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Contribution of each caffeoyl residue of the pigment molecule of gentiodelphin to blue color development

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Abstract

To clarify the function of each caffeoyl residue in the diacylated anthocyanin gentiodelphin, a pigment from the blue flower of *Gentiana makinoi*, two mono-deacyl derivatives were compared for both color development and stability. In neutral solution, 3,5-di-*O*-*β*-D-glucopyranosyl-3′-*O*-(6-*O*-caffeoyl-*β*-D-glucopyranosyl)delphinidin was both bluer and more stable than 3,3′-di-*O*-*β*-D-glucopyranosyl-5-*O*-(6-*O*-caffeoyl-*β*-D-glucopyranosyl)delphinidin. Conformational analysis of each derivative under acidic conditions revealed only the 3′-*O*-caffeoylglucopyranosyl derivative to demonstrate intramolecular stacking. Additionally, the acyl residue in the B-ring contributed more to blue color development than that in the A-ring.

Keywords: *Gentiana makinoi*; Gentiodelphin; Acylated anthocyanin; Intramolecular stacking; Flower color development; Conformational analysis

1. Introduction

Polyacylated anthocyanins, which contain two or more aromatic acid residues, are a unique group of flower pigments (Goto et al., 1982; Goto et al., 1986; Goto, 1987; Brouillard, 1988; Goto and Kondo, 1991; Brouillard and Dangles, 1994). They are very stable in neutral or weakly acidic aqueous solutions (Saito et al., 1971; Yoshitama and Hayashi, 1974; Goto et al., 1982; Goto and Kondo, 1991), whereas simple anthocyanins are quickly decolorized by hydration at the 2-position of the anthocyanidin nucleus (Brouillard and Dubois, 1977; Brouillard and Delaporte, 1977; Brouillard, 1982; Mazza and Brouillard, 1987). Since polyacylated anthocyanins become unstable on removal of acyl residues, these aromatic acids must play an important role in both color development and stability (Brouillard, 1981, 1982; Goto et al., 1982; Goto and Kondo, 1991; Figueiredo et al., 1996). It has been suggested that the latter is due to intramolecular stacking of the aromatic acid to the anthocyanidin nucleus by hydrophobic interactions (Hoshino et al., 1981; Goto et al., 1982; Hoshino et al., 1982; Brouillard, 1982; Goto, 1987; Goto and Kondo, 1991; Yoshida et al., 1991).

We have proposed a folding conformation for polyacylated anthocyanins and provided evidence of an intramolecular stacking conformation for gentiodelphin (**1**), a dicaffeoyl anthocyanin (Goto et al. 1982; Yoshida et al., 1992). In acidic methanol, only the caffeic acid attached to the glucose of B-ring is stacked to the anthocyanidin nucleus with both aromatic rings in parallel, strongly suggesting that the roles of each acyl residue differ in intramolecular stacking conformation and flower color development. However, only kinetic and equilibrium studies of structurally diverse monoacyl anthocyanins from *Pharbitis nil* have thus far been

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Table 1
Assignment of the ^{1}H-NMR spectra of **1**–**4** (10% TFA$_d$-CD$_3$OD at 30°C, 500 MHz)[a]

