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ACYLATED CYANIDIN GLYCOSIDES IN THE VIOLET–BLUE FLOWERS OF *IPOMOEA PURPUREA*

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Key Word Index—*Ipomoea purpurea*; Convolvulaceae; violet blue flower colour; acylated anthocyanins; di- and tri-caffeyl cyanidin 3-sophoroside-5-glucoside; 3-glucosylcaffeic acid; 4-glucosylcaffeic acid; caffeic acid.

Abstract—Six acylated cyanidin glycosides were isolated from violet blue flowers of *Ipomoea purpurea*. These anthocyanins were all based on cyanidin 3-sophoroside-5-glucoside, acylated with caffeic acid and/or *p*-coumaric acid. Three anthocyanin structures were elucidated to be cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)caffeyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside], cyanidin 3-*O*-[2-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-6-*O*-(*trans*-caffeyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside] and cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)-6-*O*-(*trans*-caffeyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside]. These three anthocyanins were present in all 12 violet–blue flower strains as major pigments. The colours of these acylated anthocyanins were stabler in neutral solution than their deacyl analogues.

INTRODUCTION

Recently, we reported the structure determination of 16 caffeyl anthocyanidin glycosides in the cultivars of Japanese morning glory (*Pharbitis nil*) [1–6]. In the continuing work on flower colour variation due to acylated anthocyanins, we are interested in the chemical investigation of acylated anthocyanins in *Ipomoea purpurea* (*P. purpurea*), since this species has not been thoroughly studied from the chemical point of view, although a number of papers regarding flower colour inheritance have been published [7–14].

RESULTS AND DISCUSSION

Six main anthocyanin peaks were observed in the violet–blue flowers of 12 strains of *I. purpurea* by HPLC. Their relative frequency of occurrence was 42–67% (pigment **1**), 8–29% (pigment **2**), 0–12% (pigment **3**), 2–5% (pigment **4**), 0–7% (pigment **5**) and 4–7% (pigment **6**). The isolation of these anthocyanins were performed by procedures similar to those previously reported [2, 5, 15]. These anthocyanins were extracted from the mixed flowers of the violet–blue strains with MAW (methanol–acetic acid–water, 10:1:9), and isolated and purified using Diaion HP-20 column chromatography, paper chromatography (*n*-butanol–acetic acid–water) (BAW, 4:1:5) and HPLC. Six anthocyanins (**1**–**6**) were obtained by the above mentioned process. The chromatographic and spectral properties of these anthocyanins are shown in Table 1. The molecular weights of these pigments were determined by FAB mass spectral measurement (Table 1). Furthermore, the structure study of three of the pigments (**1**–**3**) was performed as follows, but for the three others (**4**–**6**) that was not possible due to the small amounts obtained.

Acid hydrolysis of **1**–**6** gave cyanidin, glucose and caffeic acid, and in addition to these products, **5** and **6** gave *p*-coumaric acid. Alkaline hydrolysis of all six anthocyanins yielded only one deacylanthocyanin (**7**). On H_2O_2 degradation of this deacylanthocyanin, sophorose was produced.

Deacylanthocyanin

The FAB mass measurement of this deacylanthocyanin gave a molecular ion $[M]^+$ at 773 *m/z* (calcd for $C_{33}H_{41}O_{21}$ 773.192 *m/z*). The ^1H NMR spectrum of this pigment showed the presence of one molecule of cyanidin and three molecules of glucose. Six aromatic

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Table 1. Chromatographic and spectral properties of anthocyanins from flowers of *Ipomoea purpurea*

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	25	5	17	38	532, 320, 295	565† (595)†	106	17	+	16.7	1583
2	36	7	30	48	530, 323, 295	546† (579)†	104	19	+	12.9	1259
3	46	22	14	36	530, 329, 296	548† (577)†	117	22	+	14.5	1097
4	38	13	27	52	532, 323, 297		154	21	+	14.9	935
5	48	29	16	41	532, 316, 298		340	23	+	16.8	1081
6	41	20	4	22	533, 312, 297		175	19	+	22.3	1405
Deacyl	12	3	58	71	523, (330), 290	575†	31	19	+	4.7	773

