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FORMATION OF AN ALUMINUM COMPLEX OF 5-*O*-CAFFEOYLQUINIC ACID WITH CHIRAL MOLECULAR STACKING UNDER VACUOLAR CONDITION

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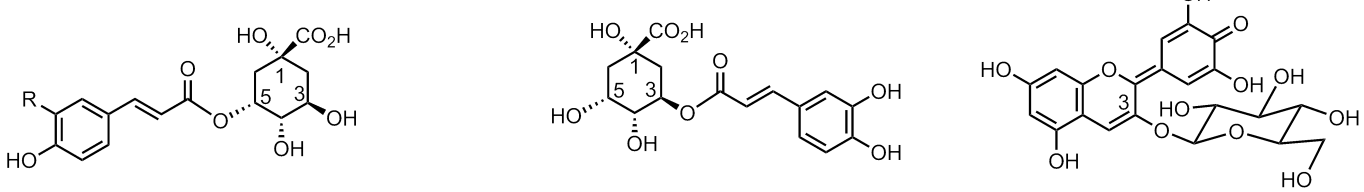
Abstract – 5-*O*-Caffeoylquinic acid (neochlorogenic acid, 5CQ) is an essential co-pigment for blue coloration of hydrangea. 5CQ solubilizes a water insoluble aluminum complex of 3-*O*-glucosyldelphinidin (Dp3G) to give a water-soluble blue complex, in which both Dp3G and 5CQ chelate a single Al^{3+} ion. However, its isomer, 3-*O*-caffeoylquinic acid (chlorogenic acid, 3CQ), has no such effect and does not give blue complex. It was clarified that 5CQ was complexed with Al^{3+} with a chiral molecular stacking under physiological vacuolar condition as aq. solution at pH 4.0. However, 3CQ did not give such complex with Al^{3+}. The difference in aluminum complexation between the isomers may result from the difference configuration of 5-OH and 3-OH group in quinic acid.

Dedicated to Professor Tohru Fukuyama on his 70th birthday

Quinic acids are common phenolic compounds in plants. Many acylquinic acids in which 3, 4 and/or 5-OH are esterified with various acyl moieties have been reported, including caffeoyl, *p*-coumaroyl, and feruloyl.[1-3] Among them 5-*O*-caffeoylquinic acid (neochlorogenic acid, 5CQ, **1**), 5-*O*-*p*-coumaroylquinic acid (5pCQ, **2**), and 3-*O*-caffeoylquinic acid (chlorogenic acid, 3CQ, **3**) are abundant in the sepals and leaves of *Hydrangea macrophylla* (Scheme 1).[4-7] These acylquinic acids (**1**-**3**) are known to be co-pigments in hydrangea sepals and play an important role in color development.[7-14] Notably, 5-*O*-acylquinic acids, **1** and **2**, are essential for blue color development in hydrangea sepals.[8-14] In addition, these acylquinic acids are thought to be important for aluminum detoxification through the formation of aluminum complexes in vacuoles.[15-17]

We have studied the mechanisms of blue coloration of hydrangea and carried out reproducing experiments by mixing natural and synthetic acylquinic acids with 3-*O*-glucosyldelphinidin (**4**) and aluminum ion (Al^{3+}). Through chemical analyses of the resulting blue complexes,[9-14] we determined the composition of the complex to be 1 : 1 : 1 of **1** or **2**, Al^{3+} and **4**.[13,14] However, the behavior of 3CQ (**3**), which is a structural isomer of **1**, was much different: **3** has no blueing effect on **4**-Al^{3+} complex. We proposed that the configurational differences between **1** and **3** may give rise to this distinct difference in co-pigmentation effect. To characterize the differences between **1** and **3** involved in the development of the blue color in sepals and the aluminum detoxification in hydrangea, we investigated their aluminum complexation in the absence of anthocyanin. Here we report the spectroscopic analysis of mixture of **1**-**3** with Al^{3+} in buffered solutions at pH 4 to mimic the conditions within blue sepal cell vacuoles.[9,12] The addition of 1 eq. of Al^{3+} to **1** produced a pale-yellow complex with chiral molecular stacking, whereas the addition to **2** and **3** did not result in complexes with Al^{3+}.