		1			**2**[b]			**3**[c]			**4**		
	4	8.74	*s*		9.13	*s*		8.86	*s*		9.17	*s*	2.0
	6	6.96	*d*	2.0	7.04	*d*	2.0	6.98	*d*	2.0	7.08	*d*	2.0
	8	6.81	*d*	2.0	7.12	*brd*	2.0	6.88	*brd*	2.0	7.17	*brd*	2.0
	2′	7.73	*d*	1.5	8.04	*brd*	2.0	7.94	*d*	2.5	8.09	*d*	2.0
	6′	7.88	*d*	1.5	8.06	*brd*	2.0	7.82	*brd*	2.5	7.97	*d*	2.0
▲	1	5.06	*d*	7.5	5.34	*d*	7.5	5.05	*d*	7.5	5.31	*d*	7.5
	2	3.75	*dd*	9.0, 7.5	3.74	*dd*	9.0, 7.5	3.74	*dd*	9.0, 7.5	3.74	*dd*	9.0, 7.5
	3	3.61	*t*	9.0	3.58	*t*	9.0	3.57	*t*	9.0	3.58	*t*	9.0
	4	3.48	*t*	9.0	3.44	*t*	9.0	3.49	*t*	9.0	3.45	*t*	9.0
	5	3.69	*ddd*	9.0, 7.0, 2.0	3.66	*ddd*	9.0, 6.5, 2.0	3.67	*ddd*	9.0, 6.5, 2.5	3.66	*ddd*	9.0, 7.0, 2.0
	6a	4.09	*dd*	12.0, 2.0	3.99	*dd*	12.0, 2.0	4.09	*dd*	12.0, 2.5	3.90	*dd*	12.0, 2.0
	6b	3.84	*dd*	12.0, 7.0	3.76	*dd*	12.0, 6.5	3.85	*dd*	12.0, 6.5	3.75	*dd*	12.0, 7.0
●	1	5.23	*d*	7.5	5.17	*d*	7.5	5.18	*d*	7.5	5.20	*d*	7.5
	2	3.81	*dd*	9.0, 7.5	3.78	*dd*	9.0, 7.5	3.72	*dd*	9.0	3.71	*dd*	9.0, 7.5
	3	3.68	*t*	9.0	3.62	*t*	9.0	3.62	*t*	9.0	3.59	*t*	9.0
	4	3.62	*t*	9.0	3.44	*t*	9.0	3.53	*t*	9.0	3.49	*t*	9.0
	5	3.88	*ddd*	9.0, 6.5, 2.0	3.88	*ddd*	9.0, 7.0, 2.0	3.63	*ddd*	9.0, 5.5, 2.5	3.60	*ddd*	9.0, 5.0, 2.0
	6a	4.66	*dd*	12.0, 2.0	4.60	*dd*	12.0, 2.0	4.06	*dd*	12.0, 2.5	3.97	*dd*	2.0, 2.0
	6b	4.41	*dd*	12.0, 6.5	4.36	*dd*	12.0, 7.0	3.84	*dd*	12.0, 5.5	3.78	*dd*	12.0, 5.0
■	1	5.21	*d*	7.0	5.06	*d*	7.5	5.27	*d*	7.5	5.04	*d*	7.5
	2	3.68	*m*		3.60	*m*		3.65	*m*		3.60	*m*	
	3	3.68	*m*		3.60	*m*		3.65	*m*		3.60	*m*	
	4	3.41	*t*	9.0	3.43	*t*	9.0	3.39	*m*		3.45	*t*	9.0
	5	3.85	*ddd*	9.5, 9.0, 2.0	3.62	*ddd*	10.0, 6.0, 2.0	3.87	*td*	9.5, 2.5	3.60	*ddd*	9.0, 5.0, 2.0
	6a	4.71	*dd*	12.0, 2.0	3.99	*dd*	12.0, 2.0	4.71	*dd*	12.0, 2.5	4.00	*dd*	12.0, 2.0
	6b	4.35	*dd*	12.0, 9.5	3.77	*dd*	12.0, 6.0	4.37	*dd*	12.0, 9.5	3.80	*dd*	12.0, 5.0
C1	α	6.23	*d*	16.0	6.20	*d*	16.0						
	β	7.47	*d*	16.0	7.44	*d*	16.0						
	2	6.98	*d*	2.0	6.97	*s*							
	5	6.74	*d*	8.0	6.75	*d*	8.5						
	6	6.86	*dd*	8.0, 2.0	6.87	*d*	8.5						
C2	α	5.91	*d*	16.0				6.02	*d*	16.0			
	β	7.05	*d*	16.0				7.12	*d*	16.0			
	2	6.37	*d*	2.0				6.42	*d*	2.0			
	5	6.51	*d*	8.0				6.53	*d*	8.5			
	6	6.34	*dd*	8.0, 2.0				6.46	*dd*	8.5, 2.0			

[a] Irr. H-1 of glucose ▲ to H-4: **1** (25°C) = −14%; **2** (0°C) = −19%; **3** (0°C) = −12%; **4** (−25°C) = −43%. Irr. H-1 of glucose ● to H-6: **1** (25°C) = −12%; **2** (0°C) = −18%; **3** (0°C) = −20%; **4** (−25°C) = −45%. Irr. H-1 of glucose ■ to H-2′: **1** (25°C) = −14%; **2** (0°C) = −16%; **3** (0°C) = −30%; **4** (−25°C) = −63%.