*1: cyanidin 3-(2-(glucosylcaffeylglucosyl)-6-(caffeylglucosylcaffeyl)-glucoside)-5-(glucoside).
2: cyanidin 3-(2-(glucosylcaffeylglucosyl)-6-(caffeyl)-glucoside)-5-(glucoside).
3: cyanidin 3-(2-(caffeylglucosyl)-6-(caffeyl)-glucoside)-5-(glucoside).
4: caffeylcyanidin 3-sophoroside-5-glucoside.
5: *p*-coumarylcaffeylcyanidin 3-sophoroside-5-glucoside.
6: *p*-coumarylcaffeylglucosylcaffeylcyanidin 3-sophoroside-5-glucoside.
7: Deacylanthocyanin: cyanidin 3-sophoroside-5-glucoside.
† λ_{max} in neutral aqueous solution (pH 6.86); ( ): inflection.
For key to abbreviations, see Experimental.

protons were assigned to be protons of cyanidin (Table 2). The proton signals of the sugar moiety appeared in the region of δ5.64–2.73. Three anomeric protons were assigned at δ5.64 (*d*, J = 7.3 Hz, Glc A), δ5.16 (*d*, J = 7.3 Hz, Glc B) and δ4.70 (*d*, J = 7.7 Hz, Glc C), and all observed vicinal coupling constants of these glucoses were J = 7.3–10.0 Hz. Therefore, these three glucose units are β-D-glucopyranose. By analysis of ^{1}H–^{1}H COSY of **7**, a proton at δ4.08 (*t*, J = 8.2 Hz, Glc G, H-2) being shifted to the lower field was directly correlated with the H-2 proton of Glc A. Supporting that, Glc C is attached to OH-1 of Glc A through a glucosidic bond. Thus, **7** is cyanidin 3-*O*-(2-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside)-5-*O*-(β-D-glucopyranoside).

Pigment **1**

The FAB mass spectrum gave a molecular ion $[M]^+$ at 1583 *m/z* in good agreement with the mass calculated for $C_{72}H_{79}O_{40}$ (1583.373), and fragment peaks, 1421 $[M - 162]^+$, 1259 $[M - 2\times162]^+$ and 1097 $[M - 3\times162]^+$. In order to elucidate the structure, the ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY and COLOC spectra of **1** were measured in CF_3CO_2D–DMSO-d_6 (1:9). Analysis of ^{1}H NMR and ^{1}H–^{1}H COSY spectra indicated the presence of one molecule of cyanidin, five molecules of glucose (A–E) and three molecules of caffeic acid (I–III). The signals of five anomeric protons appeared at δ5.69 (*d*, J = 7.7 Hz, Glc A), δ5.09 (*d*, J = 7.3 Hz, Glc B), δ4.81 (*d*, J = 7.7 Hz, Glc C), δ4.90 (*d*, J = 6.8 Hz, Glc D) and δ4.84 (*d*, J = 7.3 Hz, Glc E), and all the observed vicinal coupling constants of these five glucose units were 6.8–11.0 Hz, indicating these glucose units to be β-D-glucopyranose form. The six methylene protons of Glc A at δ4.31, 4.44, Glc C at δ3.83, 3.98 and Glc D at δ4.31, 4.44 were assigned by ^{1}H–^{1}H COSY spectrum and negative NOE difference (DIFNOE) experiments [1, 16] to be acylated with *trans*-caffeic acid, which exhibited large coupling constants (J = 15.4, 15.8 and 15.4 Hz) for the olefinic protons, respectively. All aromatic protons of cyanidin and caffeic acid moieties were assigned by analysis of ^{1}H–^{1}H COSY spectra, and confirmed by DIFNOE spectra (Table 2).

