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Essential structure of co-pigment for blue sepal-color development of hydrangea

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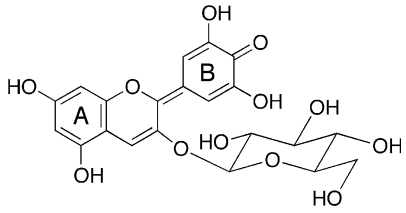
Abstract—Blue sepal-color of *Hydrangea macrophylla* might be due to a supramolecular metal-complex pigment consisting of delphinidin 3-glucoside (**1**), co-pigments (5-*O*-caffeoylquinic acid (**2**), and/or 5-*O*-*p*-coumaroylquinic acid (**3**)) and Al^{3+} in an aqueous solution around pH 4.0. To clarify the mechanism of blue sepal-color development of hydrangea, we tried to reproduce the blue color in vitro by mixing **1** with designed synthetic co-pigments in the presence of Al^{3+} at pH 4.0. We at first succeeded in clarifying the essential functional structure in the co-pigment that could form the stable blue solution. Here, we present the structure of the blue pigment caused by an Al-complex coordinating of **1** at *ortho*-dihydroxyl groups of the B-ring, 1-hydroxy, 1-carboxylic acid, and the carbonyl residue in the ester at 5-position of **2** and/or **3**. The hydrophobic interaction between the aromatic acyl residue at 5-position and the nucleus of **1** may also contribute to stabilize the complex.

Most blue, purple, and red flower colors are due to anthocyanins.[1] Generally, the chromophores of blue and red flower pigments are different; delphinidin nucleus gives a blue color and pelargonidin chromophore provides red petals.[1,2] However, hydrangea sepals, which are red, mauve, purple, violet, or blue have only one anthocyanin, delphinidin 3-*O*-glucoside (Scheme 1, **1**). Therefore, the mechanism of sepal color variation has long been attracting interest.[3–6] In the early 20th century, a correlation between blue coloration and the aluminum content of soils and sepals was clarified.[3] In the middle of the century, the structures of anthocyanin and co-pigments were determined,[4] and in the last two decades blue solution was obtained by mixing **1** (Scheme 1), Al^{3+}, and 5-acylated quinic acids (Table 1, **2** and **3**) as a co-pigment.[5] In 2003, we reported that the second layers of the sepal tissue are colored and the pigments were dissolved in the vacuoles as a clear solution.[7] We also revealed that the vacuolar pH of the blue cells is 4.0 being higher than that of red cells.[7] Nevertheless, the chemical structure of the blue pigment in hydrangea sepals is unclear. We have studied the mechanism of blue color development of the hydrangea. To obtain the structural information, we synthesized various designed co-pigments and carried out reproduction experiments of blue color by mixing them with **1** and Al^{3+} in vitro. Here, we report the essential functional structure in the co-pigment for blue color development and discuss the proposed structure of the blue pigment responsible for the blue sepal-color of the hydrangea.



delphinidin 3-*O*-glucoside (**1**)

Scheme 1.

Compound **1** was isolated from the seed coat of *Phaseolus coccineus*.[8] The following co-pigments were designed to examine which functional structure is essential for the formation of the blue pigment (Table 1, Fig. 1): (a) the linkage position of acyl moiety (**4**), (b) the number of the phenolic hydroxyl group in acyl

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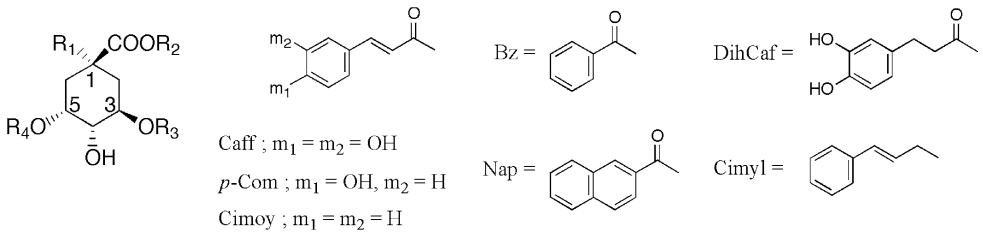
Table 1. Structure of co-pigment and blue color reproduction by mixing with **1** and Al^{3+} at pH 4.0