[b] Measured at 25°C.

[c] In **3** weak NOEs (−1 to −2%) were observe between protons of nucleus (H-4, H-8, H-2′ and H-6′) an protons of C2 (α, β, 2, 5, and 6), ▲-1 and protons of C2 (α, β, 2).

reported, and no variation in the contribution to color development was established (Dangles et al., 1993).

We therefore prepared two kinds of mono-deacyl derivatives from **1**, in order to permit a direct comparison of their respective contributions to blue color development and stability, and also undertook various NMR spectroscopic experiments in order to assess their respective conformations and degree of intramolecular stacking.

2. Results and discussion

Gentiodelphin (**1**) was purified from blue petals of *Gentiana makinoi* according to the procedure described previously (Yoshida et al., 1992) with slight modification. By controlled alkaline hydrolysis, **1** (52.9 mg) gave a reaction mixture consisting of three products (**2**–**4**) and unreacted **1**. Using preparative HPLC, the reaction mixture was separated to give **2** (4.3 mg), **3** (7.5 mg), **4** (10.0 mg) and recovered gentiodelphin (1, 4.0 mg). Compound **4** was deduced to be a fully deacylated anthocyanin by co-chromatography with an authentic sample (Yoshida et al., 1992).

High resolution FABMS of **2** and **3** gave the same molecular formula of $C_{42}H_{47}O_{25}$, corresponding to loss of one caffeoyl residue from gentiodelphin. By comparison of its NMR spectroscopic data with those in literature, **2** was identified to be albireodelphin A, previously isolated from blue flowers of *G. albireo* (Hosokawa et al., 1997; Table 1). To unambiguously determine the position of the caffeoyl residue, all the ^{1}H-NMR spectral signals of **3** were assigned by 1D-HOHAHA and NOE difference spectra with irradiation at each anomeric proton (Table 1). In **3** the 6-position of glucose ■ was esterified, because of the lower shift of the methylene protons (H-6a and 6b) of glucose ■. Thus, the structure of **3** was deduced to be 3,5-di-*O*-*β*-D-glucopyranosyl-3′-*O*-(6-*O*-caffeoyl-*β*-D-glucopyranosyl)delphinidin.

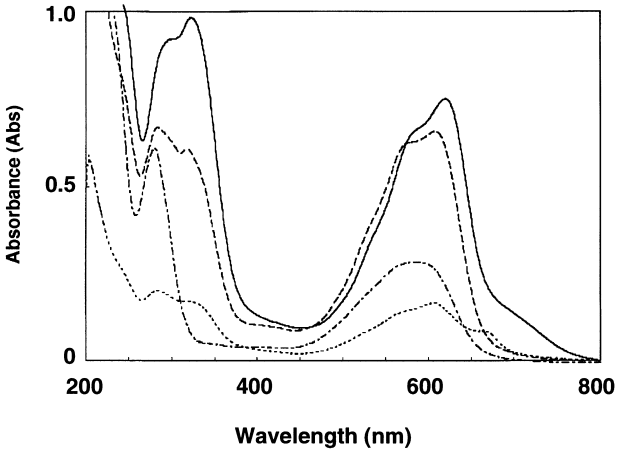
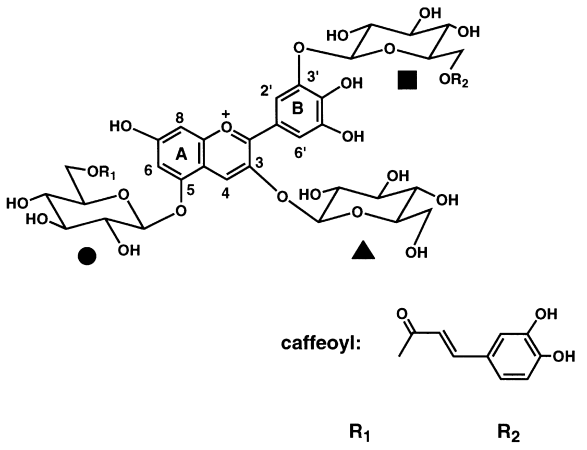


Fig. 1. UV–Vis spectra of **1**–**4** in aqueous solutions (5×10^{-5} M in 0.1 M phosphate buffer at pH 6.5, path length; 10 mm). ——: **1**, – · – · –: **2**, - - - - : **3**, · · · · **4**.

