Pergamon

0031-9422(95)00822-S

Phytochemistry, Vol. 41, No. 6, pp. 1607–1611, 1996

0031-9422/96 $15.00 + 0.00

ACYLATED PEONIDIN GLYCOSIDES IN THE SLATE FLOWERS OF *PHARBITIS NIL*

NORIO SAITO, FUMI TATSUZAWA,*† KICHIJI KASAHARA,‡ MASATO YOKOI,* SHIGERU IIDA,§ ATSUSHI SHIGIHARA‖ and TOSHIO HONDA‖

Chemical Laboratory, Meiji-Gakuin University, Totsuka, Yokohama, Japan; *Faculty of Horticulture, Chiba University, Matsudo, Chiba, Japan; §Department of Biological Science and Technology, Science University of Tokyo, Yamazaki, Noda-shi, Chiba, Japan; ‖ Institute of Medicinal Chemistry, Hoshi University, Shinagawa, Tokyo, Japan

(*Received* 15 *August* 1995)

Key Word Index—*Pharbitis nil*; Convolvulaceae; grey–purple–blue flower colour; acylated anthocyanins; caffeyl peonidin and cyanidin glucosides.

Abstract—Two new acylated and three known anthocyanins were isolated from the slate flowers of *Pharbitis nil* cultivars. The new anthocyanins were determined to be peonidin 3-*O*-[6-*O*-(*trans*-3-*O*-(β-D-glycosyl)caffeyl)-β-D-glucoside] as a major pigment, and cyanidin 3-*O*-[6-*O*-(*trans*-3-*O*-(β-D-glycosyl)caffeyl)-β-D-glucoside]-5-*O*-[β-D-glucoside] as a minor component. The three known pigments were peonidin 3-glycosylcaffeylglucoside-5-glucoside as a major pigment, and the 3- and 3,5-diglucosides of peonidin as minor pigments. A survey of five slate flower cultivars of this plant showed that peonidin 3-glucosylcaffeylglucoside and peonidin 3-glucosylcaffeylglucoside-5-glucoside were the dominant flower pigments.

INTRODUCTION

In continuing the work on flower colour variation in the various colour cultivars of *Pharbitis nil*, we have already reported the occurrence of acylated peonidin, pelargonidin and cyanidin glycosides, and also flavonols, chalcones, aurones and a flavanone in the violet–blue, red–purple, maroon, pale yellow and white flower cultivars [1–7]. In further studies on the slate (dusky violet–blue; Kuro-bato and Aka-bato) cultivars [8–11], we have now isolated and identified two new caffeyl peonidin and cyanidin glycosides along with three known anthocyanins.

RESULTS AND DISCUSSION

In a survey of *P. nil* cultivars by HPLC, nine anthocyanins were observed in the slate flowers of these plants. Five anthocyanins were isolated and identified as **1** (frequency *ca* 17.8%), **2** (*ca* 3.6%), **3** (*ca* 7.0%), **4** (23.9–68.4%) and **5** (*ca* 4.6%).

The isolation and structural determination of these anthocyanins were performed by standard procedures [2, 5]. Their R_f values, R_t (min) and spectral data are shown in Table 1. The data for pigment **2** are identical with those for peonidin 3-glucoside. Acid hydrolysis of **2** gave peonidin and glucose. The FAB mass spectrum of **2** gave its $[M]^+$ at 463 *m/z*, corresponding to the mass calculated for $C_{22}H_{23}O_{11}$ (463.123). Pigment **1** is peonidin 3,5-diglucoside, and gave peonidin and glucose by acid hydrolysis. The FAB mass spectrum of **1** gave its $[M]^+$ at 625 *m/z*, corresponding to the mass calculated for $C_{28}H_{33}O_{16}$ (625.176). The structure of **1** was confirmed by analysis of its ^{1}H NMR spectrum (Table 2). The strong intensities of UV absorption suggest that three other pigments (**3–5**) are acylated by caffeic acid (Table 1). On acid hydrolysis **3** and **4** produced peonidin, glucose and caffeic acid, while **5** produced cyanidin, glucose and caffeic acid. After deacylation, **3** gave peonidin 3-glucoside, **4** gave peonidin 3,5-diglucoside, and **5** gave cyanidin 3,5-diglucoside.

