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ACYLATED CYANIDIN 3-SOPHOROSIDES IN THE BROWNISH-RED FLOWERS OF *IPOMOEA PURPUREA*

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Key Word Index—*Ipomoea purpurea*; convolvulaceae; brownish-red flower colour; mono- and di-caffeoylcyanidin 3-sophorosides.

Abstract—Six anthocyanins were isolated from the brownish-red flowers of *Ipomoea purpurea*. Cyanidin 3-sophoroside was identified as the parent anthocyanin. The other five anthocyanins are acylated derivatives with glucosylcaffeic acid and/or caffeic acid. The structures of three were unambiguously determined to be cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl)-caffeoyl)-β-D-glucospyranosyl)-β-D-glucopyranoside], cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-β-D-glucopyranoside] and cyanidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]. The remaining two pigments could not be determined because of their small yields. In these flowers, the major pigments are cyanidin 3-glucosylcaffeoylglucosylcaffeoyl-sophoroside, cyanidin 3-sophoroside and cyanidin 3-caffeoylsophoroside. They are considered to play an important role in producing the dusky colour of these petals.

1. INTRODUCTION

The pigmentation of the dusky flower colours in Japanese morning glory (*Pharbitis nil*) is regulated by a recessive gene $+^{dusky}$ [1]. In continuing work on flower colour variation, we reported that 27 acylated anthocyanins occur in the various colour cultivars of Japanese and common morning glories (*Pharbitis nil* and *Ipomoea purpurea*) and that acylated pigments are derived from the 3-sophoroside-5-glucosides of pelargonidin, cyanidin and peonidin [2–10]. Furthermore, the phenolic acids, as acyl moieties of these acylated anthocyanins, play important roles in the control of stability and the bluing effect of flower colour in these plants. In further studies on the maroon and slate cultivars (the dusky colour flowers) of Japanese morning glory, we found the 3-glucosylcaffeoylglucosides and 3-glucosylcaffeoylglucoside-5-glucosides of anthocyanidins as dominant pigments in their flowers [11, 12]. We also found that the gene, $+^{dusky}$, of Japanese morning glory regulated the glycosylation to 2-OH of the 3-glucose residue in order to substitute the 3-hydroxyl of their anthocyanidins with sophorose and this gene might be considered to influence to a considerable extent the 5-hydroxyl glycosidation because some dusky cultivars contained only anthocyanin 3-glycosides and lacked anthocyanidin 3, 5-diglycosides in their flowers.

As part of this ongoing program, we now report that three caffeoylcyanidin 3-sophorosides and cyanidin 3-sophoroside have been isolated from the brownish-red flowers of *Ipomoea purpurea*.

2. RESULTS AND DISCUSSION

In an anthocyanin survey of *I. purpurea* brownish-red strains by HPLC, thirteen anthocyanin peaks were observed in the flower extracts and six of them (**1**–**6**) were isolated as amorphous powders from their extracts. Among them, the structures of four anthocyanins (**1**, **2**, **4** and **6**) were fully characterised, however, only the chemical composition of other two pigments (**3** and **5**) except the ester-linkages with phenolic acids could be determined at the present time because of their small amounts. The relative frequency of the pigment occurrence was **1** (~35%), **2** (~17%), **3** (~3%), **4** (~46%), **5** (~3%) and **6** (~6%) in the seven brownish-red strains by analysis of HPLC.

The isolation and the structure determination of these anthocyanins were performed by previous pro-

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Table 1. Chromatographic and spectral properties of anthocyanins from the brownish-red flowers of *Ipomoea purpurea*

Anthocyanin*	Rf values (× 100)				Spectral data in 0.1% HCl–MeOH				Rt** (min)	FAB–MS[M]$^+$
	BAW	BuHCl	1%HCl	AHW	λ_{max} (nm)	E_{acyl}/E_{max} (%)	E_{440}/E_{max} (%)	$AlCl_3$		
1	41	8	36	52	515,278	—	31	+	6.1	611
2	57	12	17	38	521,326,282	84	31	+	9.7	773
3	39	10	33	57	521,318,282	68	23	+	14.6	935
4	20	4	13	31	523,314,284	113	29	+	15.2	1259
5	39	8	6	19	527,316,286	166	28	+	19.6	1421
6	61	15	21	44	523,328,282	62	32	+	19.2	773