Compounds **1**–**4** were individually dissolved in aqueous buffer solution (5×10^{-5} M, pH 6.5 in 0.1 M phosphate buffer) and then the UV–Vis spectra of each were recorded (Fig. 1). Compound **3** showed a similar blue color as that of **1**, whereas the color of **2** and **4** in the buffer solution (pH 6.5) was purplish blue. The spectrum of **3** but not **2** was similar to that of **1**. Stability of the color in aqueous solution was recorded by monitoring the decrease in absorbance at λ_{max} (520–530 nm) at 5×10^{-5} M (Fig. 2). Gentiodelphin (**1**) was stable while the bis-decaffeoyl pigment (**4**)



	R_1	R_2
gentiodelphin (1)	caffeoyl (C1)	caffeoyl (C2)
albireodelphin (2)	caffeoyl (C1)	H
mono-deacylgentiodelphin (3)	H	caffeoyl (C2)
bis-deacylgentiodelphin (4)	H	H

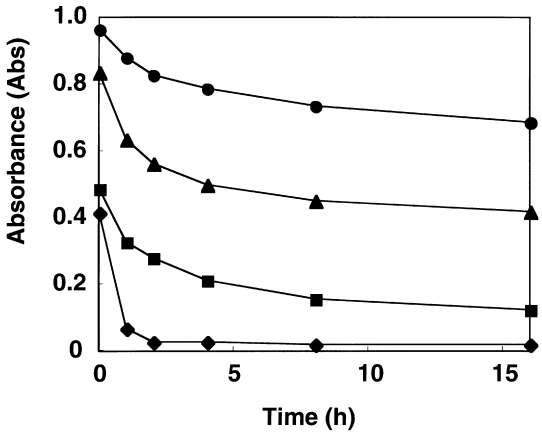


Fig. 2. Stability of **1** (●), **2** (■), **3** (▲) and **4** (◆) in aqueous solutions at 25°C (5×10^{-5} M in 0.1 M phosphate buffer at pH 6.5, path length: 10 mm). After half month, **1** and **3** retained color, but **2** an **4** were decolorized.

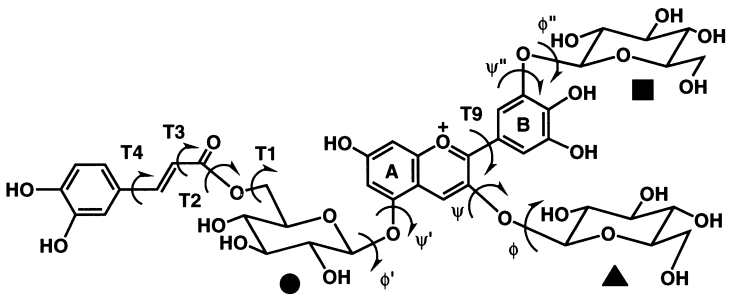
Table 2
Ratios of *gg*, *gt* an *tg* rotamers at the exo-cyclic C5–C6 bond of each glucoside in **1**–**4**[a]

Compound	Glucoside	% of each rotamer		
		gg	*gt*	*tg*
1	▲	45	55	0
	●	49	51	0
	■	22	82	−4
2	▲	49	51	0
	●	45	55	0
	■	54	46	0
3	▲	46	54	0
	●	55	45	0
	■	24	75	1
4	▲	45	55	0
	●	58	42	0
	■	58	42	0

