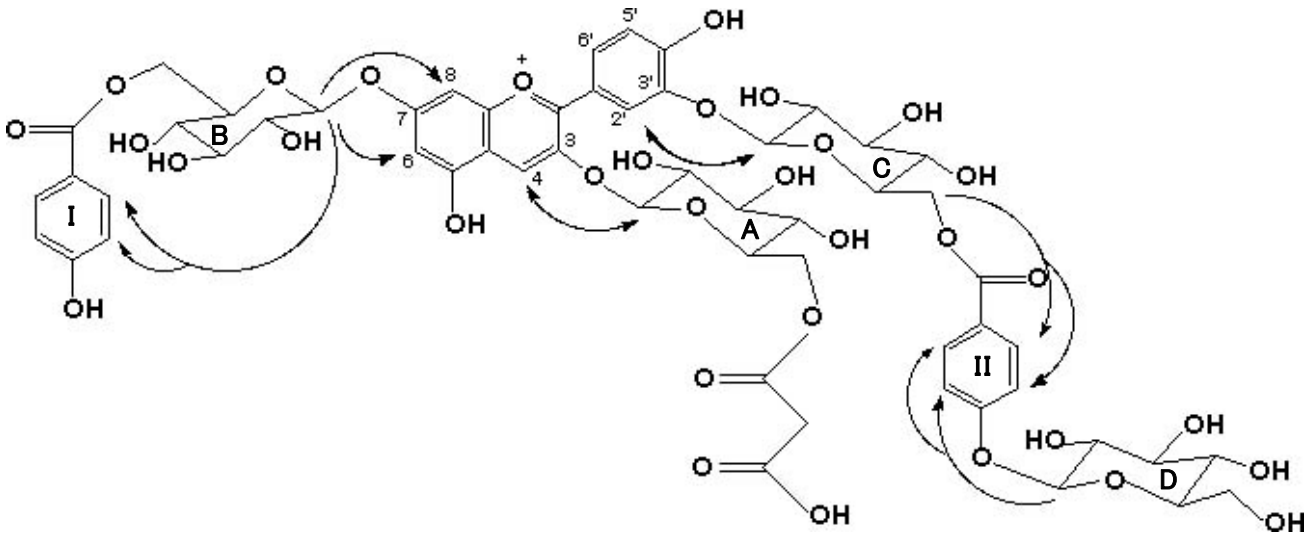

Figure 2. An acylated anthocyanin (pigment A) from the flowers of *Dendrobium*. Observed NOE's are indicated by arrows.

Pigment **B**: FAB-MS spectra of pigment **B** gave its $[M]^+$ as *m/z* 1261. This value is in good agreement with that of pigment **A**, which is composed of cyanidin with four molecules of glucose, two molecules of *p*-hydroxybenzoic acids and one molecule of malonic acid. On alkaline hydrolysis, this anthocyanin gave cyanidin 3,7,3'-triglucoside and 4-glucosyloxybenzoic acid, *p*-hydroxybenzoic acid and malonic acid as same as the result of pigment **A**. Based on the above findings, pigment **B** is considered to have similar triacyl cyanidin 3,7,3'-triglucoside, and the structure of pigment **B** is assigned by its spectral properties to be cyanidin 3,7,3'-triglucoside, in which 3'-glucosidic residue is acylated with *p*-hydroxybenzoic acid,

7-glucosidic residue is acylated with 4-glucosyloxybenzoic acid and also 3-glucosidic residue is acylated with malonic acid. This pigment is also a new anthocyanin in plants.