	2	3	4	7	8	9	10	11	12	13	14
R_1	OH	OH	OH	OH	OH	OH	OH	OCH_3	H	OH	OH
R_2	H	H	H	H	H	H	H	H	H	CH_3	H
R_3	H	H	Caff	H	H	H	H	H	H	H	H
R_4	Caff	*p*-Com	H	Cimoy	Bz	Nap	DihCaf	Cimoy	Cimoy	Caff	Cimyl
Blue color reproduction	○	○	×	○	△	◎	△	×	×	×	×

(◎): very stable blue solution; (○): stable blue solution; (△): blue solution first, after ppt appeared; (×): ppt appeared immediately.

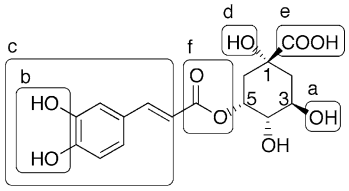
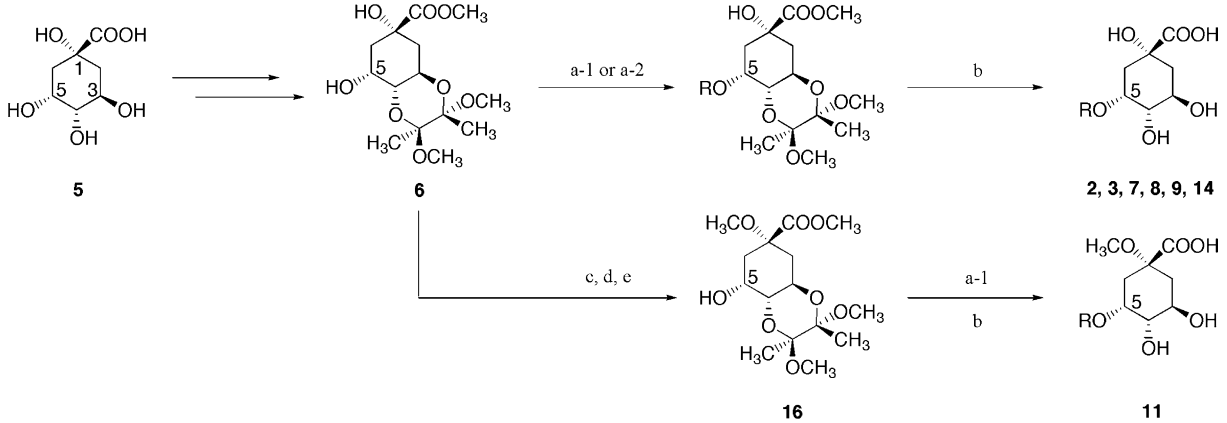


Figure 1. Modified functional structure in co-pigment for reproduction experiment.

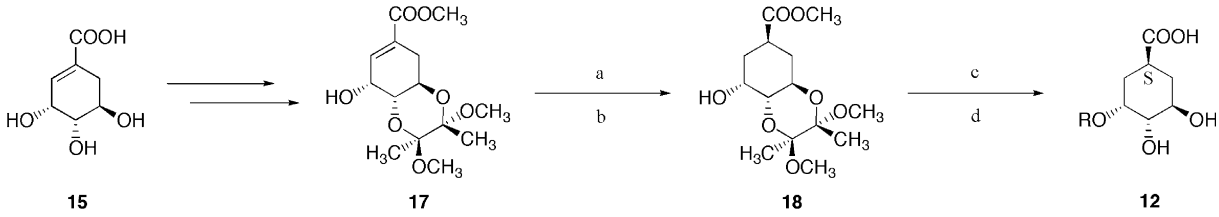
moiety (**2**, **3**, and **7**), (c) difference of aromatic part of 5-acyl moiety (**8**–**10**), (d) 1-OH of quinic acid (**11** and **12**), (e) 1-carboxyl acid of quinic acid (**13**), and (f) 5-*O*-ester residue (**14**). All co-pigments except **4**[9] were synthesized from quinic acid (**5**) and sikimic acid (**15**). 3,4-Dihydroxyl group of **5** was protected according to the procedure of Montchamp et al.[10] to give **6**. 5-OH of **6** was acylated by various acid chlorides, then deprotected in 2 N HCl to give **2**,[11,12] **3**, and **7**–**9** (Scheme 2).[13] Compound **10** was prepared by hydrogenation of **2**.[14] 5-*O*-Cinnamyl derivative, **14**, was obtained from **6** by reaction with cinnamyl bromide using sodium hydride (NaH) followed by hydrolysis (Scheme 2). Compound **11** was obtained by the same acylation reaction after methylation of **6**.[15] Compound **12** was synthesized from sikimic acid (Scheme 3).[16] Selective methylation of **2** by treatment with CH_2N_2 afforded **13**.[17]