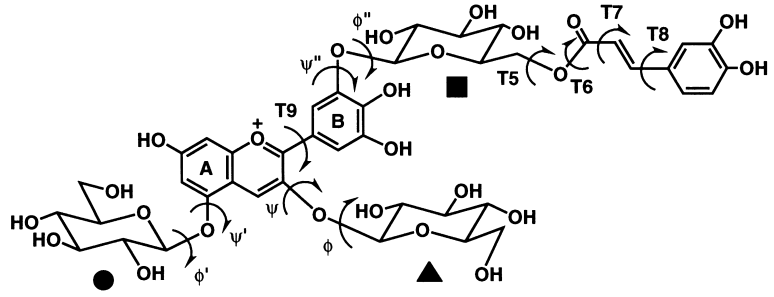
[a] NMR spectral data were measured in acidic methanol solutions and the ratios were calculated by the modified Karplus equation: $J_{5,6proS} = 1.3\,gg + 2.7\,gt + 11.7\,tg$, $J_{5,6proR} = 1.33\,gg + 11.5\,gt + 5.8\,tg$, $gg + gt + tg = 1$.

was quickly decolorized. Interestingly, the stabilities of the monocaffeoyl derivatives, **2** and **3**, were different. Compound **3** with a caffeoyl residue on the 3-*O*-glucoside was more stable than compound **2** with a caffeoyl residue on the 5-*O*-glucoside. Differences in stacking were concluded to be responsible for the variation in both the color and the stability of **2** and **3**.

To clarify the conformation of the aromatic acid residue and the anthocyanidin nucleus in **2** and **3**, a ^{1}H-NMR spectroscopic study was carried out. Since compounds **1**–**4** were slightly soluble in the buffer (pH 6.5), ^{1}H-NMR spectra were measured in 10% TFA_d-CD_3OD as described in a previous report (Yoshida et al., 1992). Shift of proton signals toward higher field under these conditions indicates the existence of face-to-face stacking with two aromatic rings in parallel (Goto et al., 1982, 1986; Goto, 1987). The signals of the delphinidin nucleus of **3** were shifted upfield 0.02–0.3 ppm more than that of **4**, while those of **2** were unaffected (Table 1). The signals of the caffeic acid residue of **3** were also shifted 0.35–0.74 ppm upfield as compared with those of methyl *E*-caffeate as a result of the large ring current (chemical shift of methyl *E*-caffeate in TFA_d-CD_3OD: H-α 6.28 ppm, H-β 7.56 ppm, H-2; 7.07 ppm, H-5; 6.78 ppm, H-6; 6.95 ppm), while those of **2** were not shifted. Therefore, the caffeoyl residue of **3** should stack face-to-face to the anthocyanidin nucleus, while that of **2** should not. The conformation was also analyzed by ^{1}H-NOE. In all pigments, **1**–**4**, strong NOEs were observed between



2



3

Fig. 3. Torsional bonds of **2** and **3** during molecular modeling.

