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Intramolecular Stacking Conformation of Gentiodelphin, a Diacylated Anthocyanin from *Gentiana Makinoi*

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Key words: gentiodelphin; polyacylated anthocyanin; sandwich-type stacking; conformational analysis

Abstract: Gentiodelphin from *Gentiana makinoi* is unusually stable in neutral aqueous solutions and may be stabilized by intramolecular sandwich-type stacking of two caffeic acids. Although in acidic methanol solution, evidence for intramolecular stacking of gentiodelphin was obtained from electronic spectra and ^{1}H NMR analysis. Using computer-assisted conformational analysis with NOE constraints and bond angle constraints, we first established the intramolecular stacking conformation of aromatic acid to the anthocyanidin nucleus. Under acidic conditions, one caffeic acid attached at the glucose of the 2-position of the B-ring was stacked to the delphinidin nucleus but the other acid attached at the glucose of the 5-position was not stacked.

INTRODUCTION

Simple anthocyanins are unstable in neutral or weakly acidic aqueous solutions and quickly decolorized by hydration at the 2-position of the anthocyanidin nucleus.[1] In 1971 Saito *et al.* isolated platyconin from purple petals of Chinese bell-flower *Platycodon grandiflorum* [2] which is unusually stable in such solution. Then, a few stable anthocyanins are found.[3-5] Since these anthocyanins contained two or more cinnamic acid derivatives, Yoshitama *et al* surmised that the existence of *ortho* dihydroxy group of the B-ring and caffeic acids may cause the stability.[3] In 1977 Abe *et al.* attributed the association to hydrogen bonding between the phenolic hydroxy groups and proposed intramolecular co-pigmentation.[6]

We have determined the structures of many complicated anthocyanins having more than two cinnamic acid derivatives, *i. e.* Heavenly Blue Anthocyanin (HBA) from *Ipomoea tricolor*,[7] gentiodelphin from *Gentiana makinoi*,[8] platyconin from *Platycodon grandiflorum*[9], etc.[10-17] In these studies we have considered that the stability of polyacylated anthocyanins may not be caused by hydrogen bonding but by hydrophobic interaction between anthocyanidin nuclei and aromatic acids. Since self-association has been proven a hydrophobic vertical stacking of anthocyanidin nuclei by us,[18-21] the same hydrophobic interaction between the anthocyanidin nucleus and two aromatic acids may occur, which we called sandwich-type stacking (Fig. 1).[15-17] This hypothesis was supported by NMR experiments with HBA in acidic solution; long distance nuclear Overhauser effects (NOEs) between the anomeric protons of the glucoses and the acyl protons were observed.[15-17] At the same time Brouillard also proposed the same stacking model without experimental evidence.[22, 23]

In this paper we describe the stacking conformation analysis of a dicaffeyl anthocyanin, gentiodelphin in acidic methanol. We first obtained evidence of the intramolecular hydrophobic stacking of an aromatic acid to the anthocyanidin nucleus.

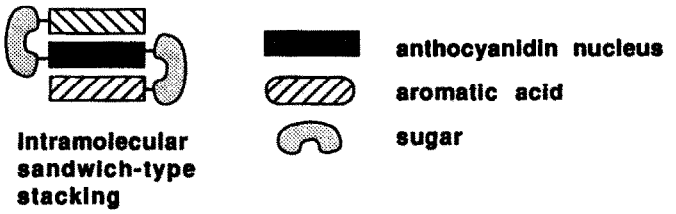
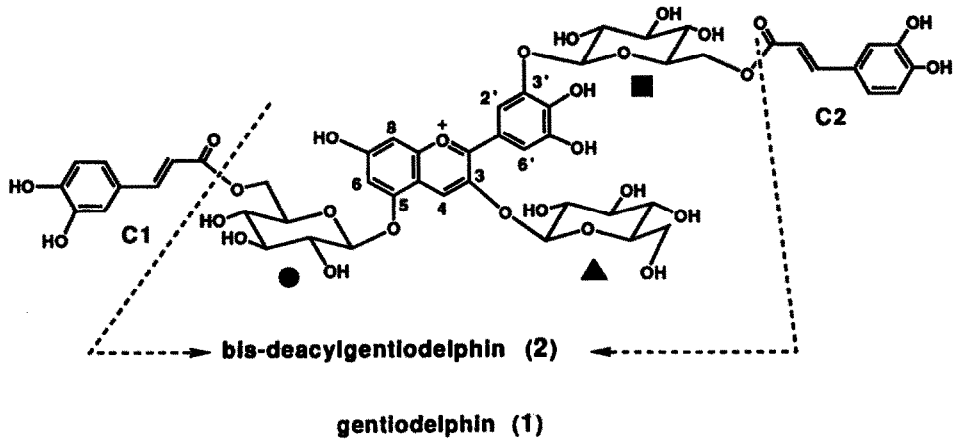


Fig. 1. Schematic presentation of intramolecular-sandwich type stacking for polyacylated anthocyanin.