For key to abbreviations, see Experimental.
* 1: cyanidin 3-sophoroside.
2: cyanidin 3-[2-(caffeoylglucosyl)-glucoside].
3: cyanidin 3-dicaffeoylsophoroside.
4: cyanidin 3-[2-(glucosylcaffeoylglucosylcaffeoylglucosyl)-glucoside].
5: cyanidin 3-[(tri-(glucosylcaffeoyl))-sophoroside].
6: cyanidin 3-[2-(glucosyl)-6-(caffeoyl)-glucoside].
** HPLC retention time (min).

cedures [11, 12]. Their Rf values, Rt(min) and spectral data are shown in Table 1. Acid hydrolysis of **1** gave cyanidin and glucose, but the other pigments **2–6** produced caffeic acid as well as cyanidin and glucose by acid hydrolysis. Furthermore, alkaline hydrolysis of **2–6** yielded only one pigment as their deacyl anthocyanin, which is identical with the pigment **1**.

The FAB mass spectrum of **1** gave its [M]$^+$ at 611 *m/z*, corresponding to the mass calculated for $C_{27}H_{31}O_{16}$, indicating the presence of one molecule of cyanidin and two of glucose. The structure of **1** was elucidated by analysis of its ^{1}H NMR spectrum (Table 2). Six aromatic protons were assigned to be protons of cyanidin. The proton signals of the sugar moieties appeared in the region of δ5.66–2.98. Two anomeric protons were assigned at δ5.66 (*d*, J=7.6 Hz, Glc A, Fig. 1) and δ4.65 (*d*, J=7.9 Hz, Glc B), and all observed vicinal coupling constants of these glucose were J=7.6–10.4 Hz, suggesting that these glucose moieties are in the β-D-glucopyranose form. By analysis of the ^{1}H–^{1}H COSY spectrum of **1**, a proton signal at δ3.97 (*t*, J=8.2 Hz, H-2 of Glc A) being shifted to a lower magnetic field was directly correlated to an H-1 proton of Glc B. Thus, this proton was assigned to H-2 of Glc A and Glc B is deduced to be attached to the 2-hydroxyl of Glc A through a glycosidic bond. Glc A was confirmed to be bonded with the 3-hydroxyl of Glc A through a glycosidic bond. Glc A was confirmed to be bonded with the 3-hydroxyl of cyanidin through a glycosidic bond because of the observation of the negative NOE (DIFNOE) [2] between H-1 of Glc A and H-4 of cyanidin. Therefore, **1** is cyanidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside].

2.1. *Pigment* **4**

The FAB mass spectrum of **4** indicated a [M]$^+$ at 1259 *m/z* corresponding to $C_{57}H_{63}O_{32}$ and two fragment ion peaks 1097 [M−162]$^+$ and 935 [M−2×162]$^+$. The ^{1}H NMR spectrum of **4** showed the presence of one molecule of cyanidin, two of caffeic acid having *trans*-olefins with the coupling constants (J=15.9 Hz) and four of glucose, all of which are in the β-D-glucopyranose form with their vicinal coupling constants (J=7.3–10.7 Hz) (Table 2, Fig. 1). By the application of DIFNOE method [2] the linkages and/or the positions of attachments of the glucose and caffeic acid units in **4** were determined to show that cyanidin is bonded with Glc A at OH-3 also Glc A is attached with Glc B at OH-2 of Glc A to form sophoroside. By irradiation at H-1 of Glc B, NOEs were observed at H-α, -β, -2, -5 and -6 of caffeic acid I as well as the strong NOE at H-2 of Glc A. Therefore, Glc B was deduced to be acylated with caffeic acid I at OH-6 of Glc B. Irradiation of H-1 of Glc C gave a DIFNOE spectrum in which NOEs were observed at H-5 of caffeic acid I with a strong doublet signal (δ7.06, *d*, J=8.9 Hz). Thus, Glc C was attached to OH-4 of caffeic acid I. Similar irradiation of H-1 of Glc D gave a strong NOE to H-2 of caffeic acid II, indicating that Glc D is bonded to the 3-hydroxyl of caffeic acid II. Since the characteristic four methylene protons of Glc B (δ3.90, 3.95) and Glc C (δ4.28, 4.44) were shifted to a low magnetic field, both OH-6 of Glc B and Glc C are acylated with caffeic acid I and II, respectively. Thus, pigment **4** is cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl)-caffeoyl)-

Table 2. ^{1}H NMR spectral data of anthocyanins isolated from the brownish-red flowers of *Ipomoea purpurea* (500 MHz, CF3CO2D-DMSO-*d*6, 1:9 at 25°C, standard TMS)