Scheme 1. Structures of acylquinic acids and anthocyanin found in sepals of *Hydrangea macrophylla*

To investigate the formation of the aluminum complexes of 5CQ (**1**), **1** (0.05 to 5 mM in aq. buffer solution at pH 4.0) was mixed with 0 – 50 eq. of Al^{3+}, then, UV and circular dichroism (CD) were measured (Figure 1). Initially, we experimented using 5 mM of **1**, which was close to the concentrations within sepal vacuoles (Figure 1, A).[9,12] Without Al^{3+}, **1** showed a UV spectrum with λmax at 326 nm and by addition of Al^{3+} to **1** λmax shifted toward longer wavelength to 329 nm (0.5 eq. to **1**), 344 nm (1.0 eq. to **1**), and 350 nm (2.0 eq. to **1**). The color of the solution became pale-yellow with the existence of Al^{3+}. The isosbestic point occurred at approximately 330 nm, indicating that complexed **1** and Al^{3+} have a linear stoichiometric relationship. No Cotton effect was observed for CD spectra of **1** without Al^{3+}, whereas addition of Al^{3+} gave an exciton-type positive Cotton effect (Figure 1, A), which indicates that the caffeoyl residues were stacked in a clockwise manner in the aluminum complex.

Similar phenomena were observed for **1** at 0.5 mM (Figure 1, B). In the absence of Al^{3+}, a λmax at 324 nm was observed and by addition of Al^{3+} to **1** λmax shifted to 350 nm (0.5 eq. to **1**), 350 nm (1.0 eq. to **1**) and 352 nm (2.0 eq. to **1**), respectively. A positive Cotton effect was also observed following the addition

of Al^{3+} to 0.5 mM **1**. However, at 0.05 mM **1**, the addition of 1-2 eq. of Al^{3+} did not produce an aluminum complex, and neither shift in λ_{max} nor exciton-type Cotton effect was observed. Conversely, the addition of 5 eq. of Al^{3+} resulted in a shift of λ_{max} with 6 nm, and the addition of 50 eq. of Al^{3+} resulted in a pale-yellow solution with λ_{max} = 350 nm and a positive Cotton effect in the CD spectra (Figure 1, C). The intensities of the Cotton effect for the solutions of 0.05 mM **1** with 50 eq. Al^{3+} added was lower than those of **1** at 5 and 0.5 mM with 1 eq. of Al^{3+} added; however, some aluminum complexes may have been present. Such a concentration effect in molecular association was observed in the case of self-association of flavocommelin.[18] At the concentrations of 5 mM and 0.5 mM, flavocommelin showed exciton-type negative Cotton effect due to the chiral molecular stacking of the chromophores with anti-clockwise manner, but at the concentration of 0.05 mM no Cotton effect was observed indicating that self-association disappeared. It is generally assumed that at 0.05 mM each molecule in the solution behaves as a monomer without any intermolecular interactions,[18,19] but intermolecular stacking was observed in very special case as alatanin C with 0.05 mM in aqueous buffer at pH 5.0.[20] The results of the present study were consistent with those of previous reports, in stating that a mixture of **1** and Al^{3+} in aqueous buffers that are at pH 4.0 forms a complex with chiral stacking at equilibrium, even for very low concentrations, provided the concentration of Al^{3+} is sufficiently high.

