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SEVEN ACYLATED ANTHOCYANINS IN BLUE FLOWERS OF *GENTIANA*

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

Abstract—Three novel anthocyanins, delphinidin 3,3′-di-*O*-*β*-D-glucoside-5-*O*-(6-*O*-caffeoyl-*β*-D-glucoside), delphinidin 3,3′-di-*O*-*β*-D-glucoside-5-*O*-(6-*O*-*trans*-*p*-coumaroyl-*β*-D-glucoside) and delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-*trans*-*p*-coumaroyl-*β*-D-glucoside), named albireodelphin A, B and C, respectively, were isolated from the blue flowers of *Gentiana* cv. Albireo, along with two known anthocyanins, gentiodelphin and gentiocyanin A. The complete structure of each anthocyanin was unambiguously determined by one- and two-dimensional NMR and other spectral methods. Two other novel anthocyanins, delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-caffeoyl-*β*-D-glucoside)-3′-*O*-(6-*O*-*trans*-*p*-coumaroyl-*β*-D-glucoside) and delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-*trans*-*p*-coumaroyl-*β*-D-glucoside)-3′-*O*-(6-*O*-caffeoyl-*β*-D-glucoside), named albireodelphin D and E, respectively, were obtained as a mixture and the structures estimated by one- and two-dimensional NMR and other spectral methods.

INTRODUCTION

Gentiana sp. (Gentianaceae), which has blue, pink or white flowers, is a popular ornamental plant in Japan. The acylated anthocyanins from the blue and pink flowers of gentian have been shown to be gentiodelphin [1] and gentiocyanins A–C [2], respectively. In this study, we determined the complete structures of seven acylated anthocyanins, including five novel ones, present in the blue flowers of gentian.

RESULTS AND DISCUSSION

Compounds **1**–**7** of the blue flowers of gentian were isolated by column chromatography on Amberlite XAD-7, followed by preparative HPLC. The UV-visible and fast atom bombardment (FAB) mass spectral data of five novel antocyanins (**1**–**3**, **6** and **7**) are shown in Table 1.

Compound **2** has a molecular ion at *m/z* 935 by FAB-MS, which is in good agreement with the mass calculated for $C_{42}H_{47}O_{24}$. In the UV-visible spectra, $E_{acyl}/E_{vis.max}$ is 0.75, indicating the presence of one molecule of hydroxycinnamic acid [3]. Fragment peaks were also observed at *m/z* 773 [M − 162 (hexose or coumaric acid)]$^+$, 611 [M − 324 (hexose and coumaric acid)]$^+$ and 303 [aglycon]$^+$.

Analysis of the ^{1}H NMR spectrum of **2** indicated the presence of delphinidin, and three hexose and one coumaric acid residues (Table 2). In a coumaric acid moiety, 7″- and 8″-protons had large coupling constants (J = 16 Hz). The olefinic bond of each coumaric acid moiety was thus concluded to have a *trans* configuration. Signals from hexoses A and B were observed in the region δ 3.42–5.31, and the vicinal coupling constants observed in the two hexose moieties were 6.8–9.7 Hz. The chemical shifts of two anomeric protons with large coupling constants were δ 5.31 (*d*, J = 7.9 Hz, glucose A) and 5.23 (*d*, J = 7.5 Hz, glucose B), thus clearly showing hexoses A and B to be *β*-D-glucopyranoside. Hexose C was also estimated to be *β*-D-glucopyranoside from the analysis of the chemical shifts in the ^{1}H and ^{13}C NMR spectra. Analysis of the proton network of glucose moieties by two-dimensional H–H COSY, indicated anomeric protons (δ 5.31, 5.23 and 5.02) of glucoses A, B and C to be correlated with the non-equivalent methylene protons of C-6 at δ 3.72–3.73 and 3.99, at δ 4.33 and 4.59, and at δ 3.77 and 3.99, respectively. Downfield shifts of the methylene signals of glucose B suggested the *trans*-*p*-coumaroyl moiety to be attached to 6-OH of glucose B.