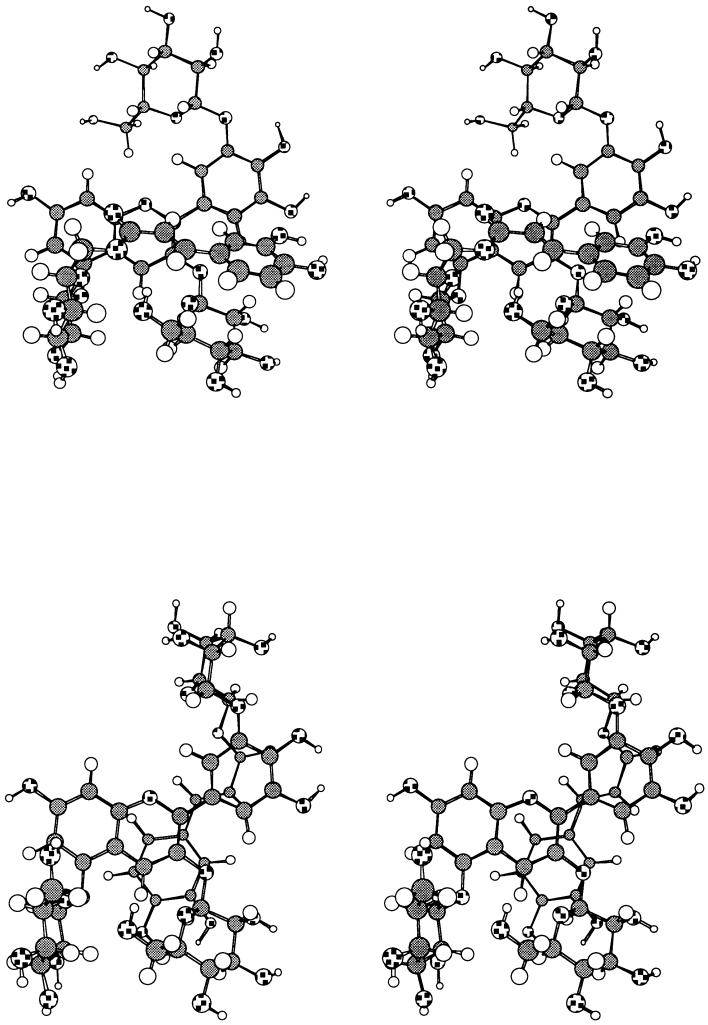


Fig. 4. Stereo-views (parallel view) of the optimized conformers in acidic methanol, obtained by molecular modeling. Upper: optimized conformer of **2**. Lower: optimize conformer of **3**.

▲-1 and H-4, ●-1 and H-6, and ■-1 and H-2′, respectively, but no NOE was detected between ▲-1 and H-2′, 6′, or ●-1 and H-4, indicating that the anomeric linkage conformation of each glucoside was fixed. Since weak NOEs give distance information useful for analyzing molecular stacking interactions, the NOE difference spectra of **2** and **3** were measured at 10°C and the long distance NOEs between the signals of chromophore and caffeic acid residue were analyzed (Table 1). NOEs of negative 1–2% were observed for compound **3** between the signals of the nucleus, H-4, H-8, H-2′ and H-6′, and the signals of the caffeic acid residue, indicating an intramolecular stacking conformation, but no long distance NOE was observed in **2**. From the coupling constant between H-5 and H-6 of glucoside, $J_{5,\,6a}$ and $J_{5,\,6b}$, the ratio of each rotamer of the exo-cyclic C5–C6 bonds was calculated (Table 2, Bruyn and Anteunis, 1976; Nishida et al., 1988). The *gt* rotamer was predominant in the ■ glucose of **3**, similar to that in **1**. However, the other glucosides, ▲, ● and ■ in **2** and ▲ and ● in **3**, showed equal rotamer ratios (*gg:gt* = ca. 1:1), therefore, acylation of 6-OH of the glucoside ■ forms the C5–C6 bond *gt* conformation. These results are consistent with those reported for gentiodelphin (**1**) (Yoshida et al., 1992). Thus, the caffeic acid residue in the B-ring has the greater contribution to formation of face-to-face intramolecular stacking.

To further investigate the conformations of **2** and **3**, computer-assisted molecular modeling was performed using QUANTA97/CHARMm software with various constraints deduced from NMR spectroscopic analysis. Number of 5158 initial conformers that were generated by grid rotation of the bonds (Fig. 3), and then each conformer was optimized by conjugate gradient energy minimization processes

using various constraints. Bond angle constraints were adopted at the exo-cyclic C5–C6 bonds of all the glucoside residues following the rotamer ratios shown in Table 2. Since the dihedral angle between the A- and B-ring, and the glucosidic bonds of ▲-glucose were not so diverse by analysis of the NMR spectroscopic data, those dihedral angles were fixed at the same conformation as that obtained from the X-ray data of commelinin (Kondo et al., 1992). Between those protons for which a strong NOE was observed, a 2.5 ± 0.5 Å distance constraint was adopted, and between the protons for which a weak NOE was observed, a 4.0 ± 1.0 Å distance constraint was adopted. From all the generated and minimized conformers, 1000 numbers were selected, then the Adopted-Basis Newton–Raphson method was used to obtain an optimized conformation. As shown in Fig. 4, the caffeoyl residue of **3** stacked to the chromophore at a distance approaching the van der Waal's distance. On the other hand, the caffeic acid residue in **2** had no interaction with the chromophore nucleus. To determine whether the caffeoyl residue of **2** stacks to the nucleus in water, we carried out molecular modeling with the dielectric constant $\varepsilon = 70$ for water, using the same procedure described above. Under these conditions the acyl moiety in **2** also stacked to the nucleus with two aromatic rings in parallel from the front side (Fig. 5).