Pigment 3

The FAB mass spectrum of **3** showed a molecular ion peak $[M]^+$ at 787 *m/z*, corresponding to the molecular formula $C_{37}H_{39}O_{16}$ (787.207). The ^{1}H NMR spectrum of **3** showed the presence of one molecule of peonidin, two molecules of glucose and one molecule of caffeic acid. The protons of peonidin and caffeic acid were assigned by analysis of the ^{1}H-^{1}H COSY spectrum of **3** (Table 2). Two anomeric protons were assigned at δ5.49 (*d*, *J* = 7.5 Hz, Glc A, Fig. 1) and 4.80 (*d*, *J* = 7.1 Hz, Glc C), and all the observed vicinal coupling constants of both glucose A and C were 7.1–9.5 Hz, indicating that these glucoses are β-D-glucopyranose. The two characteristic protons at δ4.23 and 4.48 were assigned to the methylene protons of Glc A by analyses of the ^{1}H-^{1}H COSY and the

† Author to whom correspondence should be addressed.
‡Permanent address: 1128 Moro-oka, Koho-ku, Yokohama, Japan.

Table 1. Chromatographic and spectral properties of anthocyanins from grey-purple flowers of *Pharbitis nil*

Anthocyanin	R_f values (×100)†				Spectral data in 0.1% HCl–MeOH				R_t* (min)	FAB-MS $[M]^+$
	BAW	BuHCl	1% HCl	AHW	λ_{max}(nm)	E_{acyl}/E_{max} (%)	E_{440}/E_{max} (%)	$AlCl_3$		
1 (deacyl-**4**)	22	4	10	30	524, 278	—	13	0	7.7	625
2 (deacyl-**3**)	28	18	4	16	527, 280	—	28	0	11.3	463
3	37	14	6	29	539, 317, 293, 283	79	35	0	20.6	787
4	23	4	20	48	526, 315, 293, 280	75	19	0	15.3	949
5	16	5	13	37	526, 317, 292, 280	81	21	+	11.8	935

*Column: waters C18 (4.6 ϕ × 250 mm); for other details see Experimental.
†See Experimental for solvent abbreviations.

negative difference NOE (DIFNOE) spectra, indicating the caffeic acid unit is attached to the OH-6 of Glc A. The application of the DIFNOE method to **3** revealed that Glc A was bonded to the OH-3 of peonidin and Glc C was attached to the OH-3 of caffeic acid (Fig. 1). By irradiation of H-1 of Glc A the occurrence of a NOE was observed at the H-2 of caffeic acid. Therefore, **3** is peonidin 3-*O*-[6-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranoside], which is a new anthocyanin [11, 12].

Pigment **4**

This component is a major pigment. The FAB mass spectrum of **4** gave its molecular ion at 949 *m/z*, corresponding to $C_{43}H_{49}O_{24}$. This pigment is identical with peonidin 3-glucosylcaffeylglucoside-5-glucoside, which has been isolated and identified in the maroon flowers of this plant [5]. The structure was confirmed by analyses by ^{1}H NMR spectrometry including ^{1}H–^{1}H COSY and DIFNOE spectra (Table 2).

Pigment **5**

The FAB mass spectrum of **5** gave its molecular ion at 935 *m/z*, corresponding to $C_{42}H_{47}O_{24}$. The ^{1}H NMR spectrum showed the presence of one molecule of cyanidin and three molecules of glucose, and one molecule of caffeic acid (Table 2). The proton signals of sugar moieties appeared in the region δ5.52–3.20. Three anomeric protons were assigned at δ5.52 (*d*, *J* = 7.9 Hz, Glc A), 5.12 (*d*, *J* = 7.5 Hz, Glc B) and 4.80 (*d*, *J* = 7.1 Hz, Glc C) and all the observed vicinal coupling constants of glucose were *ca.* 7.1–9.0 Hz. Therefore, these glucose units are β-D-glucopyranose. The DIFNOE spectrum of **5** like that of **3** revealed that the OH-3 and OH-5 of cyanidin were bonded to Glc A and Glc B, respectively, through glycosidic bonds, and also the OH-3 of caffeic acid was linked to Glc C through a glucosidic bond. The two methylene protons were shifted to a low magnetic field (δ4.24 and 4.52), and assigned to be the methylene protons of Glc A by the ^{1}H–^{1}H COSY and DIFNOE spectrum analyses [1], indicating that the OH-6 of Glc A is acylated with caffeic acid (Fig. 1). Therefore, **5** is cyanidin 3-*O*-[6-*O*-(*trans*-3-*O*-(β-D-glycopyranosyl)caffeyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside], which is a new anthocyanin [11, 12].