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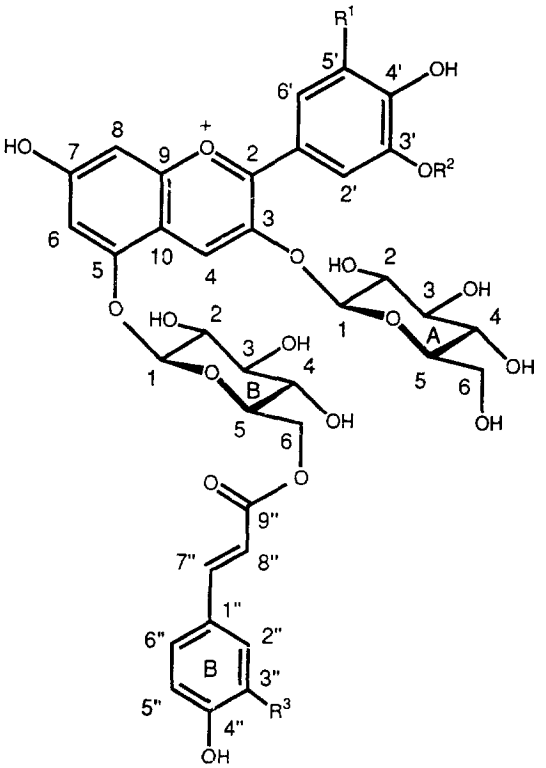
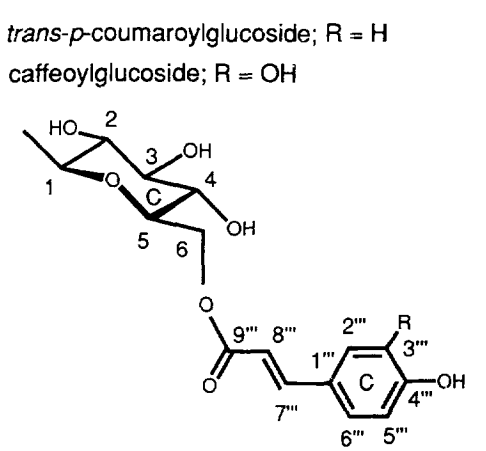
Table 1. Spectral properties of anthocyanins **1**–**3**, **6** and **7** from blue flowers of gentian

Anthocyanin	UV-visible (0.1% HCl–MeOH) $\lambda_{vis,max}$ (nm)	$\lambda_{acyl,max}$ (nm)	$E_{acyl}/E_{vis,max}$	$E_{440}/E_{vis,max}$	$AlCl_3$ shift	FAB-MS $[M]^+$ and fragment ions
1	529	332	0.59	0.14	+	951
2	530	301	0.73	0.15	+	935, 773, 611, 303
3	539	318	1.49	0.13	+	773, 611, 303
6, **7**	538	300	1.01	0.14	+	1097, 935, 757, 611, 449, 303





	R^1	R^2	R^3
1	OH	glucoside	OH
2	OH	glucoside	H
3	OH	H	H
4	OH	caffeoylglucoside	OH
5	H	H	OH
6	OH	*trans-p*-coumaroylglucoside	OH
7	OH	caffeoylglucoside	H

To confirm the position of the ester linkage, the HMBC spectrum was determined. Correlations between H-6 (δ 4.33) of glucose B and the carbonyl carbon (δ 169.0) of *trans-p*-coumaroyl residue was observed, indicating the acylating position to be 6-OH of glucose B.

Positions of the glucosidic linkage were determined by HMBC and NOE difference spectra. In the HMBC spectrum, correlations between the anomeric proton (δ 5.31) of glucose A and C-3 (δ 146.8), that (δ 5.23) of glucose B and C-5 (δ 156.7), and that (δ 5.02) of glucose C and C-3′ (δ 147.7) were observed, indicating glucoses A, B and C to be attached to 3-, 5- and 3′-OH of delphinidin, respectively. This was also confirmed by NOE experiments, in which negative NOEs were observed for the anomeric protons of glucoses A, B and C upon irradiation of H-4 (δ 9.12), H-6 (δ 7.04) and H-2′ (δ 8.05) of delphinidin, respectively. Compound **2** was thus concluded to be delphinidin 3,3′-di-*O*-β-D-glucoside-5-*O*-(6-*O*-*trans-p*-coumaroyl-β-D-glucoside), named albireodelphin B. Complete assignments of the ^1H and ^{13}C signals of **2** are shown in Tables 2 and 3, respectively.

Compound **1** was found to have a molecular ion at *m/z* 951 by FAB-MS, this being 16 mass units larger than that of **2**, which is in good agreement with the mass calculated for $C_{42}H_{47}O_{25}$. In the UV-visible spectra, $E_{acyl}/E_{vis,max}$ is 0.59, indicating the presence of one molecule of hydroxycinnamic acid [2]. Although each peak in the ^1H NMR spectrum of **1** was observed as a broad peak, the spectrum was similar to that of **2**, except for the signals of acyl moieties. In the ^1H NMR

Table 2. ^{1}H NMR spectral data of gentian anthocyanins **1**–**3**, **6** and **7** (MeOH-d_4 containing 10% TFA-*d*)

