

HETEROCYCLES, Vol. 68, No. 2, 2006, pp. 381 - 386. © The Japan Institute of Heterocyclic Chemistry  
Received, 28th November, 2005, Accepted, 29th December, 2005, Published online, 13th January, 2006. COM-05-10637

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

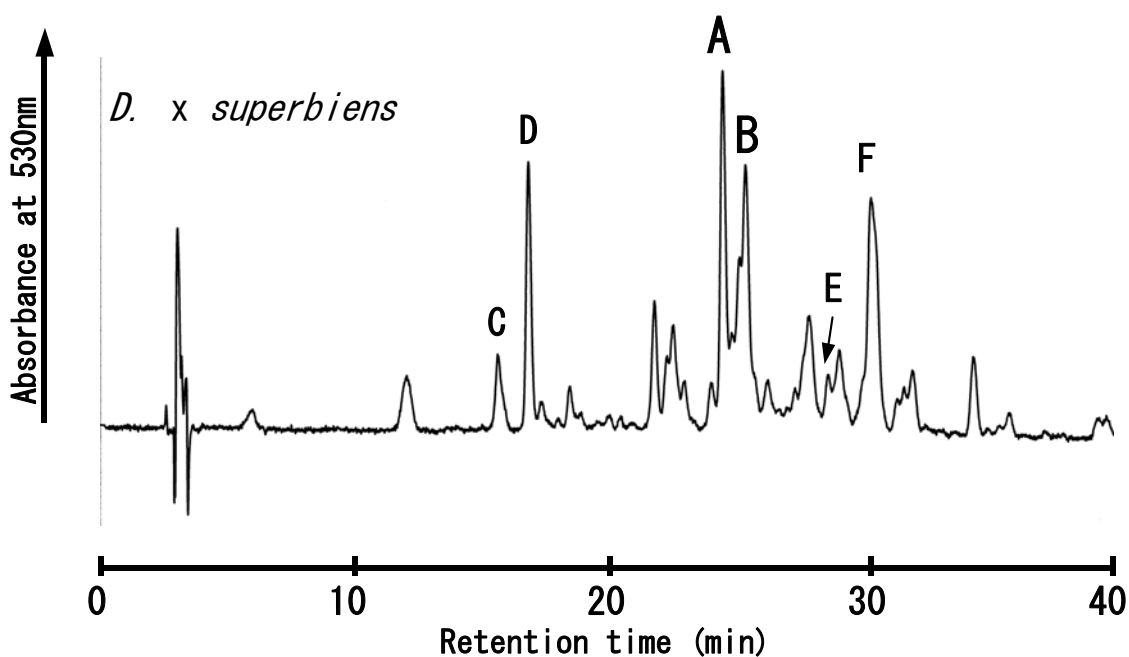
**Fumi Tatsuzawa, Norio Saito,\* Tomohisa Yukawa,\*\* Koichi Shinoda,#  
Atsushi Shigihara,\* and Toshio Honda\***

Department of Agro-Environmental Science, Hokkaido Junior College, Takushoku University, Fukagawa, Hokkaido 074-8585, Japan; \* Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41, Shinagawa, Tokyo 142-8501, Japan; \*\* Tsukuba Botanical Garden, National Science Museum, Tsukuba, Ibaraki 305-0005 Japan; # National Agricultural Research Center for Hokkaido Region, Sapporo, Hokkaido, 062-8555.

**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)- $\beta$ -D-glucopyranoside]-7-*O*-[6-*O*-(*p*-hydroxybenzoyl)- $\beta$ -D-glucopyranoside]-3'-*O*-[6-*O*-(4-( $\beta$ -D-glucopyranosyl)-*p*-hydroxybenzoyl)- $\beta$ -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*,<sup>1-3</sup> *× Laeliocattleya*, *Cattleya*, *Laelia*,<sup>4,5</sup> *Bletilla*,<sup>6</sup> *Cymbidium*,<sup>7</sup> *Phalaenopsis*,<sup>8</sup> *Sophranitis*,<sup>9</sup> and *Vanda*.<sup>10</sup> 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<sup>11</sup> were immersed in 5% HOAc for 24 h at room temperature and extracted. By the analysis of HPLC,<sup>12</sup> 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**,<sup>13</sup> cyanidin 3-malonylglucoside-7,3'-disinapoylglucoside for **F**,<sup>14</sup> and their demalonyl derivatives for **C**<sup>15</sup> and **E**<sup>16</sup> described previously<sup>3</sup> (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<sub>2</sub>O, 4:1:2, v/v/v) and 5% HOAc according to the process described previously.<sup>1-3</sup> The fractions containing two anthocyanins were further purified by preparative HPLC,<sup>12</sup> and precipitated with Et<sub>2</sub>O to afford pure pigments; pigment **A** (*ca.* 5 mg)<sup>17</sup> and pigment **B** (*ca.* 2 mg).<sup>18</sup>

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,<sup>19</sup> 4-glucosyloxybenzoic acid,<sup>20</sup> *p*-hydroxybenzoic acid and malonic acid.