By irradiation at H-4 of cyanidin, NOE was observed at H-1 of Glc A, and also at 2-, 5-, α- and β-proton signals of caffeic acid I. Supporting that, Glc A was attached to OH-3 of cyanidin through a glycosidic bond. Moreover, Glc A was presumed to be acylated with caffeic acid I [1-5]. By analysis of the ^{1}H–^{1}H COSY spectrum it was revealed that OH-2 of Glc A is bound to OH-1 of Glc C through a glycosidic bond, and this result was also confirmed by the observed DIFNOE between H-1 of Glc C and H-2 of Glc A. The irradiation of H-1 of Glc C assigned the NOEs to signals of H-2, -3 and -6 of Glc C as well as H-2 and -1 of Glc A, and also at α-, β-, 2-, 5- and 6-H of caffeic acid II. Thus, Glc C is determined to be attached to OH-2 of Glc A, and also acylated with caffeic acid II like the *Pharbitis* anthocyanins [2, 3]. Glc B was attached to OH-5 of cyanidin because of the occurrence of NOE between H-6 of cyanidin and H-1 of Glc B (Fig. 1).

Table 2. NMR spectral data for *Ipomoea purpurea* anthocyanins (400 MHz, CF_3CO_2D–DMSO-d_6, 1:9 at 25°, standard TMS)

	1		2		3	4
	δC	δH	δC	δH	(δH)	(δH)
Cyanidin						
2	162.3		162.3			
3	146.3		145.6			
4	132.8	8.81 *s*	132.8	8.80 *s*	8.81 *s*	8.90 *s*
4a	111.7		111.7			
5	155.2		155.2			
6	105.0	6.96 *br s*	105.0	6.98 *br s*	6.92 *br s*	7.04 *br s*
7	167.8		167.8			
8	96.3	6.97 *br s*	96.3	6.95 *br s*	6.95 *br s*	7.16 *br s*
8a	155.2		155.2			
1′	119.4		121.4			
2′	117.7	8.05 *d* (2.0)	117.7	8.05 *d* (2.1)	8.05 *d* (2.2)	8.11 *d* (2.0)
3′	147.0		146.3			
4′	155.2		155.2			
5′	117.0	7.10 *d* (9.0)	117.1	7.11 *d* (8.5)	7.11 *d* (8.8)	7.13 *d* (9.0)
6′	127.5	8.25 *dd* (2.0, 9.0)	127.5	8.25 *dd* (2.1, 8.5)	8.26 *dd* (2.2, 8.8)	8.29 *dd* (2.0, 9.0)
Caffeic acid*						
(I)						
1	121.4		124.8			
2	115.2	7.51 *br s*	115.3	6.98 *br s*	6.96 *br s*	
3	144.2		145.4			
4	147.4		148.4			
5	115.9	6.85 *d* (8.1)	115.9	6.76 *d* (8.1)	6.76 *d* (8.4)	
6	120.7	7.22 *d* (8.1)	124.2	6.87 *d* (8.1)	6.87 *br d* (8.4)	
α	116.2	6.44 *d* (15.4)	115.3	6.19 *d* (15.8)	6.19 *d* (15.8)	
β	144.3	7.56 *d* (15.4)	144.2	7.32 *d* (15.8)	7.32 *d* (15.8)	
C=O	166.1		166.3			
(II)						
1	125.9		125.8			
2	116.4	6.96 *br s*	115.9	7.36 *br s*	6.96	
3	145.6		145.6			
4	149.6		149.6			
5	116.4	6.76 *d* (8.2)	116.4	6.84 *d* (8.1)	6.76 *d* (8.1)	
6	124.5	6.87 *d* (8.2)	125.6	7.04 *br d* (8.1)	6.82 *d* (8.1)	
α	114.9	6.18 *d* (15.8)	114.6	6.12 *d* (15.8)	5.96 *d* (15.8)	
β	145.1	7.32 *d* (15.8)	144.8	7.24 *d* (15.8)	7.18 *d* (15.8)	
C=O	166.6		166.6			
(III)						
1	128.8					
2	115.2	7.02 *br s*				
3	145.4					
4	148.5					
5	115.9	7.07 *d* (8.2)				
6	125.6	6.89 *d* (8.2)				
α	113.7	6.07 *d* (15.4)				
β	145.7	7.21 *d* (15.4)				
C=O	166.6					
Glucose*†						
(A)						
1	99.4	5.69	99.3	5.69	5.68	5.64
2	81.8	4.07	81.8	4.07	4.07	4.08
3	74.1	3.75	74.1	3.75	3.75	3.69
4	69.8	3.47	69.3	3.47	3.49	3.43
5	76.1	3.98	76.1	3.97	3.93	3.53
6	62.6	4.31 (a), 4.44 (b)	63.1	4.33 (a), 4.40 (b)	4.33 (a), 4.41 (b)	3.63 (a), 3.79 (b)