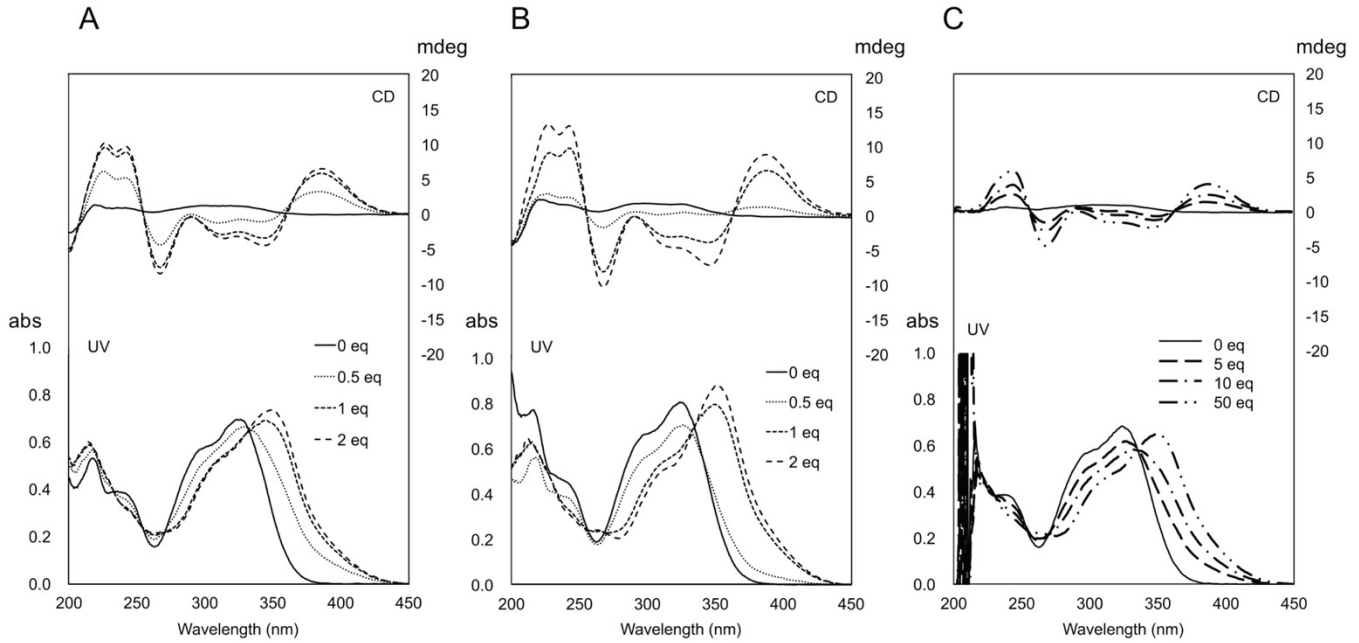


Figure 1. UV spectra and CD of 5CQ (**1**) with Al^{3+} (0 – 50 eq. to **1**) in aq. buffer solution at pH 4.0. (A): **1**: 5 mM, cell length: 0.1 mm, (B): **1**: 0.5 mM, cell length: 1.0 mm, (C): **1**: 0.05 mM, cell length: 10 mm.

Next, we investigated the aluminum complexation of 5-*O*-*p*-coumaroylquinic acid (5pCQ, **2**) and 3-*O*-caffeoylquinic acid (3CQ, **3**) at 5 mM (Figure 2, A, B). In contrast to 5CQ (**1**), **2** and **3** did not form

aluminum complexes with chiral molecular association under the same conditions. The solution of **2** showed no changes in UV or CD spectra following the addition of 2 eq. of Al^{3+} (Figure 2, A). For **3**, a very small increase in absorbance at approximately 360–420 nm, but no change in λ_{max} was observed; 326 nm (Al^{3+}: 0 eq.) and 325 nm (Al^{3+}: 2 eq.). In CD a very small Cotton effect was observed around 270-370 nm when 2 eq. of Al^{3+} was added, but the intensity was less than 1 mdeg. These results indicate that **2** does not form an Al^{3+} complex, whereas **3** may form an Al^{3+} complex without any chiral molecular associations between chromophores. Based on these findings, we suspect that Al^{3+} might complex with the catechol moiety of the caffeoyl residues of **1** and **3**. However, the structural differences between **1** and **3** produce differences in the aluminum complex structure, which account for the different colors of the solutions of these complexes.