Using the prepared co-pigments, we carried out reproduction experiments of blue sepal-color. The direct pH measurement of the blue colored vacuole showed that the average pH value was 4.0.[7] Our preliminary analysis of colored protoplasts revealed that the concentration of **1** in colored cells is around 1 mM and the ratio of co-pigment, **2**–**4** to **1** was 1–5 equiv. The content of Al^{3+} was varied depending on the sepal color[18] and the molar ratio of Al^{3+} to **1** in blue colored cells was ca. 1 equiv. As previously reported, we have already realized that **1** with **2** and Al^{3+} in aqueous solution at pH 4.0 gave blue solution.[7] The spectrum of blue sepals and blue protoplast showed a λ_{max} at 585–595 nm[7] and that of the solution mixing **1** (1 mM), **2** (3 mM), and Al^{3+} (1 mM) at pH 4.0 was identical to them (Fig. 2). Therefore, we determined the mixing condition of the reproduction of blue color to be **1**:co-pigment:Al^{3+} = 1 mM:3 mM:1 mM.[19]

First, we examined natural co-pigment, **2**–**4**. 5-*O*-Acyl quinic acid derivatives, **2**, gave a very stable blue solu-



Scheme 2. Reagents and conditions: (a-1) acyl chloride, pyridine, 0–40 °C, 2–5 h, 72–99%; (a-2) cinnamyl bromide, NaH, DMSO, rt, 0.5 h, 63%; (b) 2 N HCl, CH_3CN, 45–70 °C, 4–6.5 h, 15–48%; (c) TBDMS-Cl, imidazole, DMF, rt, 5 h, 96%; (d) CH_3I, NaH, DMF, 40 °C, 2 h, quant; (e) tetrabutyl ammonium fluoride, THF, 40 °C, 15 h, 30%.



Scheme 3. Reagents and conditions: (a) Pd–C, H_2, AcOEt–MeOH (1:2), rt, 13 h, 29%; (b) $NaOCH_3$, CH_2Cl_2–MeOH (1:4), 40 °C, 17 h, 32%; (c) cinnamoyl chloride, pyridine, 30 °C, 1.5 h, 40%; (d) 2 N HCl, CH_3CN, 60 °C, 6 h, 33%.