Continued overleaf

Table 2. *Continued*

	1		2		3	4
	δC	δH	δC	δH	(δH)	(δH)
(B)						
1	102.1	5.09	102.1	5.05	5.09	5.16
2	73.3	3.55	73.3	3.55	3.56	3.53
3	75.9	3.47	76.1	3.40	3.41	3.43
4	69.9	3.38	69.9	3.19	3.25	3.32
5	77.4	3.52	77.3	3.39	3.52	3.51
6	61.0	3.75 (a), 3.83 (b)	61.0	3.49 (a), 3.73 (b)	3.58 (a), 3.83 (b)	3.60 (a), 3.78 (b)
(C)						
1	104.5	4.81	104.6	4.81	4.81	4.70
2	74.7	3.13	74.7	3.13	3.14	3.00
3	76.1	3.26	76.1	3.25	3.28	3.12
4	70.2	3.36	70.2	3.35	3.41	3.06
5	77.7	3.55	77.7	3.55	3.20	2.73
6	63.3	3.83 (a), 3.98 (b)	65.0	3.83 (a), 3.95 (b)	3.87 (a), 3.96 (b)	3.09 (a), 3.19 (b)
(D)						
1	101.6	4.90				
2	73.3	3.43				
3	74.2	3.50				
4	69.4‡	3.34				
5	76.2	3.73				
6	63.0	4.31 (a), 4.44 (b)				
(E)						
1	102.1	4.84	102.1	4.82		
2	73.5	3.35	73.5	3.40		
3	75.7	3.19	75.9			
4	69.9‡	3.25	69.8	3.9–3.1		
5	77.4		77.3			
6	61.1	3.9–3.5	61.0			

*Assigned by ^{1}H–^{1}H-COSY.
†Assigned by DIFNOE (H).
‡Values may be exchangeable.
Coupling constants (J in Hz) are given in parentheses.

Irradiation of H-1 of Glc D gave a DIFNOE spectrum in which NOEs to a strong doublet signal of H-5 and rather weak signals of α- and β-H of caffeic acid I were observed. Therefore, Glc D is attached to the OH-4 of caffeic acid I. Furthermore, the signals of H-6a (δ4.31) and H-6b (δ4.44) of Glc D were confirmed by this irradiation. Similar irradiation of H-1 of Glc E assigned the NOEs to H-2 and α- proton signals of caffeic acid II. Thus, Glc E was attached to the OH-3 of caffeic acid II. As the methylene proton signals of Glc E were not shifted to the lower magnetic field, Glc E is a terminal residue in this pigment. On the other hand, because of the methylene proton signals of Glc D being shifted to the lower magnetic field the OH-6 of Glc D is to be esterified with caffeic acid III. Therefore, **1** is cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)caffeyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside], which is a new pigment. This structure was also confirmed by the analysis of ^{13}C NMR, ^{1}H–^{13}C COSY and COLOC spectra as shown in Table 2.