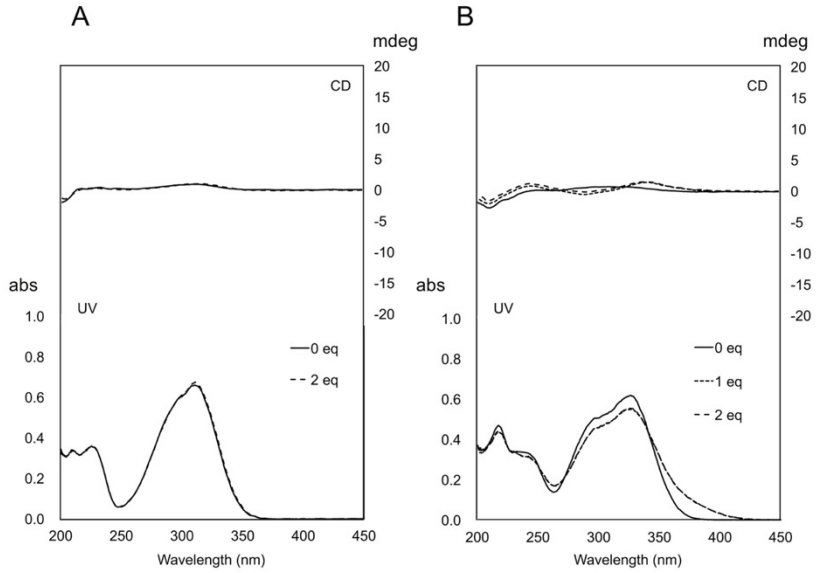


Figure 2. UV spectra and CD of 5pCQ (**2**) and 3CQ (**3**) with Al^{3+} (0 – 2 eq. to **1**) in buffer solution at pH 4.0. (A): **2**: 5 mM, cell length: 0.1 mm, (B): **3**: 5 mM, cell length: 0.1 mm.

To clarify the conformation of **1** under physiological conditions, ^{1}H NMR spectra were measured in D_2O at pD 4.0 (Table 1, Figure 3, A). The coupling constants of the ring protons of the quinic acid residue of **1** were as follows: $J_{3,4}$ = 9.5 Hz and $J_{4,5}$ = 3.5 Hz, indicating that **1** is in a chair conformation with equatorial 3-OH and 4-OH groups and axial 5-OR in weakly acidic D_2O.

Next, ^{1}H NMR spectra of **1** with Al^{3+} in D_2O at pD 4.0 were measured (Figure 3, B, C). By addition of 0.5 eq. of Al^{3+} to **1** the spectra became more complex and the signals at the higher field around 2 ppm and the lower field below 5.5 ppm were changed. With 1.0 eq. of Al^{3+}, the spectra converged. Using COSY and other 2D NMR measurements, all the signals were assigned (Table 1). It was deduced that the spectrum 2B (**1** with 0.5 eq. of Al^{3+}) was in fact a 1:1 mixture of 2A (**1**) and 2C (**1** with 1.0 eq. of Al^{3+}). This

indicates that **1** formed a 1:1 complex with Al^{3+}, and addition of a half equivalent of Al^{3+} to **1** gave a mixture with a half amount of a specific aluminum complex of **1** and remaining amount existed as free **1**. In other words, the formation of aluminum complex of **1** may be very specific and strong, therefore, the complex forms as much as the Al^{3+} content. This result is consistent with the results of our UV and CD data (Figure 1, A). In the aluminum complex of **1**, the signals attributable to the caffeoyl residue are shifted upfield by 0.47–0.81 ppm (Table 1), indicating the existence of molecular stacking between caffeoyl residues. However, almost no changes in chemical shift were observed for the signals attributed to the 3-H and 4-H of the quinic acid moiety. Methylene protons of at the 2 and 6-positions of the quinic acid were shifted downfield.

The ^{1}H NMR spectra of **3** showed that the quinic acid residue of this compound was also in a chair conformation with the 3-*O*-acyl group in the equatorial position. Unlike the spectra for **1**, no changes were observed in the ^{1}H NMR spectra of **3** in the presence of Al^{3+}, which is also consisted with our UV and CD spectra (2 eq. to **3**). The ^{1}H NMR spectrum of **2** also gave the similar results of **3**; no change was observed by addition of 2 eq. of Al^{3+}. These results were consisting with the results of UV and CD.

