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THREE ACYLATED CYANIDIN GLUCOSIDES IN PINK FLOWERS OF *GENTIANA*

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Key Word Index—*Gentiana*; Gentianaceae; pink flowers; acylated anthocyanin; cyanidin; *trans-p*-coumaric acid; caffeic acid.

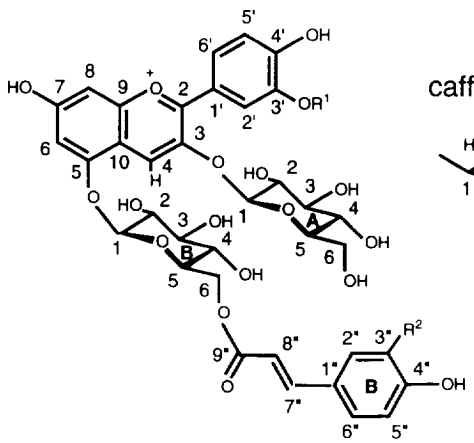
Abstract—Three novel anthocyanins, cyanidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-caffeoyl-β-D-glucoside), cyanidin 3-*O*-β-D-glucoside-5,3′-bis-*O*-(6-*O*-caffeoyl-β-D-glucoside) and cyanidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside), gentiocyanin A, B and C, respectively, were isolated from pink flowers of gentian, along with a known anthocyanin, cyanidin 3-*O*-β-D-glucoside. The complete structure of each anthocyanin was unambiguously determined by 1D- and 2D-NMR and other spectral methods.

INTRODUCTION

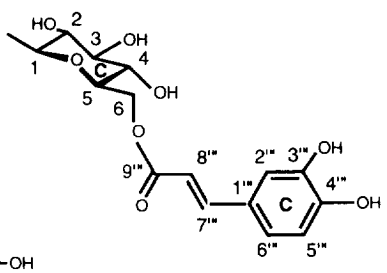
Gentiana sp. is a popular ornamental plant with blue, pink or white flowers in Japan. The anthocyanin from the blue flowers of *G. makinoi* has been shown to be gentiodelphin [1]. In this study, the complete structures of three novel anthocyanins, present in the pink flowers of gentian were determined.

RESULTS AND DISCUSSION

Anthocyanins (**1**–**4**) of the pink flowers of gentian were isolated by column chromatography on Amberlite XAD-7, followed by preparative HPLC. UV-Vis and FAB-mass spectra of three anthocyanins (**2**–**4**) are shown in Table 1. Anthocyanin **3** has the highest M_r of those isolated in the experiment. In the UV-Vis spectrum,



caffeoylglucose :



	R^1	R^2
2	H	OH
3	caffeoylglucose	OH
4	H	H

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Table 1. Spectral properties of anthocyanins (**2**–**4**) from pink flowers of gentian

Anthocyanin	UV-V is (0.1% HCl–MeOH)					FAB-mass spectra
	$\lambda_{vis,max}$ (nm)	$\lambda_{acyl,max}$ (nm)	$E_{acyl}/E_{vis,max}$	$E_{440}/E_{vis,max}$	$AlCl_3$ shift	$[M]^+$ and fragment ions
2	526	331	0.52	0.12	+	773, 611, 449, 287
3	526	330	1.02	0.17	0	1097, 935, 773, 611, 449, 287
4	527	296	0.74	0.11	+	757, 595, 449, 287

Table 2. ^1H NMR spectral data of gentian anthacyanins (**2**–**4**, in MeOH-d_4 containing 10% TFA-*d*)