Pigment **2**

The FAB mass spectrum of **2** gave its molecular ion at 1259 *m/z* $[M]^+$, corresponding to $C_{57}H_{63}O_{32}$ (1259.297), and a fragment peak 1097 $[M - 162]^+$. The structure of **2** was mainly determined by the analysis of ^{1}H and ^{13}C NMR spectra in a way similar to the one performed for pigment **1**. The ^{1}H NMR spectrum of **2** showed the presence of one molecule of cyanidin, two of caffeic acid and four of glucose, all of which are of the β-D-glucopyranose form by their vicinal coupling constants (J = 7.0–10.0 Hz). By analysis of its ^{1}H–^{1}H COSY spectrum, six protons of cyanidin and 10 protons of caffeic acid I and II were assigned, as shown in Table 2. The four characteristic protons, being shifted to a lower magnetic field at δ4.33, 4.40 (Glc A) and δ3.83, 3.95 (Glc C), were assigned to be two methylenes of glucose units A and C. The H-1 (δ5.69) of Glc A was finally correlated with the protons of δ4.33 and 4.40, and also the H-1 (δ4.81) of Glc C was correlated with the protons of δ3.83 and 3.95 by analysis of the ^{1}H–^{1}H COSY spectrum. Thus, both glucose units were acylated with caffeic acid I and II.

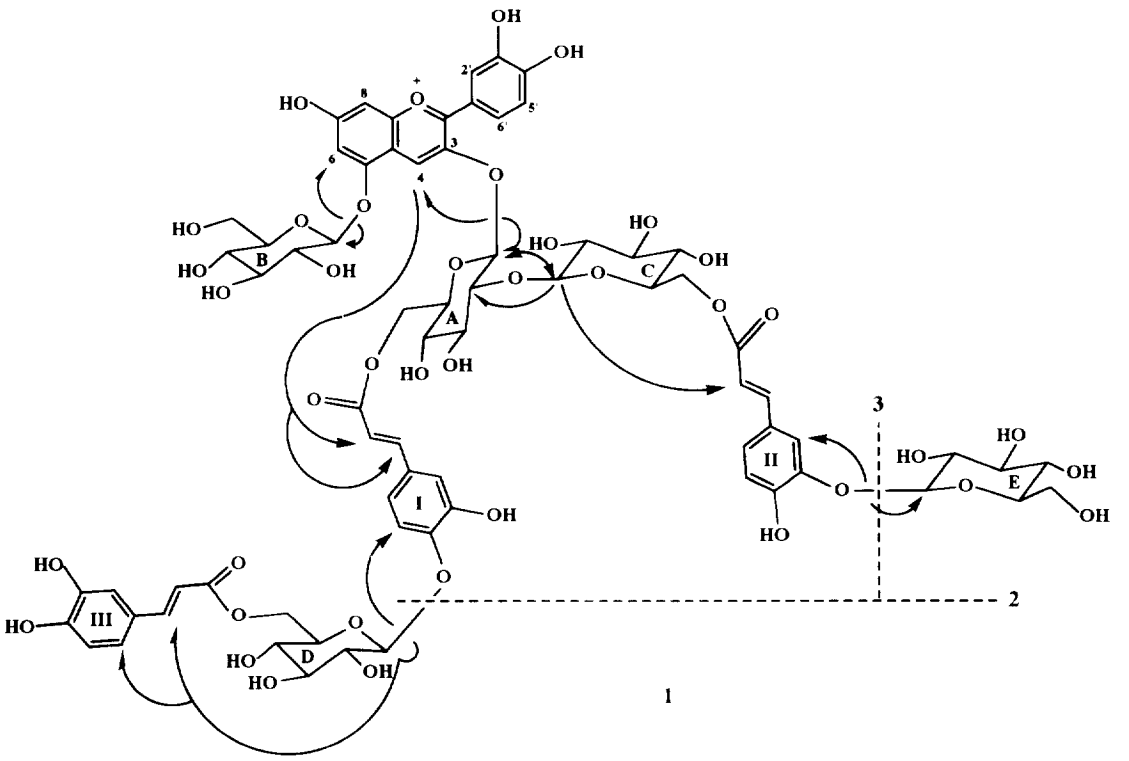

Fig. 1. *Ipomoea purpurea* anthocyanins. Observed NOEs are indicated by arrows.