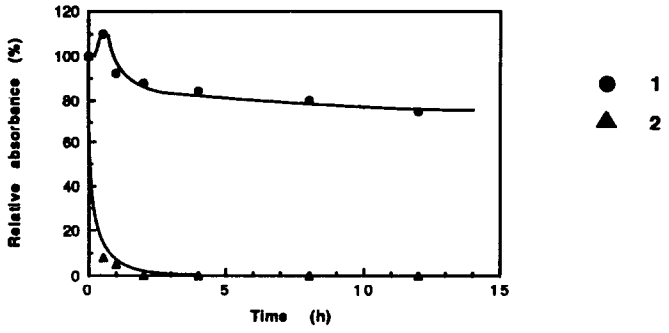


Fig. 2. Stability of **1** and **2** in an aqueous solution (pH 6.5, 5 x 10^{-5}M).

RESULTS AND DISCUSSION

Isolation and stability of gentiodelphin

Blue flower petals (2.4 Kg) of *Gentiana makinoi* were frozen with liquid nitrogen, pulverized with a blender, and extracted with 20% aqueous methanol (MeOH) containing trifluoroacetic acid (TFA). The extract was evaporated under reduced pressure and purified repeatedly by column chromatography using Amberlite XAD-7 and preparative ODS; then pure gentiodelphin (**1**) was yielded as dark red TFA salts (260 mg, FABMS *m/z* 1113; M^+). **1** was hydrolyzed by treatment with alkaline MeOH to give bis-deacylgentiodelphin (**2**, FABMS *m/z* 789; M^+). As shown in Fig.2, gentiodelphin was very stable in an aqueous solution (pH 6.5), but its deacylate was unstable and decolorized quickly. So the acyl groups must be essential to stabilize anthocyanin.

Assignment of ^{1}H NMR of gentiodelphin

We have reported the structure of **1** to be 3-*O*-(β-D-glucopyranosyl)-5-*O*-(6-*O*-(*E*)-caffeyl-β-D-glucopyranosyl)-3'-*O*-(6-*O*-(*E*)-caffeyl-β-D-glucopyranosyl)delphinidin.[8] However no detailed assignment of the ^{1}H NMR signals to distinguish the caffeyl groups from each other has been carried out. Since full assignment of the signals is necessary for the conformational analysis of **1**, we measured the ^{1}H NMR spectra of gentiodelphin involving HMBC using 500 MHz NMR. On the basis of the lowest field signal (8.74 ppm, H-4)[24] in the flavylium form the proton signals of the anthocyanidin nucleus were assigned by COSY; then the existence of delphinidin was ascertained. The remaining signals at the aromatic proton's region were completely assigned to two molecules of (*E*)-caffeic acids. By 1D-HOHAHA spectra with irradiation at the anomeric protons, the three sugars were all deduced to be β-glucopyranosides.[25,26] The positions of glucosidic linkage were determined by NOE difference spectra with irradiation at each anomeric proton (Fig. 3). The 6-positions of the glucose ● and ■ were determined to be esterified; the methylene protons (H-6a and 6b) of both glucoses were shifted lower than that of glucose ▲ (Table 1). In order to distinguish the linkages of the two caffeic acids, HMBC[27] was measured (Fig. 4). The signals of 6a and 6b of glucose ● and ■ were correlated to the signals of the α and β protons of C1 and C2, respectively, through the ^{13}C signals of the carboxyl carbon of each caffeic acid. By HMBC those glucosidic linkages were also confirmed; the anomeric protons of ▲, ● and ■ were correlated to H-4, H-6 and H-2', respectively, through the ^{13}C signals of C-3, C-5, and C-2'. Thus, assignment of the ^{1}H NMR signals has been completed as shown in Table 1.

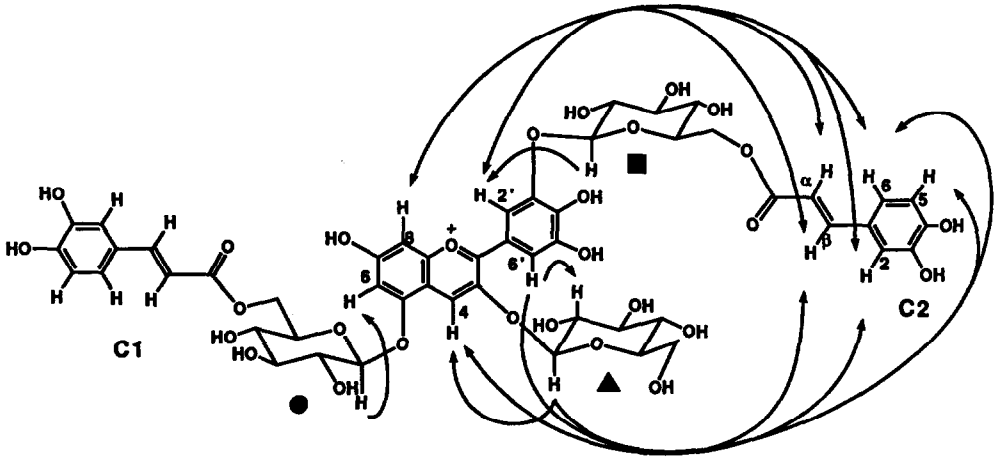


Fig. 3. The NOE network of gentiodelphin (**1**, 2.7×10^{-2}M) in 10% TFA*d*-CD3OD at 10°C (500 MHz).

