DIACYLATED MALVIDIN 3-RUTINOSIDE-5-GLUCOSIDES FROM THE FLOWERS OF *PETUNIA GUARAPUAVENSIS*

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Abstract —— Two novel diacylanthocyanins were isolated from the flowers of *Petunia guarapuavensis* T. Ando *et* Hashim. and determined to be malvidin 3-*O*-[6-*O*-(4-*O*-(4-*O*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)-*trans*-*p*-coumaryl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside] and 3-*O*-[6-*O*-(4-*O*-(4-*O*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)-*trans*-caffeyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside] .

From the flowers of *Petunia hybrida* cultivars, at least, twenty five anthocyanins were isolated and their structures were determined to be 3-glucosides, 3,5-diglucosides, 3-rutinosides, 3-rutinoside-5-glucosides

and 3-*p*-coumarylrutinoside-5-glucosides of cyanidin, peonidin, petunidin and malvidin.[1] Recently, Griesbach *et al.* found additionally three 3-caffeylrutinoside-5-glucosides of peonidin, petunidin and malvidin in the flowers of *P. hybrida* cultivars.[2,3] In this paper we wish to report the structure elucidation of two novel diacylated anthocyanins in the flowers of *P. guarapuavensis* T. Ando *et* Hashim., a species recently described from southern Brazil.

Seeds of *Petunia guarapuavensis* was collected from an isotype specimen (B65).[4] These seeds were sown in mid January, and the plants were grown in a green house of Chiba University (Matsudo). The fresh corollas of these plants were collected in June - August 1995 - 1996, dried overnight at 37°C, and kept in the refrigerator at 10°C. Dried corollas (*ca.* 50 g) of *P. guarapuavensis* was extracted with MeOH-HOAc-H_2O (2L, MAW, 4:2:14) at room temperature overnight. The extract was concentrated to *ca.* 100 mL and adsorbed on Diaion HP-20, washed with 10% HOAc and eluted with HOAc-MeOH (1:9). The eluate was concentrated, and purified by PC and HPLC as the previous procedure.[5] Solvents used were *n*-BuOH-HOAc-H_2O (BAW, 4:1:2) and 15% HOAc for paper chromatography. Preparation HPLC was run on a Waters C_{18} (19ϕ×150 mm) column at 40°C with a flow rate of 4 ml/min and monitoring at 530 nm for anthocyanins. A solvent systems used was linear gradient elution for 18 min from 30 to 60% 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). Consequently, two diacylated anthocyanins, pigment (**1**) (*ca.* 50 mg)[6] and pigment (**2**) (*ca.* 30 mg)[7] were obtained as major pigments.

Pigments (**1**) and (**2**) were hydrolyzed to give malvidin, glucose and rhamnose, whereas **1** gave *p*-coumaric and caffeic acids and **2** gave only caffeic acid as the components of acylating acids. Both anthocyanins yielded only one pigment (**3**) as their deacyl anthocyanins by alkaline hydrolysis with NaOH under nitrogen. This deacylanthocyanin was determined to be malvidin 3-rutinoside-5-glucoside by chemical and spectral methods (Table 1, Figure 1).[8] The FAB mass spectra of **1** and **2** gave their molecular ions $[M]^+$ at 1271 and 1287 *m/z* in good agreement with the masses calculated for $C_{59}H_{67}O_{31}$ and $C_{59}H_{67}O_{32}$.

Analysis of the ^{1}H NMR spectr of **1** and **2** revealed that the presence of three molecules of glucose, one molecule of rhamnose, one molecule of malvidin and two molecules of hydroxycinnamic acids in both pigments. The aromatic proton signals of anthocyanidin, *p*-coumaric and caffeic acids of **1** and **2** were assigned by ^{1}H-^{1}H COSY and DIFNOE spectral methods as shown in Table 1. All eight olefinic proton signals of *p*-coumaric acid and caffeic acids of **1** and **2** had large coupling constants (J=16.0 Hz), indicating these four hydroxycinnamic acids to have the *trans* configurations. The signals of four anomeric protons of **1** appeared at δ 5.68 (*d*, J=7.0 Hz, Glc A), δ 5.12 (*d*, J=7.5 Hz, Glc B), δ 5.04 (*d*, J=7.6 Hz, Glc C) and δ 4.61 (*s*,