	2	**3**	**4**
Aglycone			
4	9.07 *s*	8.78 *s*	9.08 *s*
6	7.01 *d* (2.0)	6.96 *d* (1.9)	7.02 *d* (2.1)
8	7.00 *d* (2.0)	6.85 *d* (1.8)	7.00 *d* (2.1)
2′	8.02 *d* (2.3)	8.02 *d* (1.9)	8.02 *d* (2.4)
5′	7.01 *d* (8.7)	7.19 *d* (8.9)	7.01 *d* (8.8)
6′	8.26 *dd* (8.7, 2.3)	8.45 *dd* (8.1, 1.8)	8.27 *d* (8.8, 2.4)
3-*O*-Glucose A			
1	5.30 *d* (7.8)	5.01 *d* (7.8)	5.29 *d* (7.9)
2	3.71 *dd* (9.2, 7.8)	3.68 *dd* (9.2, 7.8)	3.70 *dd* (9.3, 7.9)
3	3.55 *dd* (9.2, 9.0)	3.57 *dd* (9.2, 9.1)	3.55 *dd* (9.3, 9.1)
4	3.41 *dd* (9.7, 9.0)	3.43 *dd* (9.7, 9.1)	3.41 *dd* (9.6, 9.1)
5	3.65 *ddd* (9.7, 6.8, 2.3)	3.62–3.68 *m*	3.64 *ddd* (9.6, 6.9, 2.1)
6	3.73 *dd* (12, 6.8)	3.79 *dd* (12, 7.3)	3.70–3.75 *m*
	3.98 *dd* (12, 2.3)	4.06 *dd* (12, 2.3)	3.98 *dd* (12, 2.1)
5-*O*-Glucose B			
1	5.21 *d* (7.6)	5.22 *d* (7.2)	5.20 *d* (7.7)
2	3.74 *dd* (8.8, 7.6)	3.79 *dd* (8.4, 7.2)	3.74 *dd* (8.7, 7.7)
3	3.61 *dd* (8.8, 8.8)	3.61–3.64 *m*	3.61 *dd* (9.0, 8.7)
4	3.53 *dd* (9.9, 8.8)	3.60 *dd* (9.7, 8.9)	3.53 *dd* (9.5, 9.0)
5	3.85 *ddd* (9.9, 7.1, 2.3)	3.87 *ddd* (9.7, 6.7, 2.2)	3.85 *ddd* (9.5, 6.9, 2.4)
6	4.34 *dd* (12, 7.1)	4.37 *dd* (12, 6.7)	4.34 *dd* (12, 6.9)
	4.58 *dd* (12, 2.3)	4.64 *dd* (12, 2.2)	4.59 *dd* (12, 24)
3′-*O*-Glucose C			
1		5.23–5.24 *m*	
2		3.63–3.67 *m*	
3		3.63–3.67 *m*	
4		3.37–5.41 *m*	
5		3.87 *ddd* (9.5, 9.5, 2.1)	
6		4.35 *dd* (12, 9.5)	
		4.70 *dd* (12, 2.1)	
Aromatic acid moiety B			
2″	6.98 *d* (2.0)	6.94 *d* (2.0)	7.39 *d* (8.7)
3″			6.78 *d* (8.7)
5″	6.74 *d* (8.3)	6.70 *d* (8.2)	6.78 *d* (8.7)
6″	6.88 *dd* (8.3, 2.0)	6.84 *dd* (8.1, 2.0)	7.39 *d* (8.7)
7″	7.46 *d* (16)	7.43 *d* (16)	7.54 *d* (16)
8″	6.23 *d* (6)	6.20 *d* (16)	6.30 *d* (16)
Aromatic acid moiety C			
2‴		6.30 *d* (2.2)	
5‴		6.53 *d* (8.7)	
6‴		6.30 *dd* (7.9, 2.1)	
7‴		7.02 *d* (16)	
8‴		5.87 *d* (16)	

Coupling constants (*J* in Hz) in parentheses.

$E_{acyl}/E_{vis,max}$ was 1.02, indicating the presence of two molecules of hydroxycinnamic acid [2]. The FAB-mass spectrum gave the molecular ion at m/z 1097, in good agreement with the mass calculated for $C_{51}H_{53}O_{27}$. Fragment peaks were also observed at m/z 935 [M − 162 (hexose or caffeic acid)]$^+$, 773 [M − 324 (2 × [hexose and/or caffeic acid])$^+$, 611 [M − 486 (3 × [hexose and/or caffeic acid])]$^+$, 449 [M − 486 ([2 × hexose and 2 × caffeic acid])]$^+$ and 287 [aglycone]$^+$, indicating **3** to be comprised of cyanidin, three molecules of hexose and two molecules of caffeic acid.