In order to determine the linkages and/or the positions of attachment of glucoses and caffeic acids in this pigment, DIFNOE spectra were measured. Four anomeric protons were assigned to be δ5.69 (*d*, J = 7.3 Hz, Glc A), δ5.05 (*d*, J = 7.7 Hz, Glc B), δ4.81 (*d*, J = 7.7 Hz, Glc C) and δ4.82 (*d*, J = 7.0 Hz, Glc E). The presence of negative NOEs between H-1 of Glc A and H-4 of cyanidin indicated that Glc A is attached to OH-3 of cyanidin through a glycosidic bond. Also, Glc A is substituted with Glc C at OH-2 of Glc A because of the observation of NOEs between H-2 of Glc A and H-1 of Glc C. This glycosidic bond (2 → 1) between Glc A and Glc C was also confirmed by the analysis of its ^{1}H–^{1}H COSY spectrum. Glc B was determined to be glycosylated at OH-5 of cyanidin, because of the presence of NOEs between H-6 of cyanidin and H-1 of Glc B. Similarly Glc E was attached to OH-3 of caffeic acid II through a glycosidic bond, because of the presence of NOEs between H-2 of caffeic acid II and H-1 of Glc E. By irradiation at H-4 of cyanidin, rather weak NOEs were observed at the signals of H-α, -β, -2 and -6 of caffeic acid I, while strong NOEs at those of H-1, -2 and -3 of Glc A were measured. Therefore, caffeic acid I is bonded with Glc A at OH-6 of Glc A. Thus, pigment **2** is cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-6-*O*-(*trans*-caffeyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside], which is a new pigment [17, 18]. This structure was also confirmed by the analysis of ^{13}C NMR and ^{1}H–^{13}C COSY spectra (Table 2).

Pigement **3**

The FAB mass spectrum of **3** gave its molecular ion $[M]^+$ at 1097 *m/z*, corresponding to the mass calculated for $C_{51}H_{53}O_{27}$ (1097.249). The fragment peak was also observed at 953 $[M - 162]^+$. The ^{1}H NMR signals of **3** were assigned as shown in Table 2. They indicated the presence of one molecule of cyanidin, two molecules of caffeic acid and three molecules of glucose. By analysis of the ^{1}H–^{1}H COSY spectrum of **3**, three anomeric protons were assigned at δ5.68 (*d*, J = 7.7 Hz, Glc A), δ5.09 (*d*, J = 7.7 Hz, Glc B) and δ4.81 (*d*, J = 7.3 Hz, Glc C), respectively, and all observed vicinal coupling constants of these three glucose units were 7.3–11.0 Hz. Therefore, these glucose units are of the β-D-glucopyranose form. As four characteristic protons, being shifted to the lower magnetic field at δ4.33, 4.41 (Glc A) and δ3.87, 3.96 (Glc C), were assigned to the C-6 methylenes of Glc A and C, two caffeic acids I and II, were determined to be bonded to the OH-6 of both Glc A and C, similar to pigments **1** and **2**. Moreover, the glycosidic linkage (2 → 1) between Glc A and Glc C was deduced by the observation of the low-field shift (at δ4.07) of H-2 (Glc A), suggesting that OH-1 of Glc C was bonded to OH-2 of Glc A. Therefore, **3** is cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)-6-*O*-(*trans*-caffeyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside], which is a new pigment [17, 18].