H	1	2	6	4	
Cyanidin					
4	8.86 *s*	8.91 *s*	8.82 *s*	8.90 *s*	
6	6.72 *d* (2.1)	6.67 *d* (2.0)	6.69 *brs*	6.66 *d* (1.5)	
8	6.93 *d* (2.1)	6.91 *d* (2.0)	6.97 *brs*	6.82 *d* (1.5)	
2′	8.03 *d* (2.4)	8.00 *d* (2.0)	7.98 *brs*	8.01 *d* (2.1)	
5′	7.07 *d* (8.6)	7.07 *d*(8.5)	7.05 *d* (8.5)	7.06 *d* (8.9)	
6′	8.21 *dd* (2.4,8.6)	8.21 *dd* (2.0,8.5)	8.20 *brd* (8.5)	8.18 *dd* (2.1,8.9)	
Caffeic acid					
(I)					
2		6.83 *brs*	6.88 *brs*	7.01 *brs*	
5		6.74 *d* (8.2)	6.76 *d* (7.9)	7.06 *d* (8.9)	
6		6.80 *brd* (8.2)	6.87 *brd* (7.9)	6.86 *brd* (8.9)	
α		5.94 *d* (15.9)	6.21 *d* (15.9)	6.03 *d* (15.9)	
β		7.16 *d* (15.9)	7.38 *d* (15.9)	7.17 *d* (15.9)	
(II)					
2				7.48 *brs*	
5				6.84 *d* (8.6)	
6				7.02 *d* (8.6)	
α				6.44 *d* (15.9)	
β				7.52 *d* (15.9)	
Glucose*†					
(A)				(A)	(C)
1	5.66	5.56	5.69	5.56	4.90
2	3.97	3.90	4.01	3.90	3.37
3	3.64	3.68	3.69	3.64	3.35–3.28
4	3.33	3.33	3.38	3.32	
5	3.59	3.52	3.96	3.50	3.73
6a	3.52	3.49	4.19	3.49	4.28
6b	3.73	3.70	4.46	3.69	4.44
(B)				(B)	(D)
1	4.65	4.77	4.66	4.77	4.82
2	2.98	3.08	2.99	3.08	3.32
3	3.05	3.21	3.06	3.21	3.18
4	2.72	3.34	2.75	3.32	
5	3.10	3.64	3.11	3.65	3.48–3.32
6a	3.19	3.91	3.18	3.90	
6b	3.33	3.95	3.38	3.95	3.56

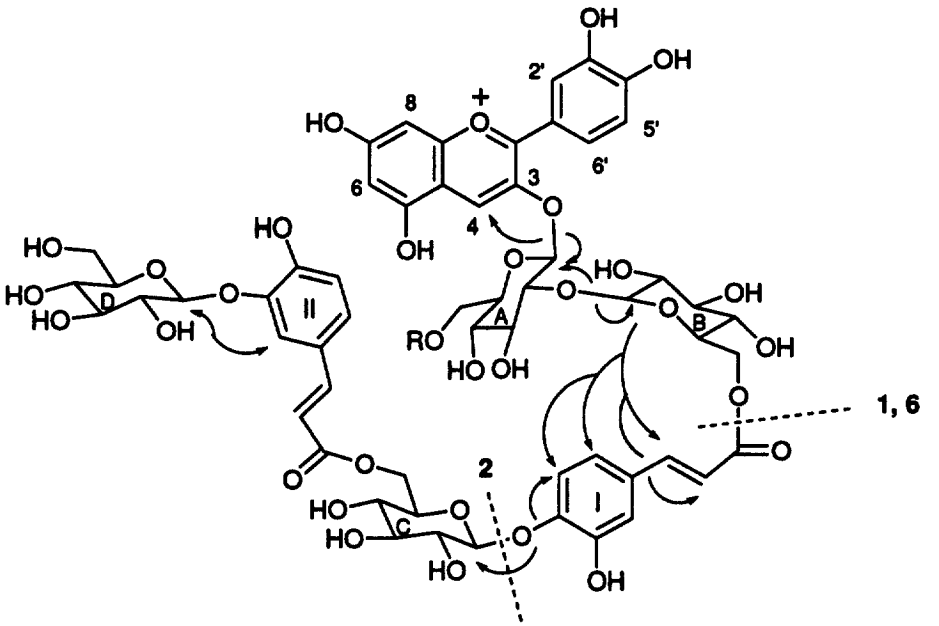
* Assigned by ^{1}H–^{1}H COSY.
† Assigned by DIFNOE.
Coupling constants (*J* in Hz) in parentheses.