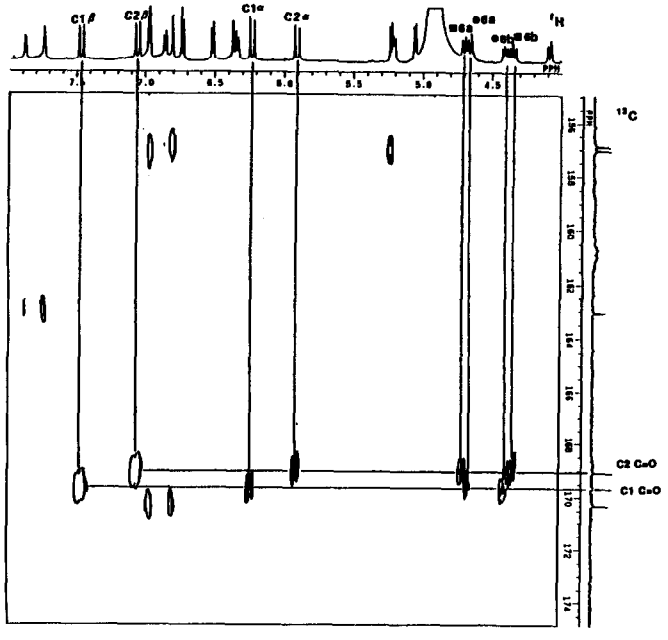


Fig. 4. The HMBC spectrum of gentiodelphin (**1**) in 10% TFA*d*-CD3OD at 25°C. (^{1}H: 500 MHz, ^{13}C: 125 MHz).

Table 1. Assignment of the ^{1}H NMR Spectra of 1and 2 (10% TFA*d*-CD_3OD at 30°C, 500MHz)

		1			2	
	4	8.74	s	9.17	s	
	6	6.96	d	2.0	7.08	d	2.0
	8	6.81	d	2.0	7.17	br.d	2.0
	2'	7.73	d	1.5	8.09	d	2.0
	6'	7.88	d	1.5	7.97	d	2.0
▲	1	5.06	d	7.5	5.31	d	7.5
	2	3.75	dd	9.0, 7.5	3.74	dd	9.0, 7.5
	3	3.61	t	9.0	3.58	t	9.0
	4	3.48	t	9.0	3.45	t	9.0
	5	3.69	ddd	9.0, 7.0, 2.0	3.66	ddd	9.0, 7.0, 2.0
	6a	4.09	dd	12.0, 2.0	3.90	dd	12.0, 2.0
	6b	3.84	dd	12.0, 7.0	3.75	dd	12.0, 7.0
●	1	5.23	d	7.5	5.20	d	7.5
	2	3.81	dd	9.0, 7.5	3.71	dd	9.0, 7.5
	3	3.68	t	9.0	3.59	t	9.0
	4	3.62	t	9.0	3.49	t	9.0
	5	3.88	ddd	9.0, 6.5, 2.0	3.60	ddd	9.0, 5.0, 2.0
	6a	4.66	dd	12.0, 2.0	3.97	dd	12.0, 2.0
	6b	4.41	dd	12.0, 6.5	3.78	dd	12.0, 5.0
■	1	5.21	d	7.0	5.04	d	7.5
	2	3.68	m		3.60	m	
	3	3.68	m		3.60	m	
	4	3.41	t	9.0	3.45	t	9.0
	5	3.85	ddd	9.5, 9.0, 2.0	3.60	ddd	9.0, 5.0, 2.0
	6a	4.71	dd	12.0, 2.0	4.00	dd	12.0, 2.0
	6b	4.35	dd	12.0, 9.5	3.80	dd	12.0, 5.0
C1	α	6.23	d	16.0			
	β	7.47	d	16.0			
	2	6.98	d	2.0			
	5	6.74	d	8.0			
	6	6.86	dd	8.0, 2.0			
C2	α	5.91	d	16.0			
	β	7.05	d	16.0			
	2	6.37	d	2.0			
	5	6.51	d	8.0			
	6	6.34	dd	8.0, 2.0			