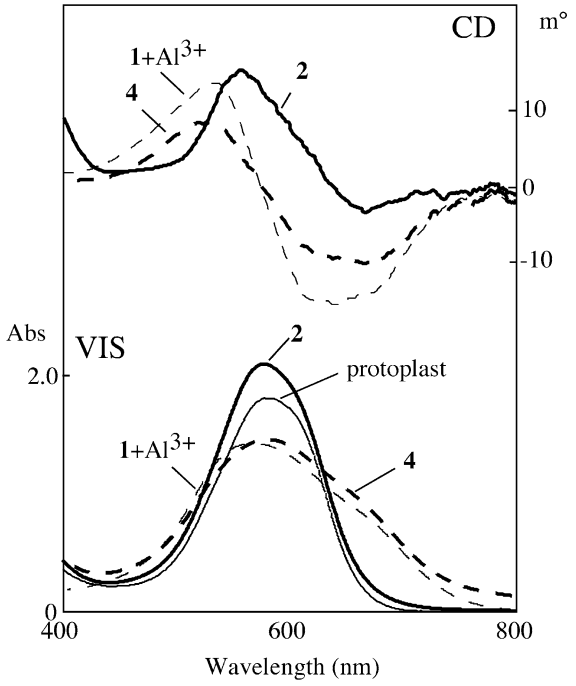


Figure 2. Vis spectra and CD of the blue *Hydrangea* protoplast and the reproduction solution just after mixing with **1**, with/without co-pigment and Al^{3+}. (——): protoplast, (– – – –): **1** and Al^{3+}, (——): **1**, **2**, and Al^{3+}, (- - -): **1**, **4**, and Al^{3+}.

tion, the same as **3**. However, 3-*O*-acyl quinic acid derivative, **4**, gave a bluish-purple colored solution first (Fig. 2), then quickly became colorless to give dark blue precipitate. The CD of the blue solution obtained by addition of **2** showed a single peak at 590 nm, while that of **4** gave a negative exiton-type Cotton effect around the λ_{max} indicating the self-association of the chromophores of **1**. Compound **3** showed the same CD as that of **2**. These results suggested that in the blue stable solution **1** and co-pigment may stack each other. The precipitate was composed of **1** and Al^{3+} and no **4** was detected.[20] Furthermore, the addition of Al^{3+} to the aqueous solution of **1** at pH 4.0 gave a precipitate whose vis spectrum and CD were similar to that of the mixture with **1**, **4**, and Al^{3+} (Fig. 2). These data strongly indicated that aluminum complex of **1** is hardly soluble in water. When **2** was added to the suspension of the dark blue precipitate, the same blue solution, which was obtained by mixing **1**, **2**, and Al^{3+}, formed again. Therefore, 5-*O*-acyl quinic acid derivative, **2**, should have a co-pigment effect on water-insoluble **1**–Al^{3+} complex to give a stable blue solution. **3** showed the same effect, while, the 3-*O*-acyl derivative, **4**, did not.

In order to obtain structural information of the blue metal-complex pigment composed of **1**, **2**, or **3**, and Al^{3+}, we measured the ^1H NMR spectra of the mixed solution in deuteriorized buffer. However, the obtained signals were very broad and we could not analyze the spectra.[21] So, we investigated the precipitate obtained by the addition of EtOH to the blue solution. The resulting precipitate was dark blue and composed of only **1** and Al^{3+} without any co-pigments being the same precipitate obtained by **1**, **4**, and Al^{3+} mixture.[20] Therefore, we concluded that the blue color of hydrangea could exist only in aqueous solution co-existing with co-pigments and Al^{3+}.

Finally, we carried out a reproduction experiment using a variety of the synthesized quinic acid derivatives with **1** and Al^{3+} at pH 4.0 (Table 1). 5-*O*-Cinnamoylquinic acid (**7**) gave the same stable blue solution as **2** and **3**, indicating that the number of the hydroxyl group at cinnamoyl residue does not contribute to the co-pigmentation effect. 5-*O*-Benzoyl (**8**) and 5-*O*-naphthoyl (**9**) derivatives also gave a blue solution, but the stability of the solutions was much different. Compound **9** showed the strongest stabilizing effect on blue color development and the effect of **8** was weaker than that of **2** and **3**. 5-*O*-Dihydrocaffeoylester (**10**) which has been broken conjugated system, gave a blue solution first, but the precipitates appeared gradually (Fig. 3). The CD of the blue solutions with **7** or **9** were much similar to that with **2** or **3**, while the solution with **8** or **10** showed a little exiton-type Cotton similar to that of **4**.[22] These results suggest that the large planner aromatic acyl residue at the 5-position may have an important co-pigmentation effect by hydrophobic interaction with the anthocyanidin nucleus. On the other hand, 1-*O*-methyl ether (**11**) and the 1-deoxy (**12**)[23] derivatives afforded a purple colored solution, not blue, which became colorless quickly to give dark blue precipitates. Methyl ester of 1-carboxyl (**13**) and 5-*O*-ether (**14**) derivatives showed the same behavior as **4** (Table 1).