EXPERIMENTAL

Plant material. Six cultivars, a group of Kuro-bato: Kagurahen, Unzen no Takigi, Cha no Orido and Kotokagami, and another group of Aka-bato: Sakura no Sui and Aka-bato strain were grown in the garden of one of the authors, (K.K.). Fresh corollas of the cultivars were collected in August–October 1994.

Isolation of anthocyanins. Mixed fresh slate corolla limbs (*ca* 2 kg) were extracted with MAW (MeOH–HOAc–H_2O, 9:1:10) (6 l). The extract was concd to *ca* 500 ml. The concd extract was purified by

Table 2. ^{1}H NMR data for anthocyanins in the grey–purple flowers of *Pharbitis nil*

H	2	3	4	5	Deacyl-3	Deacyl-4 (1)
Peonidin or cyanidin						
4	9.00 *s*	8.93 *s*	8.91 *s*	8.85 *s*	8.99 *s*	9.05 *s*
6	7.03 *br s*	6.72 *d* (2.0)	7.03 *d* (2.0)	7.03 *d* (2.0)	6.95 *d* (1.8)	7.09 *d* (1.6)
8	7.25 *br s*	7.01 *d* (2.0)	7.19 *d* (2.0)	7.08 *d* (2.0)	7.09 *d* (1.8)	7.30 *d* (1.6)
2′	8.23 *d* (2.1)	8.17 *d* (2.4)	8.20 *d* (2.0)	8.06 *d* (2.4)	8.20 *d* (2.5)	8.23 *d* (2.0)
5′	7.14 *d* (9.0)	7.11 *d* (8.7)	7.13 *d* (8.7)	7.09 *d* (8.8)	7.18 *d* (8.5)	7.18 *d* (8.7)
6′	8.38 *dd* (2.1, 9.0)	8.27 *dd* (2.4, 8.7)	8.33 *dd* (2.0, 8.7)	8.28 *dd* (2.4, 8.8)	8.27 *dd* (2.5, 8.5)	8.37 *dd* (2.0, 8.7)
$-OCH_3$	3.97	3.95	3.96	—	3.95	3.97
Caffeic acid						
2		7.39 *d* (1.6)	7.43 *d* (2.0)	7.36 *d* (2.0)		
5		6.86 *d* (8.3)	6.88 *d* (7.9)	6.88 *d* (8.3)		
6		7.11 *dd* (1.6, 8.3)	7.22 *dd* (2.0, 7.9)	7.23 *dd* (2.0, 8.3)		
α		6.34 *d* (15.9)	6.35 *d* (15.9)	6.37 *d* (15.9)		
β		7.43 *d* (15.9)	7.41 *d* (15.9)	7.43 *d* (15.9)		
Glucose*†						
(A)						
1	5.41	5.49	5.53	5.52	5.42	5.46
2	3.51	3.54	3.57	3.65	3.53	3.53
3	⎡	3.47	3.39	3.46	3.37	⎡
4	⎢	3.35	3.24	3.35	3.23	⎢
5	3.82–3.15	3.87	3.44	3.72	3.48	3.80–3.20
6a	⎢	4.23	3.94	4.24	3.50	⎢
6b	⎣	4.48	4.38	4.52	3.72	⎣
(B)						
1			5.13	5.12		5.15
2			3.53	3.54		3.51
3			3.41	⎡		⎡
4			3.33	⎢		⎢
5			3.59	3.80–3.20		3.80–3.20
6a			3.76	⎢		⎢
6b			3.81	⎣		⎣
(C)						
1		4.80	4.90	4.80		
2		3.34	3.33	3.35		
3		3.19	⎡	⎡		
4		3.39	⎢	⎢		
5		3.48	3.85–3.20	3.80–3.20		
6a		3.55	⎢	⎢		
6b		3.75	⎣	⎣		

*Assigned by ^{1}H–^{1}H COSY.
†Assigned by DIFNOE.
Coupling constants (*J* in Hz) in parentheses.