NOE	1 (25°C)	2 (-25°C)
irr. 1-H of glucose ▲ to H-4	-14%	-43%
irr. 1-H of glucose ● to H-6	-12%	-45%
irr. 1-H of glucose ■ to H-2'	-14%	-63%

Table 2. Electronic Spectral Data of **1** and **2** in 0.01% HCl-MeOH at 20°C (λ_{max} (ε))

1	**2**
540 (22,100)	529 (20,100)
331 (20,600)	276 (10,100)
300 (19,100)	
284 (18,600)	

Table 3. Upfield Shift (ppm) of Protons of **1** Compared with Methyl Caffeate.
(2.7 x 10^{-2}M in 10% TFA*d*-CD_3OD at 30°C, 500MHz)

	C1	C2
α	0.05	0.36
β	0.09	0.50
2	0.09	0.69
5	0.04	0.29
6	0.09	0.59

Conformation in solution

1 showed an electronic absorption maximum at 540 nm in acidic MeOH (2.7x10^{-5}M). A concentration dependent change in the electronic spectrum was not practically observed from 2.7x10^{-5}M to 2.7x10^{-3}M and the CD spectrum of **1** (2.7x10^{-3}M) showed no exciton type Cotton effect in the visible region, indicating that the delphinidin nuclei did not self-associate with each other. Since **2** showed an electronic absorption maximum at 529 nm in the same concentration, the 11 nm bathochromic shift of **1** may be caused by intramolecular stacking of acyl moieties such as a co-pigmentation effect.[28-30]

In ^{1}H NMR spectra the chemical shift of signals of the delphinidin nucleus of **1** (2.7x10^{-2}M at 10°C) was observed by 0.02-0.3 ppm higher field than that of **2** (Table 1). This indicates that the protons of the nucleus are influenced by the anisotropic effect of the aromatic ring current. Comparing the chemical shift of signals of the two caffeic acids, C1 and C2, with those of methyl *E*-caffeate, only the signals of C2 were shifted toward 0.35-0.74 ppm upfield (Table 3), suggesting that the protons of C2 are affected by ring current anisotropy and C1 is not affected. Thus, C2 could be stacked to the delphinidin nucleus and C1 could not. Otsubo *et al.* reported the ring current anisotropic effect of paracyclophane.[31] The up-field shift of signals of a benzene ring stacking in parallel to another benzene ring at 4Å distance is about 0.69 ppm. Since the up-field shifts of the aromatic signals of C2 in **1** were about 0.6 ppm, the distance between the delphinidin nucleus and C2 may be about 4Å.

The NOE difference spectra of gentiodelphin were measured in CD_3OD containing 10% TFA (20 mg TFA salts of **1** in 0.6 ml, 2.7x10^{-2}M) at various temperatures. At 40 °C almost no NOE was observed, whereas negative NOEs were detected at 25°C. At 10°C not only direct strong NOEs but also long distance NOEs were observed. Further lowering of the temperature caused broadening of the signals and spin diffusion occurred over all protons, therefore, NOE experiments were carried out at 10°C. On irradiation at the anomeric proton of glucose ▲, a strong NOE (-21%) was observed only at H-4, but little NOEs (-3% each) were observed at the other side protons, H-2' and H-6' of the B ring. Irradiation at H-1 of glucose ● a -23% NOE was observed at H-6 but no NOE at H-4. Thus, the glycosidic linkages must not rotate freely but must be fixed strictly to one side. In this conformation the π orbital of the double bond of the anthocyanidin nucleus and the lone pair of the glycosidic linkages are located in anti-periplanar relationship because the interaction of the bonds are maximized.[32-35]

The long distance NOE network of **1** at 10°C is shown in Fig. 3. Among the signals of H-4, H-8, H-2' and H-6', and the signal of C2 protons relative to each other a negative 1-2% NOE was observed, but no NOE was observed at the signals of C1. These NOEs also indicate intramolecular stacking of C2 to the delphinidin nucleus. At a concentration of 2.7×10^{-3}M, long distance NOEs between the signals of the anthocyanidin nucleus and C2 were also observed. At this concentration intermolecular interaction could not exist, as is obvious from the lack of concentration dependent differences of the ^{1}H NMR spectra from 2.7×10^{-2}M to 2.7×10^{-4}M (Fig. 5),[36] and so, these NOEs must be intramolecular. By means of ROESY [37] (2.7×10^{-2}M at 40°C), positive NOEs were observed between H-8, H-4 and the α, β proton of C2; therefore, the close relationship of the anthocyanidin nucleus and C2 was confirmed. In dimethylsulfoxide (DMSO)*d*6 - TFA*d* intermolecular hydrophobic interaction was destroyed, nevertheless the same long distance NOEs were also observed. Thus, the delphinidin nucleus is stacked with C2, with the two aromatic rings parallel.