The FAB MS spectra of pigments **A** and **B** gave the same molecular ion [M]<sup>+</sup> at 1261 *m/z*, in agreement with their mass calculated for C<sub>56</sub>H<sub>61</sub>O<sub>33</sub>.

**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  $^1H$  NMR spectral measurement including  $^1H$ - $^1H$  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  $\delta$  5.74 – 3.05 ppm. The chemical shifts of four anomeric protons were observed at  $\delta$  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  $\beta$ -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 ( $\delta$  3.98 – 4.90). These protons were assigned to the Glc A ( $\delta$  4.21 and 4.60, H-6a and 6b), Glc B ( $\delta$  4.21 and 4.90, H-6a and 6b), and Glc C ( $\delta$  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**  $^1H$  NMR spectral data of pigment **A** of *Dendrobium*  $\times$  *superbiens*.\*

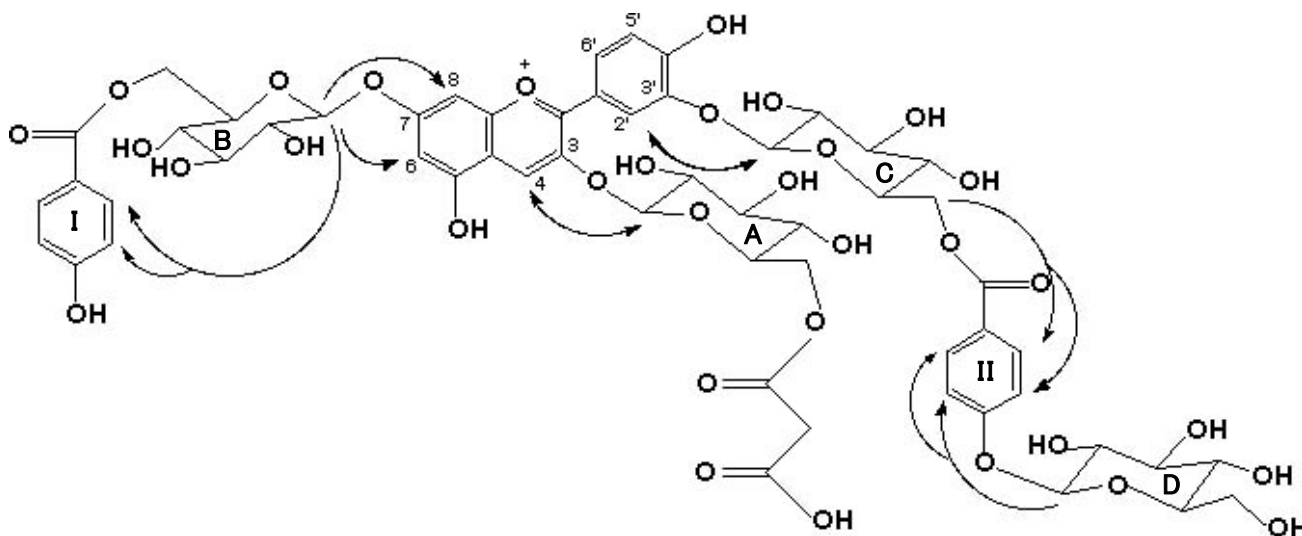
Cyanidin			Glucose A			Glucose C		
4	8.83	s	1	5.74	d (7.7)	1	4.19	d (7.6)
6	6.70	br s	2	3.78	m	2	3.26	m
8	7.71	br s	3	3.46	m	3	3.05	t (8.9)
2'	8.15	d (2.2)	4	3.33	m	4	3.31	m
5'	7.18	d (9.0)	5	3.87	m	5	3.36	m
6'	8.93	dd (2.2, 9.0)	6a	4.21	m	6a	3.98	m
			6b	4.60	d (11.6)	6b	4.54	d (9.8)
<i>p</i> -Hydroxybenzoic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

\*  $^1H$  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<sub>2</sub>O<sub>2</sub> degradation of pigment **A**, malonylglucose was determined in its product,<sup>1</sup> 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)- $\beta$ -D-glucopyranoside]-7-*O*-[6-*O*-(*p*-hydroxybenzoyl)- $\beta$ -D-glucopyranoside]-3'-*O*-[6-*O*-(4-( $\beta$ -D-glucopyranosyl)-*p*-hydroxybenzoyl)- $\beta$ -D-glucopyranoside], which is a new anthocyanin.<sup>21,22</sup>



**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

The authors thank the exhibitors of Japan Grand Prix RAN International Orchid Festival 2004 for the donation of *Dendrobium* hybrids, Mr. Kazuhiro Suzuki for skillful cultivation of the material, and also Miss Fumino Hayakawa and Miss Yukiko Toya (Chiba University) for their help in collecting plants.