Table 1. Assignment of ^{1}H NMR spectra of **1** and **1** with Al^{3+} (D_2O, pD: 4.0, 25 °C)

		1			**1** + Al^{3+} (1eq)			*Δ* (ppm)*
		δ (ppm)		*J* (Hz)	δ (ppm)		*J* (Hz)	
qunic acid	2 ax	2.01	dd	16.5, 9.5	2.21	dd	16.0, 3.0	-0.20
	2 eq	2.21	dd	16.5, 3.5	2.44	m**		-0.23
	3	4.23	td	9.5, 3.5	4.21	m**		0.02
	4	3.81	dd	9.5, 3.5	3.75	dd	9.5, 4.0	0.06
	5	5.45	brq	3.5	5.31	brd	4.0	0.14
	6 ax	2.19	dd	16.5, 3.5	2.08	dd	16.0, 3.5	0.11
	6 eq	2.31	dd	16.5, 3.5	2.62	brd	16.0	-0.31
caffeoyl	α	6.48	d	16.0	5.67	d	16.0	0.81
	β	7.70	d	16.0	7.06	d	16.0	0.64
	2'	7.26	d	1.5	6.79	brs		0.47
	5'	7.00	dd	8.5, 1.5	6.45	d	8.5	0.55
	6'	7.19	d	8.5, 1.5	6.69	brd	8.5	0.50

*: Chemical shift difference compared with **1** without Al^{3+}.

**: higher oder splitting was observed.

In conclusion, we found that 5-*O*-caffeoylquinic acid (**1**) formed an aluminum complex in an aqueous buffer at pH 4.0, which reflects the physiological conditions within hydrangea sepal vacuoles. The complex is likely composed of a 1:1 ratio of **1** and Al^{3+} with clockwise chiral molecular stacking of the

caffeoyl residues. The position of Al^{3+} might be catechol moiety of caffeoyl residue and the stereo-structure of 5-*O*-axial bond of quinic acid should be essential to produce the complex, which structure might be proposed in Scheme 2. This molecular characteristic may play a role on the blue color development of hydrangea.

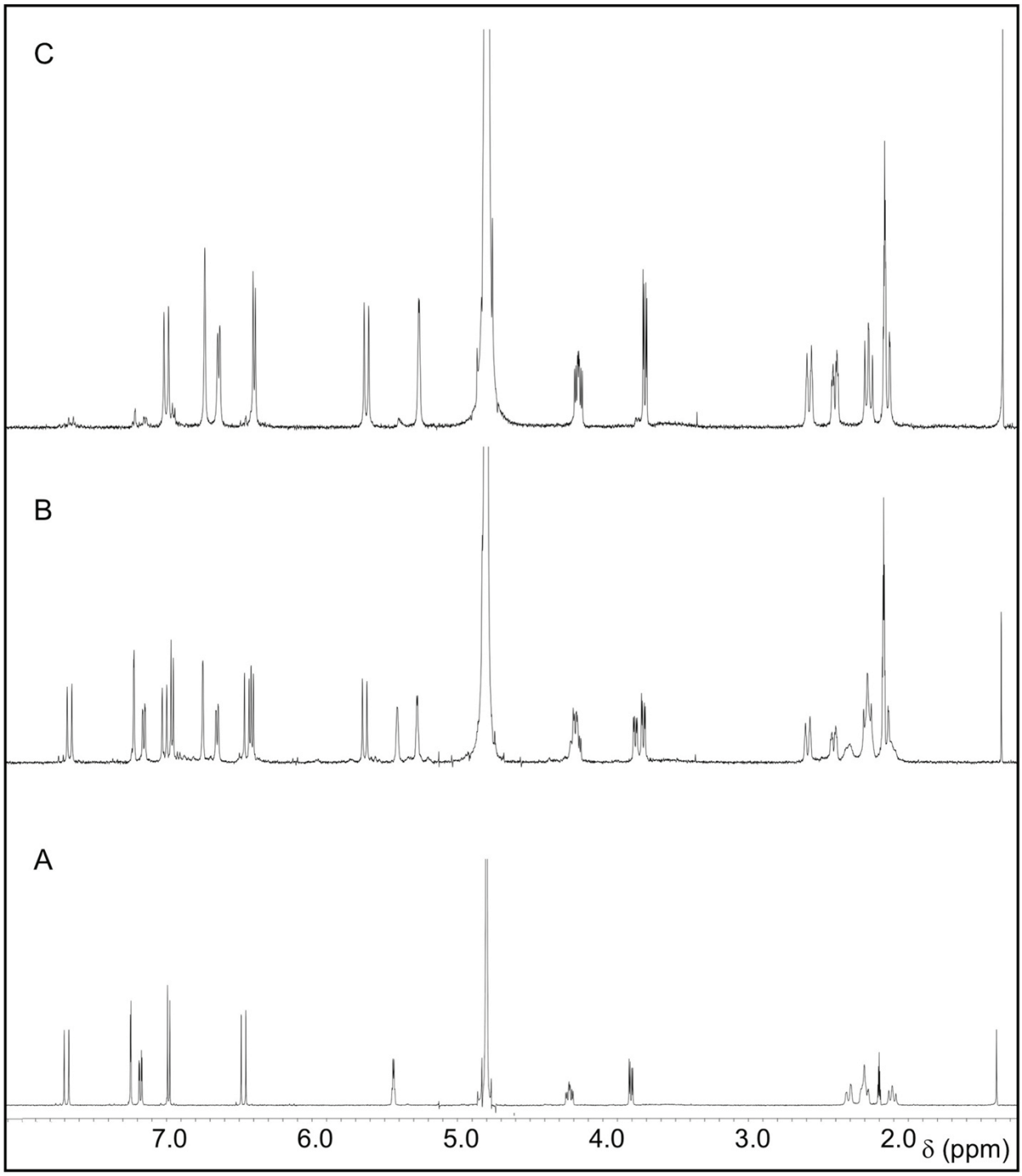
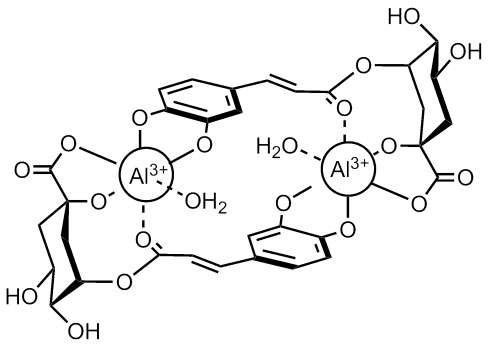