	δ (ppm); *J* (Hz)				
H	**1**	**2**	**3**	**6**	**7**
Aglycone					
4	9.10 *br s*	9.12 *s*	9.03 *s*	8.80 *s*	8.78 *s*
6	7.02 *br s*	7.04 *d*; 1.9	7.02 *d*; 1.7	6.97 *d*; 1.5	
8	7.09 *br s*	7.10 *d*; 1.9	6.97 *d*; 1.7	6.80 *d*; 1.5	
2′	8.05 *br s*	8.05 *d*; 2.3	7.77 *s*	7.83 *s*	7.79 *d*; 1.6
6′	8.01 *br s*	7.99 *d*; 2.3	7.77 *s*	7.83 *s*	7.88 *br s*
3-O-Glucose A					
1	5.29 *br d*; 7.8	5.31 *d*; 7.9	5.32 *d*; 7.8	5.03 *d*; 7.8	5.03 *d*; 7.8
2	3.72–3.77 *m**	3.72 *dd*; 9.2, 7.9	3.75 *m*	3.73 *m*	3.73 *m*
3	3.62–3.66 *m*	3.53 *dd*; 9.7; 9.3	3.65 *m*	3.57 *m*	3.57 *m*
4	3.43 *br dd*; 9.8, 9.5	3.42 *dd*; 9.7; 9.3	3.57 *m*	3.46 *m*	3.46 *m*
5	3.50–3.60 *m*	3.65 *ddd*; 9.7; 6.8, 2.1	3.75 *m*	3.66 *m*	3.66 *m*
6	3.72–3.77 *m*	3.72–3.73 *m*	3.98 *m*	3.82 *m*	3.82 *m*
	3.98 *br dd*; 12, 4.6	3.99 *dd*; 12, 2.1	4.04 *m*	4.04 *dd*; 12, 1.7	4.04 *dd*; 12, 1.7
5-O-Glucose B					
1	5.23 *brd*; 7.0	5.23 *d*; 7.5	5.21 *d*; 7.8	5.21 *d*; 7.4	5.21 *d*; 7.4
2	3.73–3.78 *m*	3.74 *dd*; 8.8, 7.5	3.75 *m*	3.77 *m*	3.77 *m*
3	3.62–3.66 *m*	3.61 *dd*; 9.0, 8.0	3.65 *m*	3.64 *m*	3.64 *m*
4	3.58–3.64 *m*	3.53 *dd*; 9.7, 9.0	3.54 *m*	3.57 *m*	3.57 *m*
5	3.85 *br dd*, 8.1, 6.7	3.86 *ddd*; 9.7, 7.2, 2.3	3.83 *m*	3.87 *m*	3.86 *m*
6	4.35 *br dd*; 12, 7.0	4.33 *dd*; 12, 7.2	4.35 *m*	4.41 *dd*; 12, 6.4	4.39 *m*
	4.58 *br d*; 12	4.59 *dd*; 12, 2.3	4.56 *m*	4.66 *dd*; 12, 2.1	4.65 *m*
3′-O-Glucose C					
1	5.03 *br d*; 6.4	5.02 *d*; 7.6		5.21 *d*; 7.1	5.21 *d*; 7.1
2	3.50–3.60 *m*	3.57 *m*		3.65 *m*	3.65 *m*
3	3.50–3.60 *m*	3.55 *m*		3.65 *m*	3.65 *m*
4	3.41 *br dd*; 9.2, 8.0	3.39–3.43 *m*		3.38 *m*	3.38 *m*
5	3.74–3.77 *m*	3.58–3.64 *m*		3.83 *m*	3.83 *m*
6	3.73–3.78 *m*	3.77 *dd*; 12, 6.4		4.26 *dd*; 12, 9.3	4.34 *dd*; 12, 9.3
	3.98 *br dd*; 12, 4.6	3.99 *dd*; 12, 2.1		4.75 *dd*; 12, 2.2	4.69 *dd*; 12, 2.2
Aromatic acid moiety B					
2″	6.95 *br s*	7.39 *d*; 8.7	7.40 *d*; 7.9	6.97 *d*; 2.0	7.38 *d*; 8.5
3″		6.79 *d*; 8.7	6.79 *d*; 7.9		6.78 *d*; 8.5
5″	6.73 *br d*; 8.0	6.79 *d*; 8.7	6.79 *d*; 7.9	6.73 *d*; 8.3	6.78 *d*; 8.5
6″	6.85 *br d*; 8.0	7.39 *d*; 8.7	7.40 *d*; 7.9	6.86 *dd*; 8.3, 2.0	7.38 *d*; 8.5
7″	7.42 *br d*; 16	7.53 *d*; 16	7.55 *d*; 16	7.46 *d*; 16	7.56 *d*; 16
8″	6.17 *br d*; 16	6.29 *d*; 16	6.31 *d*; 16	6.24 *d*; 16	6.32 *d*; 16
Aromatic acid moiety C					
2‴				6.84 *d*; 8.5	6.38 *br s*
3‴				6.53 *d*; 8.5	
5‴				6.53 *d*; 8.5	6.51 *d*; 8.0
6‴				6.84 *d*; 8.5	6.35 *dd*; 8.0, 1.5
7‴				7.17 *d*; 16	7.07 *d*; 16
8‴				5.96 *d*; 16	5.92 *d*; 16