Analysis of the ^{1}H NMR spectrum of **3** indicated the presence of cyanidin, three glucose residues and two caffeic acid residues (Table 2). In each caffeic acid moiety, 7″- and 8″-protons had large coupling constants (J = 16 Hz). Therefore, the olefinic bond of each caffeic acid moiety had a *trans* configuration. Signals from glucoses A and B were observed in the region of δ3.43–5.22 and all vicinal coupling constants of both these glucose moieties were at 7.2–9.7 Hz. The chemical shifts of two anomeric protons with large coupling constants were δ5.01 (d, J = 7.8 Hz, glucose A) and δ5.22 (d, J = 7.2 Hz, glucose B), thus clearly showing glucose A and B to be β-D-glucopyranoside. In the ^{1}H NMR spectrum of the third hexose, glucose C, protons on C-1 to C-4 were observed as multiplets. Coupling patterns were examined by spin simulation using the PANIC program (Bruker Aspect 3000 NMR Software Manual, Bruker). The chemical shift of the anomeric carbon of glucose C was δ101.1. $J_{C,H}$ between the anomeric proton and carbon of glucose C was 162 Hz. Those of the glucose A and B were the same, 164 Hz, thus demonstrating glucose C to be β-D-glucopyranoside. Analysis of the proton network of glucose moieties indicated that the anomeric protons (δ5.01, 5.22 and 5.23) of glucoses A, B and C were ultimately correlated to the non-equivalent methylene protons of C-6 at δ3.79 and 4.06, at δ4.37 and 4.64, and at δ4.35 and 4.70, respectively. Downfield shifts of these methylene signals of glucose B and C indicated the caffeoyl moieties to be attached to OH-6 of glucoses B and C.

To confirm the position of the ester linkage, the heteronuclear multiple-bond correlation (HMBC) spectrum was determined. Correlations between H-6 (δ4.37 and 4.64) of glucose B and carbonyl carbon (δ169.1) of caffeic acid B and between H-6 (δ4.35 and 4.70) of glucose C and carbonyl carbon (δ168.5) of caffeic acid C were observed, indicating caffeic acids B and C to be attached to OH-6 of glucose B and glucose C via an ester bond, respectively. Positions of the glucosidic linkage were determined by HMBC and nuclear Overhauser effect (NOE) difference spectra. In the HMBC spectra, correlations between the anomeric proton (δ5.01) of glucose A and C-3 (δ146.6), that (δ5.22) of glucose B and C-5 (δ156.7), and that (δ5.23) of glucose C and C-3′ (δ146.9) were observed, indicating glucoses A, B and C to be attached to OH-3, OH-5 and OH-3′ of cyanidin, respectively. This was also confirmed by NOE experiments, in which negative NOEs were observed for anomeric protons of glucoses A, B and C upon irradiation of H-4 (δ8.78), H-6 (δ6.96) and H-2′ (δ8.02) of cyanidin, respectively. Thus the structure

Table 3. ^{13}C NMR spectral data of gentian anthacyanins (**2**–**4**, in MeOH-d_4 containing 10% TFA-d)

C	2	3	4
Aglycone			
2	164.8	162.8	164.9
3	146.7	146.6	146.7
4	135.8	135.7	135.9
5	156.7	156.7	156.7
6	106.1	106.5	106.1
7	169.5	169.9	169.5
8	97.3	97.5	97.3
9	157.1	156.5	157.1
10	113.5	113.9	113.5
1′	121.1	120.9	121.1
2′	118.7	118.3	118.7
3′	147.6	146.9	147.6
4′	156.7	157.1	156.7
5′	117.6	118.5	117.6
6′	129.1	131.5	129.1
3-*O*-Glucose A			
1	103.9	104.2	104.0
2	74.7	74.6	74.7
3	78.3	78.3	78.3
4	71.4	71.5	71.4
5	78.9	79.0	79.0
6	62.7	62.9	62.7
5-*O*-Glucose B			
1	102.4	102.4	102.4
2	74.5	74.6	74.5
3	77.7	77.7	77.7
4	71.7	71.5	71.7
5	76.0	76.1	76.1
6	64.4	64.1	64.5
3′-*O*-Glucose C			
1		101.1	
2		74.6	
3		77.3	
4		72.5	
5		76.4	
6		64.8	
Aromatic acid moiety B			
1″	127.7	127.6	127.1
2″	115.4	115.3	131.3
3″	146.7	146.7	116.9
4″	149.6	149.5	161.3
5″	116.5	116.5	116.9
6″	123.1	123.2	131.3
7″	147.3	147.2	147.0
8″	114.7	114.6	114.7
9″	169.1	169.1	169.1
Aromatic acid moiety C			
1‴		127.0	
2‴		114.5	
3‴		146.3	
4‴		149.3	
5‴		116.8	
6‴		122.9	
7‴		146.0	
8‴		115.5	
9‴		168.5	

of **3** is cyanidin 3-*O*-β-D-glucoside-5,3′-bis-*O*-(6-*O*-caffeoyl-β-D-glucoside).