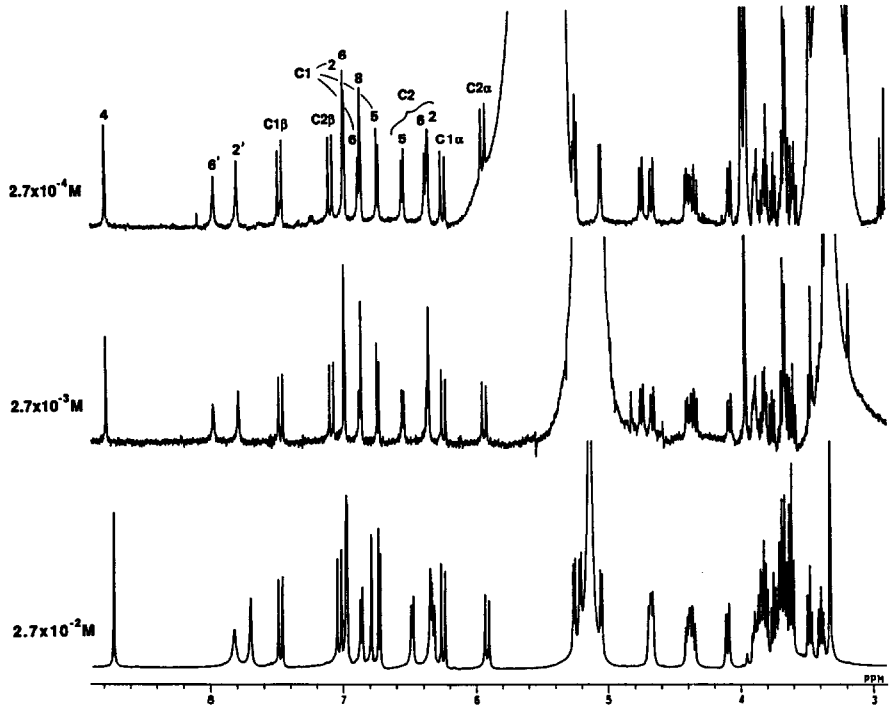


Fig. 5. ^{1}H NMR of gentiodelphin (**1**) at various concentrations in 10% TFA*d*-CD_3OD at 25°C (500 MHz).

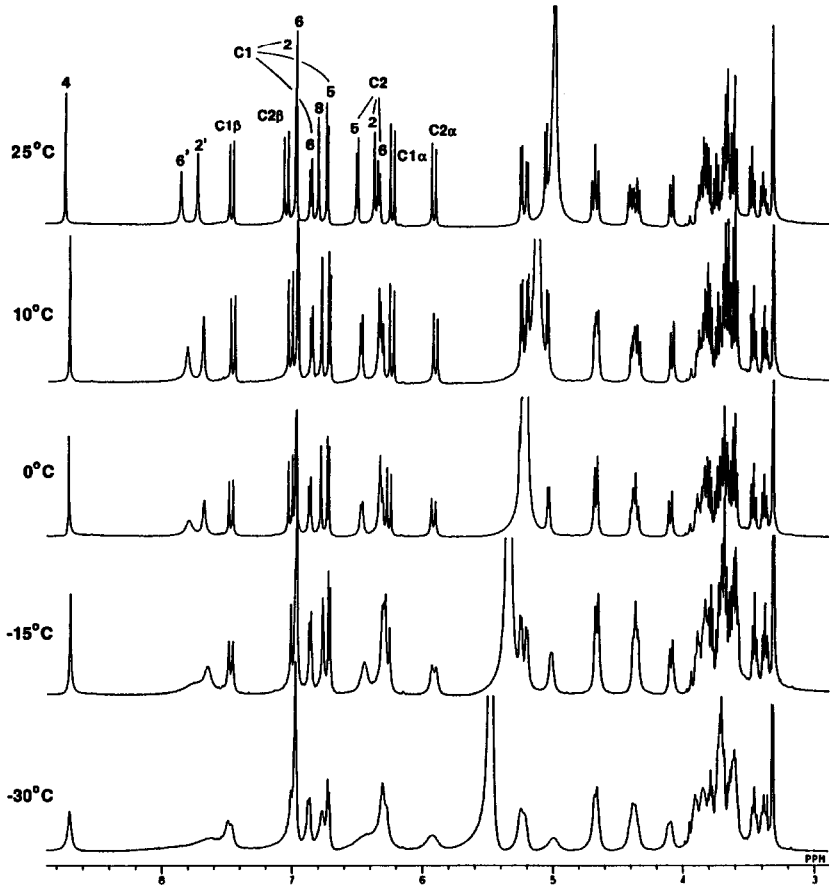


Fig. 6. ^{1}H NMR of gentiodelphin (**1**, 2.7x10^{-2}M) at various temperatures in 10% TFA*d*-CD$_3$OD (500 MHz).