Other anthocyanins (**4**–**6**)

These pigments (**4**–**6**) were composed of cyanidin 3-sophoroside-5-glucoside, confirmed by alkaline hydrolysis, and their spectral and chromatographic data are shown in Table 1. By alkaline hydrolysis, **4** produces caffeic acid, **5** caffeic and *p*-coumaric acids, and **6** also caffeic and *p*-coumaric acids, as acylated substituents. From the analytical results and also FAB mass data, the structures of **4**–**6** were tentatively attributed to be monocaffeyl cyanidin 3-sophoroside-5-glucoside as **4**, caffeyl-*p*-coumaryl cyanidin 3-sophoroside-5-glucoside as **5** and caffeylglucosylcaffeyl-*p*-coumaryl cyanidin 3-sophoroside-5-glucoside as **6**.

To date, two anthocyanidin types have been reported to be present in the blue–violet flowers of *Pharbitis* plants and related ones. The first group is a cyanidin type characteristic of *I. purpurea*, *I. batatas* and *I. cairica* [19], and the second is a peonidin type which groups *I. tricolor* [16], *I. congesta* [20] and *P. nil* [1, 3]. In *I. purpurea* the main anthocyanin **1** has the characteristic side chain at the 3-hydroxyl which closely resembles those of Heavenly Blue Anthocyanin (HBA) from *I. tricolor* [16] and *Pharbitis* anthocyanins (PA) [2, 3]. From the chemotaxonomic point of view, the structures of these unusual branched side chains may be related to those of the flower pigments in the genus *Ipomoea* and its related plants.

The relative colour stabilities of **1**–**3** were compared with their deacylated pigment in neutral solution by the process described previously [2, 3]. The pigment **1** is fairly stable in neutral solution, and **2** and **3** are also stable, but the deacylanthocyanin is very unstable. The stabilities of the pigments (half-lies in buffer solution; absorbance 570 nm, pH 6.86, *ca* 5.6×10^5 ml at *ca* 20°) decreased in the order: **1** (> 8 days), **2** (1.5 days), **3** (2.0 days) and deacylanthocyanin (0.4 hr). These results show that the acyl groups are effective in maintaining colour stability, such as the acylated anthocyanins of *Pharbitis* flowers [2, 3, 21, 22]. Therefore, these acylated cyanidin glycosides may have intramolecular stacking structures when these pigments are present in neutral solution and *in vivo* [21–25].

EXPERIMENTAL

Plant material. Seeds were collected from wild types of *I. purpurea* at Sendai and Matsudo in Japan, at Katmandu in Nepal, at Salta in Argentina and at Rabaul in Papua New Guinea. These plants were grown on the farm of Chiba University, and also in the private garden of one of us (K.K). These plants have similar violet–blue flowers (Violet–Blue 89B ~ 90C by R.H.S. colour chart). Fresh corollas of these strains were collected in August–October 1993 and 1994.

Isolation of anthocyanins. Fresh corolla (1,500 g) of violet–blue strains were extracted with MAW (10 l, 10:1:9). The extract was concd to 500 ml. The concd extract was purified by Diaion HP-20, CC, PC and HPLC as described previously [1–3, 15]. Solvents used were 15% HOAc, BAW (4:1:5), 5% HOAc–MeOH and MAW for CC and PC. HPLC was performed on a LC-6A system (Shimadzu). Prep. HPLC was run on a Waters C_{18} (19ϕ × 150 mm) column at 40° with a flow rate of 4 ml min^{-1} and monitored at 530 nm for anthocyanins. Solvent systems used were: linear gradient elution for 30 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 to dryness *in vacuo*. The residues were dissolved in a small vol. of 5% HOAc–EtOH followed by addition of excess of Et_2O, and then dried to give pigment powder (pigment **1**, *ca* 50 mg; pigment **2**, *ca* 30 mg; pigment **3**, *ca* 10 mg; pigment **4**, *ca* 3 mg, pigment **5**, 3 mg; pigment **6**, 2 mg).