*Multiplet.

spectrum of the aromatic acyl moiety, the signals of the 1,4-disubstituted benzene ring found in **2** were replaced with those of a 1,2,4-trisubstituted benzene ring.

Compound **3** has a molecular ion at *m/z* 773 by FAB-MS, in good agreement with the mass calculated for $C_{36}H_{37}O_{19}$. The ^{1}H NMR spectrum of **3** was also similar to those of **2**, except for the lack of the signals

Table 3. ^{13}C NMR spectral data of gentian anthocyanins **2**, **6** and **7** in MeOH-d_4 containing 10% TFA-*d*

	δ (ppm)		
	2	**6**	**7**
Aglycone			
2	164.3	163.0	162.8
3	146.8	146.7	146.7
4	136.4	135.8	135.6
5	156.7	156.6	156.6
6	106.3	106.6	106.6
7	169.9	169.9	169.8
8	97.5	97.5	97.4
9	157.4	156.6	156.6
10	114.3	113.9	113.9
1′	120.1	119.8	119.9
2′	114.3	112.8	111.9
3′	147.7	147.0	147.0
4′	146.3	146.1	146.0
5′	147.9	148.0	147.9
6′	115.9	115.4	115.4
3-O-Glucose A			
1	104.0	104.2	104.2
2	74.9	74.6	74.6
3	78.3	78.4	78.3
4	71.4	71.4	71.4
5	79.0	77.4	77.4
6	64.4	62.8	62.8
5-O-Glucose B			
1	102.4	102.5	102.5
2	74.5	74.6	74.6
3	77.7	79.0	79.1
4	71.7	71.6	71.5
5	76.1	76.2	76.3
6	64.4	64.2	64.3
3′-O-Glucose C			
1	104.1	101.7	101.4
2	74.7	74.7	74.7
3	77.5	77.7	77.7
4	71.5	72.6	72.6
5	78.8	76.1	76.2
6	62.8	64.8	64.7
Aromatic acid moiety B			
1″	127.1	127.7	127.1
2″	131.3	115.4	131.3
3″	116.9	146.7	116.9
4″	161.3	149.6	161.3
5″	116.9	116.6	116.9
6″	131.3	123.2	131.3
7″	146.9	147.4	147.0
8″	114.7	114.7	115.5
9″	169.0	169.2	169.2
Aromatic acid moiety C			
1‴		126.4	127.1
2‴		130.7	115.2
3‴		117.0	147.0
4‴		160.9	149.2
5‴		117.0	116.7
6‴		130.7	122.8
7‴		145.9	146.3
8‴		115.5	114.8
9‴		168.6	168.6

of glucose C. Fragment peaks could also be seen at *m/z* 611 [M − 162 (hexose or coumaric acid)]$^+$ and 303 [aglycon]$^+$. These data suggested that **3** corresponded to a deglucosyl derivative of **2**. The positions of the glucosidic linkages were determined by the NOEs.

The structures of **1** and **3** were thus concluded to be delphinidin 3,3′-di-*O*-β-D-glucoside-5-*O*-(6-*O*-caffeoyl-β-D-glucoside) and delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside), named albireodelphin A and C, respectively. Complete assignments of the ^1H signals of **1** and **3** are shown in Table 2.