## REFERENCES AND NOTES

1. N. Saito, K. Toki, K. Uesato, A. Shigihara, and T. Honda, *Phytochemistry*, 1994, **37**, 245.
2. C. A. Williams, J. Greenham, J. B. Harborne, J. -M. Kong, L. -S. Chia, N. -K. Goh, N. Saito, K. Toki, and F. Tatsuzawa, *Biochemical Systematics and Ecology*, 2002, **30**, 667.
3. F. Tatsuzawa, T. Yukawa, K. Shinoda, and N. Saito, *Biochemical Systematics and Ecology*, 2005, **33**, 625.
4. F. Tatsuzawa, N. Saito, M. Yokoi, A. Shigihara, and T. Honda, *Phytochemistry*, 1994, **37**, 1179.
5. F. Tatsuzawa, N. Saito, M. Yokoi, A. Shigihara, and T. Honda, *Phytochemistry*, 1996, **41**, 635.
6. N. Saito, M. Ku, F. Tatsuzawa, T. S. Lu, M. Yokoi, A. Shigihara, and T. Honda, *Phytochemistry*, 1995 **40**, 1523.
7. F. Tatsuzawa, N. Saito, and M. Yokoi, *Lindleyana*, 1996, **11**, 214.
8. F. Tatsuzawa, N. Saito, H. Seki, R. Hara, M. Yokoi, and T. Honda, *Phytochemistry*, 1997, **45**, 173.
9. F. Tatsuzawa, N. Saito, M. Yokoi, A. Shigihara, and T. Honda, *Phytochemistry*, 1998, **49**, 869.
10. F. Tatsuzawa, N. Saito, H. Seki, M. Yokoi, T. Yukawa, K. Shinoda, and T. Honda, *Biochemical Systematics and Ecology*, 2004, **32**, 651.
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·min<sup>-1</sup> 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<sub>3</sub>PO<sub>4</sub>, 20% HOAc, 25% MeCN in H<sub>2</sub>O) in solvent A (1.5% H<sub>3</sub>PO<sub>4</sub> in H<sub>2</sub>O). Preparative HPLC was run on a Waters C18 (19 x 150 mm) column at 40°C with flow rate of 4 mL·min<sup>-1</sup> 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):  $\lambda_{\max}$  533, 248 nm,  $E_{440}/E_{\max}$  (%) = 23, AlCl<sub>3</sub> shift 0; TLC:  $R_f$ -values BAW (*n*-BuOH/HOAc/H<sub>2</sub>O, 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<sub>2</sub>O, 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):  $\lambda_{\max}$  534, 330, 295 nm,  $E_{440}/E_{\max}$  (%) = 29,  $E_{\text{acyl}}/E_{\max}$  (%) = 154, AlCl<sub>3</sub> 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):  $\lambda_{\max}$  533, 294 nm,  $E_{440}/E_{\max}$  (%) = 24, AlCl<sub>3</sub> 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):  $\lambda_{\max}$  534, 330, 295 nm,  $E_{440}/E_{\max}$  (%) = 28,  $E_{\text{acyl}}/E_{\max}$  (%) = 121, AlCl<sub>3</sub> 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):  $\lambda_{\max}$  530, 248 nm,  $E_{440}/E_{\max}$  (%) = 26, AlCl<sub>3</sub> 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):  $\lambda_{\max}$  531, 248 nm,  $E_{440}/E_{\max}$  (%) = 29, AlCl<sub>3</sub> 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:  $\lambda_{\max}$  513, 280 nm,  $E_{440}/E_{\max}$  (%) = 36, AlCl<sub>3</sub> 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:  $\lambda_{\max}$  232, 248 nm,  $R_t$ (min): 4.6 min.
21. J. B. Harborne and H. Baxter, *The Handbook of Natural Flavonoids, Volume 2*, 1999, John Wiley & Sons, Chichester, (New York, Weinheim, Brisbane, Singapore and Toronto).
22. T. Honda and N. Saito, *Heterocycles*, 2002, **56**, 633.