Analysis of anthocyanins. Fresh corolla limbs (*ca* 0.02 g) of each strain were extracted with 20% MeOH containing 1.5% H_3PO_4 or MAW. TLC and HPLC of these extracts were carried out. The anthocyanin quantitative analysis of these extracts was performed by HPLC on a Waters C_{18} (4.6ϕ × 250 mm) column at 40° with a flow rate of 1 ml min^{-1} and monitoring at 530 nm for anthocyanins. Solvent systems used were: linear gradient elution for 30 min from 40 to 85% solvent B in solvent A. Characterization of pigments **1**–**6** and deacylanthocyanin were carried out by PC, TLC and UV–VIS spectrometry. Solvents used were BAW, BuHCl (*n*-BuOH–2 N HCl, 1:1), 1% HCl and AHW (HOAc–HCl–H_2O, 15:3:82) for anthocyanins, and *n*-BuOH–HOAc–H_2O (4:1:2), EtOAc–HOAc–H_2O (3:1:1) and EtOAc–HCO_2H–H_2O (5:2:1) for organic acids and sugars. Acid hydrolysis, alkaline deacylation, H_2O_2 oxidation and partial acid hydrolysis of anthocyanins were performed according to standard procedures [15, 26].

FAB mass and NMR measurements. FAB mass spectra were recorded on a JEOL JMS SX-102A (positive mode in 'magic bullet' and negative mode in glycerol). NMR spectra were recorded at 400 MHz for ^1H and ^{13}C spectra by JEOL JNM GX-400 in DMSO-d_6–CF_3COD (9:1). Chemical shifts are reported relative to TMS as int. standard (δ) and coupling constants are reported in Hz.

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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. Yoneda, Y. (1976) *Rep. Dep. Liberal Arts, Shizuoka Univ.* **12**, 27.
8. Imai, Y. (1972) *Jour. College Agric., Imp. Univ. Tokyo* **9**, 199.
9. Imai, Y. and Tabuchi, K. (1938) *Genetics* **35**, 433.
10. Kasahara, K. (1956) *Kyoyobu Hokoku, Hosei Univ.* **1**, 1 (in Japanese).
11. Ennos, R. A. and Clegg, M. T. (1987) *J. Hered.* **74**, 247.
12. Schoen, D. J., Giannasi, D. E., Ennos, R. A. and Clegg, M. T. (1984) *J. Hered.* **75**, 113.
13. Epperson, B. K. and Clegg, M. T. (1987) *J. Hered.* **78**, 346.
14. Epperson, B. K. and Clegg, M. T. (1988) *J. Hered.* **79**, 64.
15. Saito, N. and Harborne, J. B. (1992) *Phytochemistry* **31**, 3009.
16. Kondo, T., Kawai, T., Tamura, H. and Goto, T. (1987) *Tetrahedron Letters* **28**, 2237.
17. 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.
18. Strack, D. and Wray, V. (1994) in *The Flavonoids, Advances in Research Since 1986* (Harborne, J. B., ed.), p. 1. Chapman & Hall, London.
19. Pomilio, A. B. and Sproviero, J. F. (1972) *Phytochemistry* **11**, 1125.
20. Pomilio, A. B. and Sproviero, J. F. (1972) *Phytochemistry* **11**, 2323.
21. Dangles, O., Saito, N. and Brouillard, R. (1993) *J. Am. Chem. Soc.* **115**, 3125.
22. Dangles, O., Saito, N. and Brouillard, R. (1993) *Phytochemistry* **34**, 119.
23. Saito, N., Osawa, Y. and Hayashi, K. (1972) *Bot. Mag. Tokyo* **85**, 105.
24. Goto, T. and Kondo, T. (1991) *Angew. Chem., Int. Edn Engl.* **30**, 17.
25. Brouillard, R. and Dangles, O. (1994) in *The Flavonoids, Advances in Research Since 1986* (Harborne, J. B., ed.), p. 565. Chapman & Hall, London.
26. Harborne, J. B. (1984) *Phytochemical Methods*, 2nd Edn. Chapman & Hall, London.