ACKNOWLEDGEMENTS

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11. Fresh flowers of *Dendrobium* hybrids (*ca*. 1000 g), whose anthocyanin components are the same to those of *D.* ×*superbiens*, were gifted by the exhibitors of Japan Grand Prix RAN International Orchid Festival 2004 and 2005. *D.* ×*superbiens* was grown in the greenhouses of Tsukuba Botanical Garden (TBG), National Science Museum, Tokyo. Voucher specimen of *D.* ×*superbiens* was deposited at TBG.
12. Analytical HPLC was run on a Waters C18 (4.6 x 250 mm) column at 40°C with flow rate of 1 $mL \cdot min^{-1}$ and monitored at 530 nm for anthocyanins and 230 nm for 4-glucosyloxybenzoic acid and *p*-hydroxybenzoic acid. Solvent system used was 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). Preparative HPLC was run on a Waters C18 (19 x 150 mm) column at 40°C with flow rate of 4 $mL \cdot min^{-1}$ and monitored at 530 nm for anthocyanins. Solvent system used was linear gradient elution for

15 min from 60 to 80 % solvent B in solvent A.

13. Pigment **D**, cyanidin 3-malonylglucoside-7,3'-di(glucosyl-*p*-hydroxybenzoylglucoside); UV-VIS (in 0.1% HCl-MeOH): λmax 533, 248 nm, E_{440}/E_{max} (%) = 23, $AlCl_3$ shift 0; TLC: R_f-values BAW (*n*-BuOH/HOAc/H_2O, 4:1:2, v/v/v) 0.04, BuHCl (*n*-BuOH/2N HCl, 1:1, v/v) 0.04, 1% HCl 0.21, AHW (HOAc/HCl/H_2O, 15:3:82, v/v/v) 0.47; HPLC: R_t(min) 16.8.
14. Pigment **F**, cyanidin 3-malonylglucoside-7,3'-disinapoylglucoside; UV-VIS (in 0.1% HCl-MeOH): λmax 534, 330, 295 nm, E_{440}/E_{max} (%) = 29, E_{acyl}/E_{max} (%) = 154, $AlCl_3$ shift 0; TLC: R_f-values BAW 0.28, BuHCl 0.14, 1% HCl 0.15, AHW 0.53; HPLC: R_t(min) 30.3.
15. Pigment **C**, cyanidin 3-glucoside-7,3'-di(glucosyl-*p*-hydroxybenzoylglucoside); UV-VIS (in 0.1% HCl-MeOH): λmax 533, 294 nm, E_{440}/E_{max} (%) = 24, $AlCl_3$ shift 0; TLC: R_f-values BAW 0.05, BuHCl 0.04, 1% HCl 0.25, AHW 0.49; HPLC: R_t(min) 15.8.
16. Pigment **E**, cyanidin 3-glucoside-7,3'-disinapoylglucoside; UV-VIS (in 0.1% HCl-MeOH): λmax 534, 330, 295 nm, E_{440}/E_{max} (%) = 28, E_{acyl}/E_{max} (%) = 121, $AlCl_3$ shift 0; TLC: R_f-values BAW 0.34, BuHCl 0.15, 1% HCl 0.16, AHW 0.46; HPLC: R_t(min) 28.7.
17. Pigment **A**: UV-VIS (in 0.1% HCl-MeOH): λmax 530, 248 nm, E_{440}/E_{max} (%) = 26, $AlCl_3$ shift 0; TLC: R_f-values BAW 0.03, BuHCl 0.03, 1% HCl 0.14, AHW 0.30; HPLC: R_t(min) 24.4.
18. Pigment **B**: UV-VIS (in 0.1% HCl-MeOH): λmax 531, 248 nm, E_{440}/E_{max} (%) = 29, $AlCl_3$ shift 0; TLC: R_f-values BAW 0.04, BuHCl 0.06, 1% HCl 0.34, AHW 0.49; HPLC: R_t(min) 25.3.
19. Cyanidin 3,7,3'-triglucoside (in 0.1% HCl-MeOH); UV-VIS: λmax 513, 280 nm, E_{440}/E_{max} (%) = 36, $AlCl_3$ shift 0; TLC: R_f-values BAW 0.09, BuHCl 0.04, 1% HCl 0.30, AHW 0.62; HPLC: R_t(min) 6.2.
20. 4-glucosyloxybenzoic acid; HPLC: λmax 232, 248 nm, R_t(min): 4.6 min.
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