These results reveal that the blue sepal-color of *H. macrophylla* is developed by the supramolecular pigment consisting of delphinidin 3-glucoside (**1**), 5-*O*-acyl quinic acid (**2** and/or **3**), and Al^{3+}. The 5-*O*-ester, the 1-OH and 1-carboxyl groups in the quinic acid part are essential to the co-pigmentation effect followed by constructing the water soluble blue metal-complex pigment. Here,

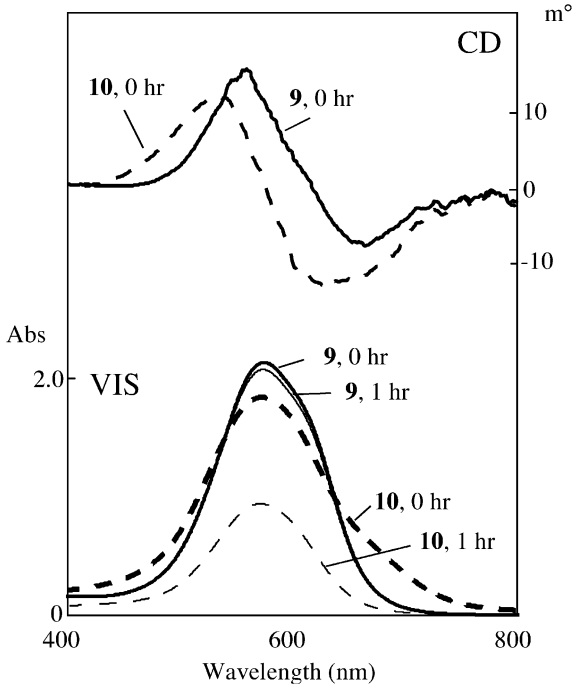


Figure 3. Vis spectra and CD of the solution mixed with **9** or **10** with **1** and Al^{3+} at pH 4.0. (——): **9**, 0 h, (– – –): **10**, 0 h, (——): **9**, 2 h, (– – –): **10**, 2 h.

we propose a structure of the blue pigment caused by an Al-complex coordinating of **1** at the *ortho*-dihydroxyl groups of the B-ring.[5c] 1-Carboxylate may also coordinate to the Al^{3+}. 1-OH and the 5-ester residue may be essential to the co-pigment effect by constructing hydrogen bond network. The hydrophobic interaction between the aromatic acyl residue at the 5-position of the co-pigment and the nucleus of **1** may also stabilize the complex. This newly elucidated metal complex-anthocyanin with non-flavonoid co-pigment exists in the vacuoles of hydrangea sepals developing a beautiful blue color. Further investigations are in progress.