β-D-glucopyranosyl)-β-D-glucopyranoside], which is a new pigment in plants [13, 14]. This structure was also confirmed by the analysis of ^{13}C NMR, ^{1}H–^{13}C COSY and HMBC spectra (see Section 3, Experimental).

2.2. *Pigments* **2** *and* **6**

The FAB mass spectra of **2** and **6** gave the same molecular ion $[M]^+$ at 773 *m/z* in good agreement with the mass calculated for $C_{36}H_{37}O_{19}$. ^{1}H NMR spectral analysis of **2** and **6** revealed the presence of one molecule of cyanidin, two of glucose and one of caffeic acid, respectively. However, their TLC and HPLC data are not in accord with each other, indicating that **2** and **6** are isomers and have different linkages between sugar and acid units (Table 1). The structures of **2** and **6** were determined by analyses of ^{1}H–^{1}H COSY and DIFNOE spectra. The proton chemical shifts of **2** were essentially the same as those of **4** except the proton chemical shifts of Glc C, D and caffeic acid II moieties (Table 2). Two characteristic methylene protons of Glc B in **2** were clearly shifted to the lower magnetic field at δ3.91 and 3.95 showing that caffeic acid is attached to OH-6 of Glc B. Therefore, **2** is cyanidin 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-β-D-glucopyranoside].

The proton chemical shifts of **6** are very similar to



4

Fig. 1. *Ipomoea* brownish-red anthocyanins. **1** R = H, **2** R = H, **4** R = H, **6** R = caffeic acid. Observed NOEs are indicated by arrows.

those of **2** (Table 2). However, the chemical shifts of four methylene protons (δ4.19, 4.46 Glc A and δ3.18 and 3.38 Glc B) of **6** were clearly different from those (δ3.49, 3.70 Glc A and δ3.91 and 3.95 Glc B) of **2**. These results show that the OH-6 of Glc A of **6** is acylated with caffeic acid and that of Glc B is free from an acid. Therefore, **6** is cyanidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-(*trans*-caffeoyl)-β-D-glucopyranoside]. The occurrence of cyanidin 3-(caffeoylsophoroside) has already been reported by Aubert and Foury [15]. However, this is the first time that the structures of **2** and **6** have been fully determined by FAB mass and NMR spectra [13, 14].

2.3. *Pigments* **3** *and* **5**

The FAB mass spectra of **3** and **5** gave their molecular ions $[M]^+$ at 935 *m/z* and 1421 *m/z* (Table 1), respectively. These values indicate that **3** is composed of cyanidin with four molecules of glucose and caffeic acid (probably glucose: caffeic acid = 2:2) and **5** is composed of cyanidin with seven molecules of glucose and caffeic acid (probably glucose: caffeic acid = 4:3). By alkaline hydrolysis, both pigments gave the same cyanidin 3-sophoroside as their deacylanthocyanin and caffeic acid was obtained by acid hydrolysis. The further structural study of **3** and **5**, however, could not be carried out due to the small amounts available for analysis. The structures of **3** and **5** were tentatively attributed to be dicaffeoylcyanidin 3-sophoroside for **3** and triglucosylcaffeoyl-cyanidin 3-sophoroside for **5**.

Acylated anthocyanidin 3-sophoroside-5-glucosides are characteristically present in the red or blue–violet flowers of *Pharbitis nil* [3, 4], *Ipomoea purpurea* [9, 10], *I. tricolor* [16], *I. congesta* [17] and *I. cairica* [18]. However, the dusky mutants of *P. nil* contain pelargonidin 3-glucosylcaffeoylglucoside as the major pigment in the maroon flowers [11] with peonidin 3-glucosylcaffeoylglucoside and peonidin 3-glucosylcaffeoylglucoside-5-glucoside as the major pigments in the slate flowers [12].

Therefore, the 2-*O*-glucosyltransferase forming the 3-sophoroside of anthocyanins is completely blocked in these dusky mutants and the enzymatic activity of 5-*O*-glucosyltransferase in these flowers is rather low or weak. In this study, the brownish-red flowers of *I. purpurea* were shown to have only a 3-sophoroside type of cyanidin, i.e., cyanidin 3-(glucosylcaffeoylglucosylcaffeoyl)-sophoroside, cyanidin 3-sophoroside and cyanidin 3-caffeoylsophoroside. Therefore, the 2-*O*-glucosyltransferase of cyanidin 3-glucoside is fully activated in these flowers, but the 5-*O*-glucosyltransferase of cyanidin is completely inhibited.