Anthocyanin **2**, a major component, was found to have a molecular ion at *m/z* 773 by FAB-mass spectrometry, in good agreement with the mass calculated for $C_{36}H_{37}O_{19}$. In the UV-Vis spectrum, $E_{acyl}/E_{vis,max}$ was 0.52, indicating the presence of one molecule of hydroxycinnamic acid [2]. Fragment peaks were observed at *m/z* 611 $[M - 162$ (hexose or caffeic acid)$]^+$, 449 $[M - 324$ (2 × [hexose and/or caffeic acid])$]^+$ and 287 [aglycone]$^+$. Anthocyanin **2** would thus appear to be cyanidin, two molecules of hexose and one molecule of caffeic acid. The ^{1}H NMR spectrum of **2** was similar to that of **3** and upfield shifts of H-5′ (δ7.01) and H-6′ (δ8.26) were observed, indicating **2** to correspond to the descaffeoylglucoside derivative at OH-3′ of **3**.

Anthocyanin **4** had a molecular ion at *m/z* 757 according to FAB-mass spectrometry, this being 16 mass units less than that of **2**, in good agreement with the mass calculated for $C_{36}H_{37}O_{18}$. In the aromatic acid region of the ^{1}H NMR spectrum of **4**, signals from a 1,4-disubstituted benzene ring were found in place of the 1,2,4-trisubstituted pattern for caffeic acid in **2**. *trans-p*-Coumaric acid should thus be contained in the molecule. The structures of anthocyanins **2** and **4** are thus cyanidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-caffeoyl-β-D-glucoside) and cyanidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside), respectively. In addition a known anthocyanin (**1**) was also isolated along with **2**–**4**. Complete assignments of the ^{1}H and ^{13}C signals, as determined by 1D and 2D NMR spectrometry, are shown in Tables 2 and 3.

Anthocyanins **2**–**4** are novel anthocyanins in which the 5-*O*-glucose attached to anthocyanidin is acylated with hydroxycinnamic acid. This type of anthocynanin containing 5-*O*-(6-*O*-hydroxycinnamoyl)glucoside has been observed only in flowers of *G. mokinoi* [1] and may be specifically synthesized in flowers of *Gentiana*.

EXPERIMENTAL

Plant material. Pink flowers of *Gentiana* unnamed cultivar, a breeding line grown at Iwate Horticultural Experiment Station, were collected and freeze-dried in October 1993.

Isolation of anthocyanins. Freeze-dried flowers (110 g) were extracted with a mixture of EtOH–HOAc–H_2O (10:1:9) at 4°. The concd extract was applied onto a column of Amberlite XAD-7 and washed with 5% HOAc. The anthocyanins were eluted by 50% MeOH containing 5% HOAc. For further purification, the crude anthocyanins were purified by prep. HPLC on a Chromatorex-ODS (20φ × 250 mm, Fuji Silysia Chemical Ltd, Aichi, Japan) column at a flow rate of 10 ml min^{-1}, while monitoring anthocyanins at 500 nm. The following solvent systems were used for elution: linear gradient elution for 50 min from 30 to 100% solvent A (25% MeCN, 20% HOAc and 0.5% TFA in H_2O) in solvent B (0.5% TFA) and isocratic solvent systems of 30, 43, 45 and 50% solvent A in solvent B for **1**, **2**, **3** and **4**, respectively. For **1**, a isocratic solvent system of HOAc–TFA–H_2O (4:1:196) was used. To replace counter anions of anthocyanins with trifluoroacetate, the concentrated fractions were adsorbed on a cartridge of activated Sep-Pak tC18 (Waters Associates, Milford, MA, U.S.A.) which was then washed with 1% aq. TFA and eluted with MeOH containing 1% TFA. The solutions of purified anthocyanins were concentrated to drying under a stream of N_2. Each residue was dissolved in a small amount of 1% TFA and freeze-dried to give a total of four anthocyanins as powders (**1**, 11.9 mg; **2**, 142.9 mg; **3**, 52.4 mg; **4**, 35.9 mg).

Spectral analysis. UV-visible spectra were recorded in MeOH containing 0.1% HCl. FAB-MS were obtained in the positive mode with glycerol as the matrix. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra were obtained in MeOH-d_4 containing 10% TFA-*d* as the solvent.

Cyanidin 3-O-β-D-glucoside (**1**). UV-Vis λ_{max}; 530: $E_{440}/E_{vis,max}$; 0.22, $AlCl_3$ shift (+). FAB-MS (*m/z*); 449 $[M]^+$, 287. ^{1}H NMR and ^{13}C NMR agreed with lit. values.

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