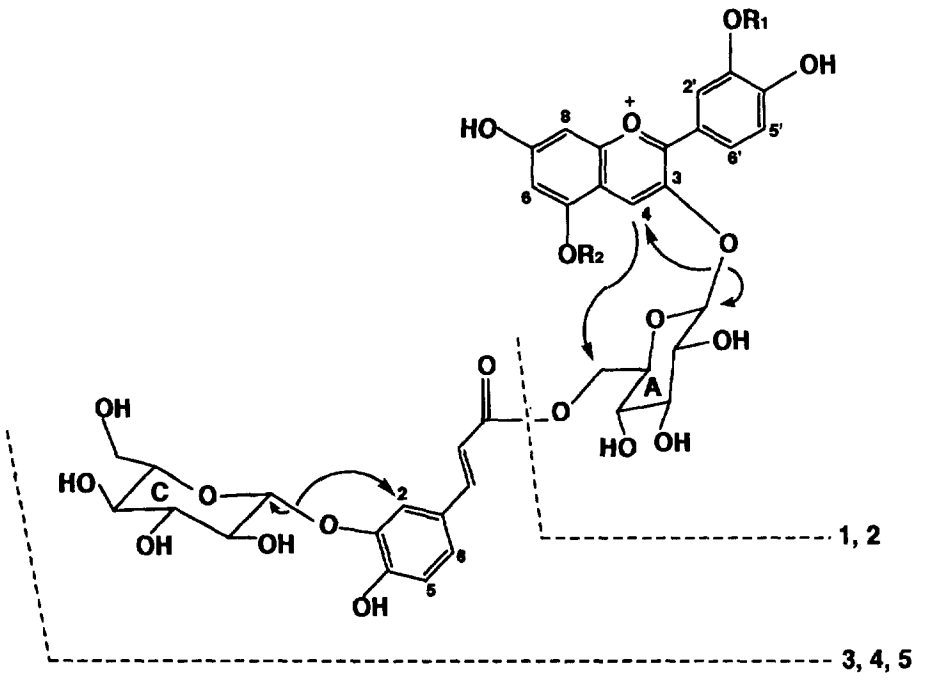

Fig. 1. Acylated anthocyanins in the slate flowers of *Pharbitis nil*. **1**: R_1 = Me, R_2 = Glc(B); **2**: R_1 = Me, R_2 = H; **3**: R_1 = Me, R_2 = H; **4**: R_1 = Me, R_2 = Glc(B); **5**: R_1 = H, R_2 = Glc(B). Observed NOEs are indicated by arrows.

Table 3. Distribution of anthocyanins in flower extracts of *Pharbitis nil* cultivars

	Anthocyanin (as %)*								
Cultivar	1	2	5	A	4	B	C	D	3
Kuro-bato									
Kagurahen	+	3.6	+	11.0	23.9	+	+	10.4	7.0
Unzen no Takigi	4.7	+	4.6	10.0	68.4	+	+	+	+
Cha no Orido	16.5	+	3.6	6.6	58.4	+	+	+	+
Kotokagami	17.8	+	+	7.8	57.5	+	4.9	+	+
Aka-bato									
Sakura no Sui	8.2	+	3.6	10.0	50.3	+	7.4	3.9	4.4
Strain	8.0	+	+	10.3	63.8	+	4.9	+	3.4

*Anthocyanin no. and HPLC conditions are the same as in Table 1. R_t(min): **1** = 7.7, **2** = 11.3, **3** = 20.6, **4** = 15.3 and **5** = 11.8; unidentified anthocyanins, A = 13.8, B = 16.6, C = 19.2 and D = 19.7. Percentage of total absorbance of all detected anthocyanins at 530 nm in HPLC analysis.
+ = under 3%.