Table 1 ^{1}H NMR spectral data of *Petunia guarapuavensis* anthocyanins (500 M Hz, DCl-DMSO-*d* 6, 1:9 at 25℃ , standard TMS)

	1 δH	2 δH	3 δH
Malvidin			
4	9.07 *s*	9.09 *s*	9.09 *s*
6	7.16 *br s*	7.11 *br s*	7.21 *d* (2.0)
8	7.39 *br s*	7.40 *br s*	7.44 *d* (2.0)
2',6'	7.98 *s*	7.99 *s*	8.02 *s*
3',5' (O-CH$_3$)	3.94 *s*	3.94 *s*	3.74 *s*
Hydroxycinnamic acid [I]			
2	7.58 *d* (8.6)	7.17 *br s*	
3	7.06 *d* (8.6)		
5	7.06 *d* (8.6)	7.08 *d* (8.4)	
6	7.58 *d* (8.6)	6.99 *br d* (8.4)	
α	6.31 *d* (16.0)	6.27 *d* (16.0)	
β	7.51 *d* (16.0)	7.47 *d* (16.0)	
[II]			
2	7.10 *d* (2.0)	7.11 *br s*	
3			
5	6.80 *d* (8.6)	6.81 *d* (8.1)	
6	6.97 *dd* (2.0, 8.6)	6.98 *d* (8.1)	
α	6.25 *d* (16.0)	6.25 *d* (16.0)	
β	7.45 *d* (16.0)	7.47 *d* (16.0)	
Glucose [A]			
1	5.68	5.71	5.67
2	3.50	3.51	3.52
3	3.38	3.39	3.43
4	3.33	3.32	3.24
5	3.74	3.71	3.55
6 (a)	3.76 - 3.83	3.75	3.61
(b)	3.84	3.84	3.82
[B]			
1	5.12	5.13	5.12
2	3.50	3.48	3.51
3	3.36	3.42	3.41
4	3.28	3.36	3.37
5	]	3.58	3.52
6 (a)	3.63 - 3.80	] 3.63 - 3.80	3.60
(b)	]	]	3.80
[C]			
1	5.04	4.90	
2	3.31	3.40	
3	3.39	3.32	
4	3.27	3.30	
5	3.66	3.75	
6 (a)	4.21	4.26	
(b)	4.41	4.43	
Rhamnose			
1	4.61	4.62	4.54
2	3.65	3.38	3.55
3	3.66	3.33	3.32
4	4.81	4.80	3.11
5	3.62	3.63	3.36
6 (-CH$_3$)	0.88	0.90	1.02

J in parentheses. The data obtained from ^{1}H NMR, ^{1}H- ^{1}H COSY, ^{13}C- ^{1}H COSY and DIFNOE spectral methods.