The temperature dependent change of the ^{1}H NMR spectra of **1** are shown in Fig. 6. The broadening of the line width of the C2 signals is more apparent than that of the signals of C1 at lower temperature. For example, at 0°C the signals of α, 2, 5 and 6 of C2 became broad, whereas, the signals of C1 were still sharp at that temperature. Since the line-broadening of signals is closely correlated to molecular movement, these broadenings may suggest that C2 is stacked more rigidly than C1.

Exo cyclic C5-C6 bonds of hexapyranoses are expected to exist *gg*, *gt* and *tg* rotamers (Fig. 7). The ratio of each rotamer can be estimated by calculation from their coupling constants, $J_{5,\,6a}$ and $J_{5,\,6b}$.[38] We estimated the ratio of *gg*, *gt* and *tg* rotamers of glucose ▲, ● and ■ in **1** according to the equation of Ohrui *et al.*[39, 40]

(Table 4). In glucose ▲ and ● the ratio of the *gg* and *gt* rotamers is ca. 50:50, no *tg*, but that of glucose ■ was different and the *gt* rotamer was predominant (82%). When C2 was removed, the ratio of the rotamers of glucose ■ was changed to *gg*: *gt* = ca.50:50, indicating that the conformation of the 6-position of glucose ■ should be related to the intramolecular stacking of C2.

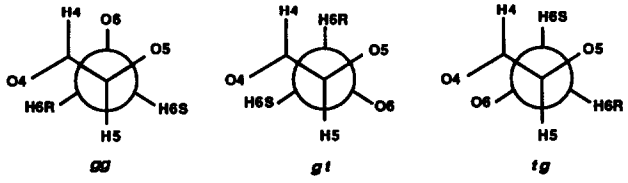


Fig. 7. *gg*, *gt* and *tg* rotamers of glucopyranoside.

Table 4. Ratio of *gg*, *gt* and *tg* Rotamers of Three Glucoses of **1** Calculated by the Modified Karplus Equation.

	Equation A			Equation B		
	% of each rotamer					
	gg	*gt*	*tg*	*gg*	*gt*	*tg*
glucose ▲	45	55	0	45	59	-4
glucose ●	49	51	0	49	54	-3
glucose ■	18	82	0	17	87	-4

Equation A : $J_{5,6proS} = 1.3gg + 2.7\,gt + 11.7\,tg$
$J_{5,6proR} = 1.33gg + 11.5gt + 5.8\,tg$
$gg + gt + tg = 1$

Equation B : $J_{5,6proS} = 2.2gg + 2.4\,gt + 11.1\,tg$
$J_{5,6proR} = 1.7gg + 10.8gt + 4.1\,tg$
$gg + gt + tg = 1$

Molecular modeling

Molecular modeling was carried out with QUANTA-CHARMm software[41-43] using an IRIS-4D workstation. At first, the conformation search for the three glucosidic linkages of **2** was done by rotating the torsional angles Φ, Ψ, Φ', Ψ', Φ" and Ψ". In the lowest energy conformation the plane of each glucose ring was nearly perpendicular to the anthocyanidin nucleus and the distances between each anomeric proton of glucose ▲, ● and ■, and the corresponding nucleus protons, H-4, H-6 and H-2', were ca. 2.5Å, which is consistent with the strong NOEs described above.

To the optimized geometry of **2** one caffeic acid, C1, was connected; then the conformation search was done by rotating the torsional angles T1, T2, T3 and T4 (Fig. 8) with distance constraints between the anomeic protons and the anthocyanidin ring protons where strong NOEs were observed relative to each other.

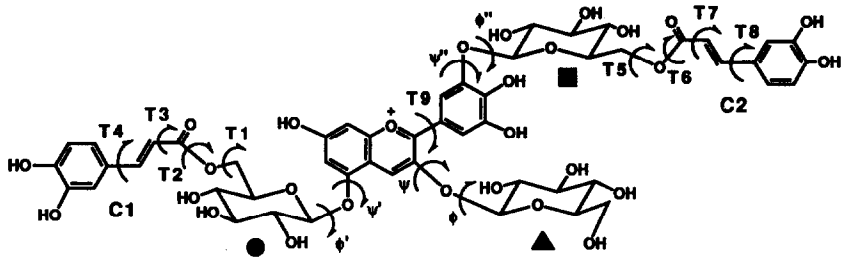


Fig. 8. Torsional bonds of **1** rotated during conformation search.

After several steps of minimization, the lowest energy conformation was selected and another caffeic acid, C2, was attached to the optimized geometry. The conformation search for **1** was done with the distance constraints between the protons of C2 and anthocyanidin nucleus, and between the anomeric protons and the anthocyanidin nucleus protons estimated by NOE experiments. During this conformation search, the dihedral angles of C5 -C6 bonds of glucose ▲ and ● were fixed as the *gg* conformation and that of glucose ■ was fixed as the *gt* conformation. A random sampling routine with rotation of the 4 torsions, Φ", Ψ", T5 and T6 was done being fixed the torsional angle of T7 and T8 at 0° or 180° each other, then several steps of minimization were performed. Finally the optimized conformation was minimized taking off all the constraints.