As shown by the UV–Vis spectra and stability in aqueous solution (pH 6.5), the contribution of each caffeoyl residue to blue color development is different. The mono-*O*-caffeoyl anthocyanin, with its acyl moiety on the glucoside in the B-ring, showed a blue color and was relatively stable under neutral conditions. Although the evidence for stacking conformation was obtained in acidic methanol only, molecular modeling studies in neutral aqueous and acidic methanol conditions suggest the same result. The difference of stability among **1**–**3** in neutral aqueous solution suggests that both the acyl moieties of **1** stack to the nucleus, but the stacking manner of the 3′-acyl residue was much closer to the nucleus than that of the 5-acyl residue. In gentiodelphin **1**, the contribution of each acyl moiety to blue color development and stability is different and must be estimated by linear calculation.

3. Experimental

3.1. General procedures

UV–Vis spectra were recorded on a JASCO Ubest-55 spectrometer. NMR spectra were obtained with a JEOL GX500 spectrometer (^{1}H: 500 MHz, ^{13}C: 125 MHz) in a 5 mm ϕ tube at variable temperature, using 10% TFA_d–CD_3OD as solvent. Chemical shifts were recorded as parts per million (ppm) with the CD_2HOD resonance set at 3.325 ppm as a standard. FABMS data were recorded on a JEOL MStation using glycerol–HCl as the matrix, and ESIMS data were recorded on a JEOL JMS-700 using the flow injection mode. Analytical and preparative HPLC were carried out using an ODS-column (Develosil ODS-HG5 4.6 mm $\phi \times 250$ mm, 20 mm $\phi \times 250$ mm and 50 mm $\phi \times 250$ mm, Nomura Chemical). HPLC was performed at 40°C with linear gradient elution from 10 to 30% aq. acetonitrile (CH_3CN) solution containing 0.5% TFA in 30 min, or isocratic elution with various concentrations of aq. CH_3CN solution containing 0.5% TFA.

3.2. Isolation of pigments

From fresh blue petals of *G. makinoi*, crude pigments were obtained using the procedure described previously (Yoshida et al., 1992). Crude pigment (9.6 g) was dissolved in 1.5 l of water containing 0.5% TFA, then poured onto an Amberlite XAD-7 column (50 mm $\phi \times 300$ mm). The column was stepwise eluted

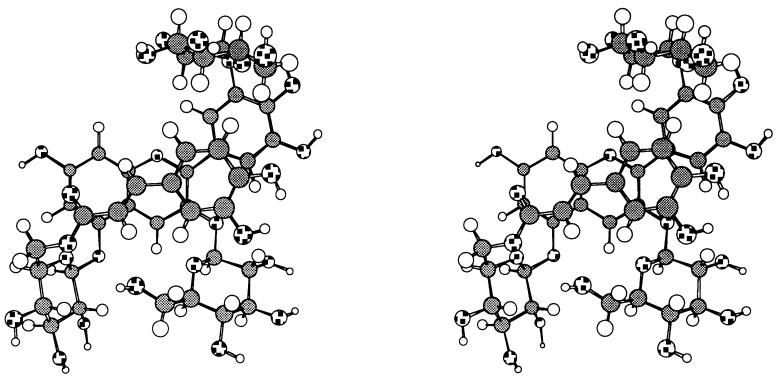


Fig. 5. Stereo-view (parallel view) of the optimized conformers of **2** obtained by molecular modeling in water.

from 20 to 60% aq. MeOH containing 0.5% TFA. The 30–50% MeOH fraction contained the major pigments. The fraction was evaporated in vacuo, dissolved into 0.5% aq. TFA solution, and then the solution was subjected to chromatography using a preparative ODS–HPLC which was eluted with 5–50% aq. CH_3CN solution containing 0.5% TFA. Gentiodelphin (**1**) was eluted in the 19% aq. CH_3CN fraction. The eluent was dried in vacuo, yielding **1** (319 mg) as a dark-red amorphous TFA salt.