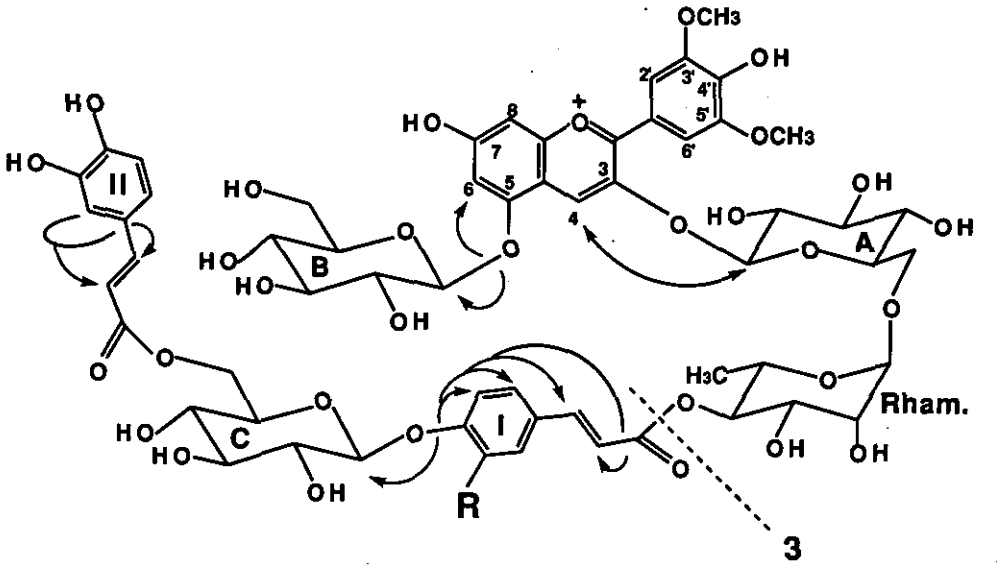

Figure 1 Anthocyanins from *Petunia guarapuavensis*.
Pigment 1 : R = H, 2 : R = OH, 3 : deacylanthocyanin (malvidin 3-rutinoside-5-glucoside).
Main NOEs are indicated by arrows.

rhamnose), and the assigned glucose protons had coupling constants J=7.0 - 11.0 Hz, indicating these glucose residues of **1** must be of β-D-glucopyranose form. In the rhamnose moiety of **1**, one singlet signal of anomeric proton (δ 4.61) and one doublet signals of methyl protons (δ 0.88, *d*, J=6.5 Hz) at C-5 suggested the existence of α-L-rhamnopyranose form. The proton signals of H-4 of rhamnose (δ 4.81, *t*, J=8.9 Hz) and H-6a and b (δ 4.21, *dd*, J=6.0, 11.0 Hz and δ 4.41, *br d*, J=11.0 Hz) of Glc C were shifted to lower magnetic fields (Figure 2), indicating that the OH-4 of rhamnose and OH-6 of Glc C are acylated with hydroxycinnamic acids. In order to determine the attachments and positions of the sugar and hydroxycinnamic acid units in the pigment molecule, DIFNOE spectra were measured. By irradiation at both anomeric protons of Glc A and B, NOEs were observed at H-4 and H-6 of malvidin, respectively, indicating Glc A is bonded with 3-OH of malvidin and Glc B is bonded with 5-OH of malvidin (Figure 1). By deacylanthocyanin of **1** and **2**, it was confirmed that rhamnose is bonded with 6-OH of Glc A and formed rutinoside in this pigment (Table 1). Furthermore, by irradiation at H-1 of Glc C of **1**, NOEs were observed at H-3,5 and H-2,6 of *p*-coumaric acid and also rather weak NOEs were observed at H-α and β

of caffeic acid. Therefore, **1** is malvidin 3-*O*-[6-*O*-[4-*O*-(4-*O*-*trans*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)-*p*-coumaryl)-α-L-rhamnopyranosyl]-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside] which is a new pigment.[3] This structure was confirmed by the analysis of its HMQC and HMBC spectra. A similar structural study of this pigment in the cultivars of *Petunia* was reported independently by Fukui *et al*., but they did not describe it in such detail.[9]

The signals of anthocyanidin and sugar moieties of **2** were good agreement with those of **1** (Table 1, Figure 2). The linkages and attachment positions of sugar and hydroxycinnamic acid units of **2** were determined by the application of DIFNOE method and the analysis of ^{1}H-^{1}H COSY spectra as the similar manner of **1**. The proton signals of H-4 of rhamnose δ 4.80, *t*, *J*=9.2 Hz) and H-6a and b (δ 4.26, *dd*, *J*=6.5, 11.1 Hz and δ 4.43, *br d*, *J*= 11.1 Hz) of Glc C were also shifted to lower magnetic fields (Figure 2). This result indicates that the OH-4 of rhamnose and OH-6 of Glc C in **2** were acylated with both caffeic acids (I and II). The glycoside bond between the OH-4 of caffeic acid (I) and the OH-1 of Glc C was confirmed by DIFNOE method as for **1**. Consequently, **2** was determined to be malvidin 3-*O*-[6-*O*-[4-*O*-(4-*O*-*trans*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)-caffeyl)-α-L-rhamnopyranosyl]-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside] which is a new pigment.[3]