Figure 3. ^{1}H NMR spectra of **1** with Al^{3+} in D_2O at pD 4.0 (^{1}H: 500 MHz, 25 °C). (A): Al^{3+}: 0 eq., (B): Al^{3+}: 0.5 eq., (C): Al^{3+}: 1 eq.



Scheme 2. Proposed structure of the aluminum complex of **1**

EXPERIMENTAL

General

UV spectra were recorded on a JASCO V-560 spectrometer. CD was recorded on a JASCO J-720 WN apparatus. NMR spectra were obtained with a JEOL ECA-500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) instrument in a 5-mm i.d. tube at variable temperature using D_2O as a solvent. Chemical shifts for ^{1}H NMR were reported on a scale relative to the signal of *t*-BuOH (δ 1.29 ppm) and the coupling constant was expressed in Hz. Measurement of pH was carried out with a D-21 pH meter (HORIBA, Japan) with a LAQUA 9618 electrode. The adjustment of the electrode was carried out using the standard buffer of pH 7 and 4 and the value of pD in deuterated buffer solution was noted as addition of 0.4 to the recorded value.

Chemicals

5-*O*-Caffeoylquinic acid (**1**) and 5-*O*-*p*-coumaroylquinic acid (**2**) were synthesized according to our procedures.[21] 3-*O*-Caffeoylquinic acid (**3**) was purchased from Tokyo Chemical Industry Co., Ltd., Japan. $AlNH_4(SO_4)_2$•$12H_2O$ was purchased From NACALAI TESQUE, INC., Japan. All commercially available reagents and solvents were used directly without further purification.

Preparation of aluminum complex of acylquinic acid for measurement of UV and CD

To a buffer solution (100 mM AcOH-AcONa, pH 4.0) was added MeOH solution of acylquinic acid at the final concentration of acylquinic acid to be 5 mM, 0.5 mM, and 0.05 mM, and aq. solution of $AlNH_4(SO_4)_2$ was added as the equivalent to the co-pigments as indicated. UV spectrum and CD were recorded in a quartz cell (path length: 0.1 mm, 1.0 mm and 10 mm, respectively) at 25 °C.

Preparation of aluminum complex of acylquinic acid for measurement of NMR

5CQ (**1**, 5 mM) was dissolved in D_2O and added Al^{3+}, then the pD of the solution was adjusted by addition of NaOD and CD_3CO_2D to be 4.0.

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