Compounds **6** and **7** were obtained as a mixture, and every effort to separate them failed. Analysis of the ^1H NMR spectrum measured as a mixture distinguished two sets of protons (Table 2), one for a major compound (**6**) and another, a minor compound (**7**). The analysis revealed the presence of delphinidin, three hexoses, *trans*-*p*-coumaric acid and caffeic acid in each compound. The FAB mass spectra also gave the molecular ion at 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 coumaric acid)]$^+$, 757 [M − 340 ([hexose or coumaric acid] and caffeic acid)]$^+$, 611 [M − 486 (3 × (hexose and/or coumaric acid))]$^+$, 449 [M − 648 (4 × [hexose and/or coumaric acid])]$^+$ and 303 [aglycone]$^+$. By the two-dimensional H–H TOCSY experiment of this mixture of **6** and **7**, each anomeric proton (δ 5.21 and 5.21) of two hexose groups of **6** was connected to each methylene proton of δ 4.41 and 4.66, and δ 4.26 and 4.75, respectively. As to the two anomeric protons of the hexose moieties of **7**, each one (δ 5.21 and 5.21) was also connected to each methylene proton of δ 4.39 and 4.65, and δ 4.34 and 4.69, respectively. The same chemical shifts were observed in the third hexose moieties of **6** and **7**, and the anomeric proton (δ 5.03) was connected to each methylene proton of δ 3.82 and 4.04. The chemical shifts of the protons of three hexose moieties of **6** and **7** were reverted by the relayed COSY (with one- to three-step relay) and the carbon atoms bonded to the protons of each hexose were reverted by HSQC. From these results, each hexose was determined to be glucose. The positions of the glucosidic linkage were determined using the NOESY spectra. The NOEs were observed between H-4 (δ 8.80), H-6 (δ 6.97) and H-2′ (δ 7.83) of delphinidin and anomeric protons of glucoses A, B and C, respectively. The downfield shift of the methylene signals of glucoses B and C indicated that aromatic acid moieties were attached to the 6-OH of both glucose moieties. The position of the ester linkages of both glucose moieties were confirmed by the HMBC.

Remarkable upfield shifts of the protons of the *trans*-*p*-coumaric acid of **6** and of the caffeic acid of **7** were observed. Yoshida *et al.* [4] reported that the signals of aromatic acid associated with the 3′-*O*-glucose of anthocyanidin were shifted to higher field than those associated with the 5-*O*-glucose in the ^1H NMR

spectrum of gentiodelphin. The same phenomena were also observed in the ^{1}H NMR spectrum of gentiocyanin B [2]. It was consequently concluded that the *trans-p*-coumaric acid of **6** and caffeic acid of **7** were attached to the 6-OH of each glucose C. The structure of **7** only differed from that of **6** with regard to the position of the ester linkages of the caffeic and *trans-p*-coumaric acids.

The structures of compounds **6** and **7** are thus delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-caffeoyl-β-D-glucoside)-3′-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside) and delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-*trans*-*p*-coumaroyl-β-D-glucoside)-3′-*O*-(6-*O*-caffeoyl-β-D-glucoside), named albireodelphin D and E, respectively.

Gentiodelphin (**4**) [1] and gentiocyanin A (**5**) [2] were also isolated along with the five novel anthocyanins **1**–**3**. **6** and **7** (albireodelphins A–E). The anthocyanins which had been found in gentian flowers were acylated with hydroxycinnamic acid, namely coumaric and caffeic acids. This type of anthocyanin containing 5-*O*-(6-*O*-hydroxycinnamoylglucoside) has been observed only in gentian flowers [1, 2] and may be specifically synthesized in flowers of *Gentiana*.

EXPERIMENTAL

Plant material. Blue flowers of *Gentiana* cv. Albireo, bred at Iwate Horticultural Experiment Station, were collected and freeze-dried in October 1993.

Isolation of anthocyanins. Freeze-dried flowers (75 g) were extracted with a mixture of EtOH–HOAc–H_2O (10:1:9) at 4°. The concd extract was applied to a column of Amberlite XAD-7 and washed with 5% HOAc. The anthocyanins were eluted with 50% MeOH containing 5% HOAc. For further purification, the crude anthocyanins were purified by prep. HPLC on a Chromatorex-ODS (20$^{\varphi}$ × 250 mm, Fuji Silysia Chemical Ltd) 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) for isolation of **1** and **4**. An isocratic solvent system of 40% solvent A followed by an isocratic solvent system of solvent C (10% MeOH, 15% HOAc and 0.5% TFA in H_2O) was used for **2** and **3**. For **5** and the mixture of **6** and **7**, an isocratic solvent system of solvent C was used. To replace counter anions of anthocyanins with trifluoroacetate, the concd fractions were adsorbed on a cartridge of an activated Sep-Pak tC18 (Waters Associates, Milford, MA) which was then washed with 1% aq. TFA and eluted with MeOH containing 1% TFA. The solns of purified anthocyanins were concentrated to dryness under a stream of N_2 gas. Each residue was dissolved in a small amount of 1% TFA and freeze-dried to give a total of seven anthocyanins as powders (**1**, 67 mg; **2**, 5.7 mg; **3**, 1.2 mg; **4**, 172 mg; **5**, 4.5 mg; **6** and **7** (mixture), 14.2 mg).

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

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