3.3. Alkaline hydrolysis

Compound **1** (52.9 mg, 0.043 mmol) was dissolved in MeOH (1.25 ml) at room temperature, bubbled with argon gas (Ar), then cooled to 0°C. To this solution, 1.0 N NaOH (1.25 ml) was added, and the mixture was allowed to stand at 0°C under Ar atmosphere for 30 min. To the reaction mixture, 6 N aq. HCl was added at 0°C, then the solution was warmed to room temperature. The mixture was evaporated in vacuo to 1/10 volume and diluted five fold with water, then subjected to preparative ODS–HPLC (20 mm $\phi \times 250$ mm). The column was eluted from 10 to 50% aq. CH_3CN solution containing 0.5% TFA. Compounds **4**, **3**, **2** and **1** were eluted in 10, 12, 15 and 19% CH_3CN fractions, respectively. Fractions were evaporated in vacuo to give **1** (4.0 mg, 7.6%), **2** (4.3 mg, 9.4%), **3** (7.5 mg, 16.4%) and **4** (10.0 mg, 25.8%).

3.3.1. 3,3′-Di-O-β-D-glucopyranosyl-5-O-(6-O-caffeoyl-β-D-glucopyranosyl)delphinidin (albireodelphin A, 2)

UV–Vis (0.01% HCl–MeOH) nm (ε: absorption coefficient): 279 (14,300), 296 (12,700), 331 (12,200), 529 (21,700); HR-FABMS calcd. for $C_{42}H_{47}O_{25}$ m/z 951.2406; found m/z: 951.2421 (+1.5 mmu).

3.3.2. 3,5-Di-O-β-D-glucopyranosyl-3′-O-(6-O-caffeoyl-β-D-glucopyranosyl)delphinidin (3)

UV–Vis (0.01% HCl–MeOH) nm (ε): 279 (18,600), 297 (15,800), 332 (15,200), 537 (30,100); HR-ESIMS calcd. for $C_{42}H_{47}O_{25}$ m/z 951.2406; found m/z: 951.2399 (−0.7 mmu).

3.4. Absorption spectra

Each isolated anthocyanin (TFA salt) was dissolved in water at a concentration of 5×10^{-2} M. Individual solutions were diluted to 5×10^{-5} M with 0.1 M phosphate buffer (pH 6.5), with UV–Vis spectra subsequently measured in a quartz cell (d = 10 mm) at 25°C.

3.5. Molecular modeling

Conformational analysis was performed on an IRIS O2/R10000 workstation (Silicon Graphics) using the molecular modeling package QUANTA97/CHARMm 23.2, with dielectric constants, $\varepsilon = 32.6$ in methanol and $\varepsilon = 70.0$ in water. The starting conformation was constructed using the X-ray data of malonylawobanin in commelinin (Kondo et al., 1992; Kondo et al., unpublished results). The conformation search for **2** was carried out using a grid scan routine with rotation of the torsional angles, Ψ', Φ', T1 and T2 by 60° increments, and rotation of T3 and T4 by 180°. All the generated conformers were minimized by conjugate gradient energy minimization until energy gradient tolerance was equal to 0.05. During minimization, dihedral constraints *gg* at C5–C6 bond of glucose ●, and NOE constraints 2.5 ± 0.5 Å between the anomeric protons and the nucleus protons were adopted. Among the minimized conformers, 1000 from the lowest energy level were selected and then the energy minimization for each was achieved using Adopted-Basis Newton–Raphson methods without any constraints but T9 to obtain the optimized conformer. The conformation search for **3** was performed with rotation of the torsional angles, Ψ'', Φ'', T5 and T6 by 60° increments, and rotate T7 and T8 by 180°. During minimization, dihedral constraints *gt* at the C5–C6 bond of glucose ■, and NOE constraints 2.5 ± 0.5 Å between the anomeric protons and the nucleus protons and 4.0 ± 1.0 Å between the protons for which weak NOEs were observed, were adopted using the same procedure.

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