The optimized conformation was shown in Fig. 9. The distances between the anomeric protons of glucose ▲, ● and ■, and H-4, H-6 and H-2', were 2.94 Å, 2.29 Å and 2.09 Å, respectively. Ring A and ring B were not on the same plane but have about 21° of torsion. C2 was stacked to the delphinidin nucleus from the back of the A-B ring co-plane in Fig. 9 and both aromatic rings of the anthocyanidin nucleus and C2 were close to each other at nearly van der Waal's distance. In this conformation, the distance between C2-α and H-2' was 4.77 Å, C2-β and H-8; 3.93 Å, C2-2 and H-4; 3.42 Å, C2-5 and H-6'; 4.06 Å, C2-6 and H-4; 5.07 Å, respectively. On the other hand, C1 was spread and had no interaction with the nucleus.

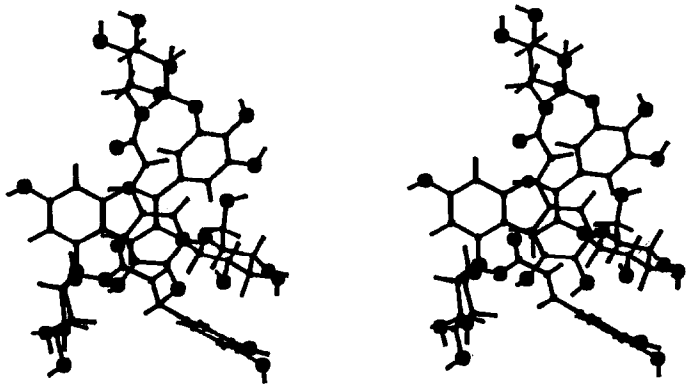


Fig. 9. Stereostructure of the optimized conformation of gentiodelphin (**1**).
● : oxygen atom.

CONCLUSION

We performed conformational analysis of gentiodelphin in acidic methanol, because of its poor solubility in neutral aqueous solutions. By ^1H NMR studies and computer-assisted molecular modeling of **1**, one of the two caffeic acid residues, C2, was certainly stacked to the anthocyanidin nucleus. It may be considered that hydrophobic interaction between the anthocyanidin nucleus and the aromatic acid stabilizes the stacking conformation.

The stability of gentiodelphin in neutral aqueous solutions is caused by the intramolecular caffeic acids. In our previous studies on polyacylated anthocyanins, at least two intramolecular cinnamic acid derivatives were necessary for stability in diluted aqueous neutral solution.[15,16] Under neutral conditions, the two aromatic acids of gentiodelphin, C1 and C2, may stack to both sides of the delphinidin nucleus preventing the hydration.[44]

EXPERIMENTAL

General procedures.

UV-VIS data were recorded on a Hitachi UV-228 spectrometer. ^1H NMR (500 MHz) spectra were obtained on a JEOL GX500 spectrometer in a 5 mm ϕ tube at variable temperature using 10% TFA*d*-CD_3OD or 30% TFA*d*-DMSO*d*6 as solvents. Chemical shifts were recorded as parts per million (ppm) and the signel of CD_2HOD was set at 3.325 ppm as a standard. FABMS data were recorded on a JEOL DX-300/DA5000 system and JEOL DX304/DA5000 system, using a 1N HCl-glycerol matrix. HPLC was carried out using a JASCO Trirotar III equipped with a UVIDEC 100-III detector (detected at 280 nm and 530 nm). Analytical HPLC was carried out using an ODS-column (Develosil ODS-5, Nomura Chemical) which was eluted with a 40% *A* solution (diluted with H_2O; *A* solution was a mixture of AcOH-CH_3CN-H_2O=20:25:55) containing 3% H_3PO_4. For preparative HPLC, an ODS glass column (24 mm ϕ x 250 mm, Develosil ODS-lop, Nomura Chemical) and an ODS stainless steel column (20 mm ϕ X 250 mm, Develosil ODS-5, Nomura Chemical) were used with various concentrated *A* solutions containing 3% H_3PO_4 as an eluent. For replacing the counter anion of anthocyanin from PO_4^{3-} to CF_3COO^- ion, the eluent was diluted four to five times with H_2O, adsorbed on an ODS-column (24 mm ϕ x 100 mm), and washed the column with 0.5% aq. TFA, and then eluted with *A* solution containing 0.5% TFA.

Isolation of pigments.