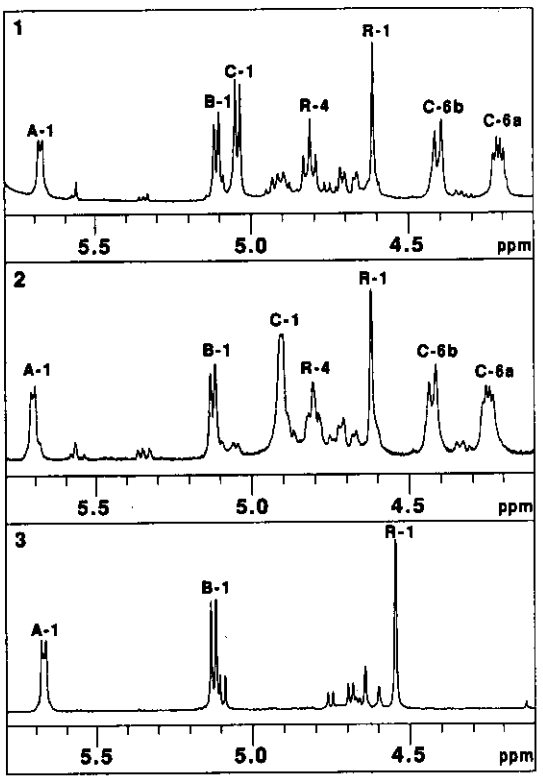

Figure 2 ^{1}H-NMR spectra of the sugar regiones of pigment 1 (upper, 1), pigment 2 (middle, 2), and deacyl pigment 1 and 2 (lower, 3) at 500 MHz in DCl/DMSO - *d*$_6$ (1:9).

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6. Pigment (**1**); UV λmax (0.1% HCl-MeOH) 543, 325, 284 nm, $E_{440}/E\text{max}$ = 0.15, $E\text{acyl}/E\text{max}$ = 0.73; TLC Rf-values (×100) BuOH-AcOH-H_2O (BAW, 4:1:2) 36, BuOH-2N HCl (BuHCl, 1:1) 2, 1% HCl 7, AcOH-HCl-H_2O (AHW, 15:3:82) 29; FAB MS *m/z* 1271; HPLC Rt (min) 28.1; ^{13}C NMR (100MHz, DCl/DMSO-*d*6, 1:9) : malvidin 162.1(C2), 147.6(C3), 133.8(C4), 154.7(C5), 104.4(C6), 168.1(C7), 96.8(C8), 155.8(C9), 112.0(C10), 118.2(C1'), 109.8(C2'), 148.3(C3'), 144.8(C4'), 148.3(C5'), 109.8(C6'), 56.6(2×CH3); *p*-coumaric acid 128.0(C1), 130.0(C2), 116.6(C3), 159.0(C4), 116.6 (C5), 130.0(C6), 116.0(Cα), 144.4(Cβ), 166.3(C=O); caffeic acid 125.6(C1), 114.6(C2), 145.0(C3), 148.5(C4), 116.0(C5), 121.6(C6), 113.6(Cα), 145,6(Cβ), 166.6(C=O); Sugars 101.3 (A-1), 101.4(B-1), 99.9(C-1), 100.5(Rham-1), 77.4, 76.4, 75.9, 75.7, 73.9(Rham-4), 73.6 (C-5), 73.2(Rham-2), 73.0(C-2), 70.3, 69.5(Rham-3), 69.3, 68.3, 66.1(Rham-5), 65.7, 65.1(A-6), 63.4(C-6), 60.5.
7. Pigment (**2**); UV λmax (0.1% HCl-MeOH) 543, 321, 283 nm, $E_{440}/E\text{max}$ = 0.13, $E\text{acyl}/E\text{max}$ = 0.88; TLC Rf-values (×100) BAW 35, BuHCl 1, 1%HCl 6, AHW 24; FAB MS *m/z* 1287; HPLC Rt (min) 27.2
8. Malvidin 3-rutinoside-5-glucoside (**3**); UV λmax (0.1% HCl-MeOH) 537, 273 nm, $E_{440}/E\text{max}$ = 0.11, TLC Rf-values (×100) BAW 29, BuHCl 1, 1%HCl 45, AHW 67; FAB MS *m/z* 801; HPLC Rt (min) 17.2.
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