Diaion HP-20 gel CC, PC and TLC [5, 7]. Solvents used were BAW (*n*-BuOH–HOAc–H_2O, 4:1:5), BuHCl (*n*-BuOH–2 M HCl, 1:1), AHW (HOAc–HCl–H_2O, 15:3:82), 1% HCl, MAW and 15% HOAc. Prep. HPLC was run on a Waters C_{18} (19ϕ × 150 mm) column at 40° with a flow rate of 4 ml min^{-1}, UV-vis monitoring at 530 nm for anthocyanins. Solvent systems used were: a linear gradient elution for 40 min from 40 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). The pigment frs were evapd *in vacuo* to dryness. After these processes each fr. of these pigments was dissolved in a small vol. of 5% HOAc–MeOH or 3% TFA–MeOH, and pptd by addition of excess Et_2O. Then the pptd pigments were dried to powders **1** (*ca* 10 mg), **2** (*ca* 5 mg), **3** (*ca* 10 mg), **4** (*ca* 50 mg) and **5** (*ca* 10 mg).

Distribution of anthocyanins in the slate (greyish) flower colours of six cultivars. There were two kinds of slate flower colour in *P. nil* cvs. One group showed grey–blue–purple flowers named the Kuro–bato, another exhibited reddish grey–blue–purple flowers named the Aka-bato.

These fresh corolla limbs were extracted with 20% MeOH containing 1.5% H_3PO_4. HPLC purification was carried out. The quantitative analysis was performed as described previously [1–7] by HPLC—a Waters C_{18} (4.6 $\phi \times 250$ mm) column at 40° with a flow rate of 1 ml min^{-1} UV-vis monitoring at 530 nm for anthocyanins. Solvent system used was: linear gradient elution for 30 min from 40 to 85% solvent B in solvent A. The results for anthocyanin components in both groups were very similar for flowers of the Kuro-bato and flowers of the Aka-bato (Table 3).

Standard analysis of anthocyanins. Pigment identifications were carried out by standard procedures involving deacylation with a base and hydrolysis with an acid [1–7, 13]. ^1H NMR (400 MHz) spectra of anthocyanins were measured in CF_3CO_2D–DMSO–d_6 (1:9).

Acknowledgements—We thank Ms K. Yamakage and Ms T. Kakefuda for their technical assistance.

REFERENCES

1. Lu, T. S., Saito, N., Yokoi, M., Shigihara, A. and Honda, T. (1991) *Phytochemistry* **30**, 2387.
2. Lu, T. S., Saito, N., Yokoi, M., Shigihara, A. and Honda, T. (1992) *Phytochemistry* **31**, 289.
3. Lu, T. S., Saito, N., Yokoi, M., Shigihara, A. and Honda, T. (1992) *Phytochemistry* **31**, 655.
4. Saito, N., Lu, T. S., Yokoi, M., Shigihara, A. and Honda, T. (1993) *Phytochemistry* **33**, 245.
5. Saito, N., Lu, T. S., Akaizawa, M., Yokoi, M., Shigihara, A. and Honda, T. (1994) *Phytochemistry* **35**, 407.
6. Saito, N., Cheng, J., Ichimura, M., Yokoi, M., Abe, Y. and Honda, T. (1994) *Phytochemistry* **35**, 687.
7. Saito, N., Tatsuzawa, F., Yoda, K., Yokoi, M., Kasahara, K., Iida, S., Shigihara, A. and Honda, T. (1995) *Phytochemistry* **40**, 1283.
8. Imai, Y. (1938) *Jpn J. Genet.* **14**, 24.
9. Hagiwara, T. (1954) *Bull. Res. College Agric Vet. Sci., Nihon Univ.* **3**, 1.
10. Hagiwara, T. (1956) *Bull. Res. College Agric Vet. Sci., Nihon Univ.* **5**, 34.
11. Harborne, J. B. and Grayer, R. J. (1988) in *The Flavonoids, Advances in Research since* 1980 (Harborne, J. B., ed.), p. 1. Chapman & Hall, London.
12. Strack, D. and Wray, V. (1994) in *The Flavonoids, Advances in Research since* 1986 (Harborne, J. B., ed.), p. 1. Chapman & Hall, London.
13. Harborne, J. B. (1984) *Phytochemical Methods*, 2nd edn. Chapman & Hall, London.