3. EXPERIMENTAL

3.1. *Plant material*

The brownish-red strains of *I. purpurea* were grown on the farm of Chiba University and also in the private garden of one of us (K.K.). These flowers exhibited brownish-red (187C and D by R.H.S. colour chart, chromaticity value $(b/a) = 0.50 \sim -0.07$). Fresh corollas were collected in August–October 1996.

3.2. *Isolation of anthocyanins*

Fresh corollas (ca. 0.5 kg) were extracted with MAW (10: 1:9, 5 l). The extract was concentrated to 1 l. The concentrated extract was purified by Diaion HP-20 CC, PC and HPLC as described previously [10, 11, 12]. Solvents used were 15% HOAc, BAW (n-BuOH-HOAc-H_2O, 4:1:5), 5% HOAc-MeOH and MAW for CC and PC. Prep. HPLC was run on a Waters C_{18} ($19\phi \times 150$ nm) column at 40°C with a flow rate of 4 ml min^{-1} and monitored at 530 nm for anthocyanins. Solvent systems used were as follows: a linear gradient elution for 30 min for 25 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 evaporation residues were dissolved in a small volume of 5% HOAc-EtOH, followed by addition of excess Et_2O, and then dried to give pigment powders (pigment **1**, ca 8 mg; pigment **2**, ca 7 mg; pigment **3**, ca 5 mg; pigment **4**, ca 20 mg; pigment **5**, ca 5 mg and pigment **6**, ca 10 mg).

3.3. *Analysis of anthocyanins*

Fresh corolla limbs (ca 0.02 g) were extracted with MAW. Quantitative analysis was performed by HPLC on a Waters C_{18} ($4.6\phi \times 250$ mm) column at 40°C with a flow rate of 1 ml min^{-1}, monitoring at 530 nm for anthocyanins. Solvent systems employed were: a linear gradient elution for 30 min from 40 to 85% solvent B in solvent A. Characterization of pigments was carried out by PC, TLC and UV–VIS spectrometry. Solvents used were BAW, BuH (n-BuOH-2N HCl, 1:1), 1% HCl and AHW (HOAc-HCl-H_2O, 15:3:82) for anthocyanins, and BAW (n-BuOH-HOAc-H_2O, 4:1:2), EtOAc-HOAc-H_2O (3:1:1) and EtOAc-HCOOH-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 the standard procedures [19].

3.4. *FAB mass and NMR measurements*

FAB MS were recorded in positive mode using the magic bullet and in negative mode in glycerol. NMR were recorded at 500 MHz for ^1H and at 125 MHz for ^{13}C in DMSO-*d*6-CF_3COOD (9:1). Chemical shifts are reported relative to TMS int. standard, (δ) and coupling constants are reported in Hz.

3.4. ^{13}C NMR of pigment **4**

Cyanidin 161.7 (C-2), 143.6 (C-3), 135.5 (C-4), 155.8 (C-5), 102.3 (C-6), 168.5 (C-7), 94.1 (C-8), 157.7 (C-9), 111.7 (C-10), 119.5 (C-1′), 117.4 (C-2′), 146.9 (C-3′), 154.4 (C4′), 115.9 (C-5′), 126.9 (C-6′); caffeic acid (I) 128.6 (C-1), 115.2 (C-2), 146.1 (C-3), 147.3 (C-4), 116.8 (C-5), 120.7 (C-6), 115.6 (C-α), 144.3 (C-β), 166.0 (C=O); caffeic acid (II) 125.8 (C-1), 116.2 (C-2), 145.6 (C-3), 149.5 (C-4), 116.3 (C-5), 124.3 (C-6), 114.8 (C-α), 145.2 (C-β), 166.6 (C=O); sugars 104.5 (B-1), 101.4 (C-1), 100.1 (A-1), 82.1 (A-2), 77.5, 77.3 (A-5), 76.0 (B-5), 75.6, 74.7, 74.0 (C-5), 73.4, 73.2, 70.1, 69.7, 69.4, 69.2, 65.1, 63.2, (C-6), 62.8 (B-6), 60.9 (A-6), 60.6 (D-6).

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