Acknowledgments

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11. To a solution of **6** (1.33 g, 4.7 mmol) in pyridine (20 mL) was added a solution of 3′,4′-di-*O*-acetylcaffeoyl chloride (1.74 g, 6.17 mmol) in CH_2Cl_2 at 0 °C and warmed to 30 °C and stood for 3 h. The reaction mixture was added saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The extracts were purified by silica gel chromatography (hexane–AcOEt 1:1) to afford the corresponding acylated compound as a white powder (1.67 g, 3.01 mmol, 72%). The acylated compound (1.55 g, 2.73 mmol) was deprotected by treatment with 2 N HCl (3 mL) at 45 °C for 4 h. To the reaction mixture was added saturated aqueous $NaHCO_3$ and extracted with AcOEt, then the aqueous layer was acidified by addition of TFA (6 mL). Compound **2** was extracted with AcOEt (10 mL × 10) and the resulting crude product was purified by HPLC (Develsil ODS-HG-5, eluent: aqueous CH_3CN) to give **2**[12] (228 mg, 0.64 mmol, 23%); ^1H NMR (CD_3OD, 600 MHz) δ 1.98 (dd, $J = 11.0$, 14.0 Hz, 1H), 2.14 (m, 1H), 2.16 (m, 1H), 2.28 (dd, $J = 3.0$, 15.0 Hz, 1H), 3.81 (dd, $J = 3.0$, 9.0 Hz, 1H), 4.22 (ddd, $J = 4.0$, 9.0, 11.0 Hz, 1H), 5.44 (q, $J = 3.0$ Hz, 1H), 6.47 (d, $J = 16.0$ Hz, 1H), 6.99 (d, $J = 8.0$ Hz, 1H), 7.18 (d, $J = 8.0$ Hz, 1H), 7.24 (s, 1H), 7.69 (d, $J = 16.0$, 1H).
12. Compound **2** was synthesized by Sefkow et al. using the similar synthetic route Sefkow, M.; Kelling, A.; Schilde, U. *Eur. J. Org. Chem.* **2001**, *14*, 2735–2742.
13. During the deprotection reaction in aqueous acidic solution 5-*O*-acyl moiety was hydrolyzed simultaneously, therefore the reaction was low yield.
14. Hydrogenation of **2** with H_2/Pd–C gave **10** in quantitative yield.
15. 5-OH in **6** was selectively silylated with TBDMS-Cl–imidazole/DMF, then 1-OH was methylated with NaH–CH_3I/DMF. After desilylation with TBAF (**16**, 32%) the compound was acylated by treatment of cinnamoyl chloride, then deprotected to give **11** (11%).
16. Hydrogenation of sikimate derivative (**17**) with H_2/Pd–C (29%) gave an undesired diastereo-isomer at 1-$COOCH_3$ ($J_{1,2eq} = 2.2$ Hz, $J_{1,2ax} = 5.1$ Hz, $J_{1,6eq} = 2.2$ Hz, $J_{1,6ax} = 6.2$ Hz). Therefore, the H-1 was epimerized with NaOMe/MeOH to give the thermodynamic product (**18**, 32%, $J_{1,2eq} = 4.4$ Hz, $J_{1,2ax} = 12.5$ Hz, $J_{1,6eq} = 3.3$ Hz, $J_{1,6ax} = 12.5$ Hz).

17. 5-*O*-Caffeoyl quinic acid methyl ester (**13**) was obtained from **2** by treatment with CH_2N_2 (71%).
18. Toyama-Kato, Y.; Yoshida, K.; Fujimori, E.; Haraguchi, H.; Shimizu, Y.; Kondo, T. *Biochem. Eng. J.* **2003**, *14*, 237–241.
19. General procedure of reproduction experiment is as follows. **1**, **2**, and Al^{3+} were mixed in 0.1 M acetate buffer at pH 4.0 at their final concentration to be 1 mM, 3 mM, 1 mM, respectively, then UV–vis spectrum and CD were recorded (cell length: 1 mm).
20. The precipitate was gathered by centrifugation, and the mass was washed with a small portion of ultra-pure water and dried under reduced pressure. The quantitative analysis of **1** and **4** by HPLC and Al by graphite furnace atomic absorption spectroscopy, respectively, showed that the mass was composed with **1** and Al. Compound **4** was not detected.
21. Compounds **1**, **2**, and Al^{3+} (1 mM, 3 mM, 1 mM) were mixed in 50 mM CD_3COOD–CD_3COONa in D_2O (pD 4.0, not adjusted) and the ^1H NMR spectrum was recorded (JEOL α-600, ^1H: 600 MHz, at 25 °C). The signal broadening may be caused by generation of phenoxy radical at the B-ring of **1**.
22. The CD of the mixture of compounds **1**, **8**, or **10**, and Al^{3+} showed just like the intermediate CD mixed with **1**, **2**, and Al^{3+} and **1**, **4**, and Al^{3+}. These mixture gave precipitates gradually. Because of the low co-pigment effect of **8** and **10**, small amount of blue hydrangea pigment may co-exist with self-associated **1**.
23. The cyclohexyl ring of **12** took a boat conformation ($J_{1,2eq} = 5.0$ Hz, $J_{1,2ax} = 10.0$ Hz, $J_{2eq,3} = 3.0$ Hz, $J_{2ax,3} = 3.0$ Hz, $J_{3,4} = 5.0$ Hz, $J_{4,5} = 3.5$ Hz, $J_{5,6eq} = 3.5$ Hz, $J_{5,6ax} = 10.0$ Hz). The lack of co-pigmentation effect of **12** may be due to the difference of the conformation.