Fresh petals (2.4 Kg) of *Gentiana makinoi* were frozen with liq. nitrogen and pulverized with a blender. To the obtained powder 20% aq. MeOH (6.5 L) containing trifluoroacetic acid (130 ml) was added with stirring and was left to stand at ambient temperature for 20 h. By continuous centrifugation, the extract was isolated, and the residue was twice extracted with 2% TFA-MeOH (3L) for 3h. The combined extract was filtered and concentrated to 2 L under diminished pressure below 40 °C. The concentrate was poured into an Amberlite XAD-7 column (70 mm ϕ x 600 mm), and the column was stepwise eluted from H_2O to 80% aq. MeOH containing 1% TFA. The 60-80% MeOH fraction contained the major pigment. The fraction was evaporated, dissolved into 3% aq. H_3PO_4, and then the solution was chromatographed on a preparative medium pressure ODS-HPLC (glass column) which was eluted from H_2O to 45% aq. *A* solution containing 3% H_3PO_4. To replace the counter anion

from PO_4^{3-} to volatile CF_3COO^-, the dark red-color fraction was diluted with a 5-fold volume of H_2O and adsorbed on a short ODS column, which was washed with 0.5% aq. TFA, then eluted out with an *A* solution containing 0.5% TFA. The solution was dried and further purified by preparative ODS-HPLC (stainless steel column) with 43% aq. *A*-solution containing 3% H_3PO_4 as an eluent. After replacement of the counter anion, the pure pigment as a dark-red amorphous TFA salt was obtained (260 mg).

Alkaline hydrolysis of 1

1 (90 mg) was dissolved in 2.5 ml of MeOH, bubbled with argon gas, then cooled to 0° C. To the solution 2.5 ml of 1.0N NaOH was added and the mixture was allowed to stand at 0° C under argon atmosphere for 1h. To the reaction mixture 6N HCl aq. was added at 0°C, then the solution was warmed to room temperature. The mixture was evaporated and diluted five times with H_2O, then adsorbed on preparative medium pressure ODS-HPLC. The column was eluted from 3% H_3PO_4 aq. to 45% aq. *A*-solution containing 3% H_3PO_4 and the counter anion replaced; then **2** was obtained in a 46 mg (69%) yield as TFA salts.

Molecular modeling

Conformational analysis was done on an IRIS 4D/25G workstation (Silicon Graphics) using a molecular modeling package, QUANTA 3.2/CHARMm 21 with constant dielectric; $\varepsilon=1$.[41] Dihedral constraints; $E=\Sigma K(\phi_i-\phi_{i0})^2$, and NOE constraints; $E=K_1(r-r_0)^2$ for $r<r_0$ ($K_1=10K_bT/2(rlow)^2$, K_b; Boltzman constant, T; absolute temperature) and $E=K_2(r-r_0)^2$ for $r>r_0$ ($K_1=10K_bT/2(rhigh)^2$) were considered during constrained minimization.[41-43] Conformation search for **2** was done using a grid scan routine with rotating the torsional angles, Φ, Ψ, Φ', Ψ', Φ" and Ψ". Each torsional angles were rotated with 30° increments and the each conformer was minimized by 100 steps of steepest descent minimization. During the conformational search and the following minimization, the distance constraints (2.5 ± 0.5 Å) between 1H of glucose ▲ and H-4, 1H og glucose ● and H-6, and1H of glucose ■ and H-2' was on. To the optimized geometry of **2** C1 was connected to the 6 position of glucose . The grid search routine was carried out by rotating the torsional angles, T1 and T2, with 30° increments and the torsional angles, T3 and T4, with 180° increments. During the conformational search and the following minimization the distance constraints between the each anomeric proton and the each proton of the anthocyanidin nucleus was also on and the the dihedral angles of C5-C6 bonds of glucose ▲, ● and ■ were fixed as the *gg* conformation. Each conformer was minimized by the same method described above, and to the lowest energy conformer one more caffeic acid, C2, was connected to the 6 position of glucose ■ . Four conformers, obtained by setting the torsional angles T7 and T8 at 0° or 180° (transiod and cisoid form), were selected as starting conformer for molecular modeling. A random sampling routine for the conformation search was carried out by changing the torsional angles Φ", Ψ", T5 and T6 by 30° each within 360°. During the conformation search and the energy minimization, the distance constraints on basis of the distance (from 3.5 to 5 Å) derived from the value of the long distance NOEs (Fig. 3), and the dihedral constraints by fixing the geometry around the C5-C6 bond of glucose ▲ and ● to *gg* -rotamer and glucose ■ to *gt* were applied to the calculation. Starting from the each conformer, 2000 conformers were generated, and then minimized with the method described above. From 8000 conformers ca. 1200 lower energy conformers was selected and each conformers were minimized by 200 steps of steepest descent minimization, followed by 200 steps of conjugate gradient minimization with dihedral and distance constraints. Finally several of the optimized conformers were selected

and each conformer was minimized using Newton Raphson minimization without any constraints. The optimized conformer was shown in Fig. 9.

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