mica plate touched the drop before the folding of the surface was established (Figure 2b). The aqueous solution in the cuvette was removed by using a Hamilton pump and the solvent inside was evaporated. The resulting film was studied by surface force microscopy. A similar process was used for the collapsed drop (Figure 2f).

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Chiral Molecular Recognition on Formation of a Metalloanthocyanin: A Supramolecular Metal Complex Pigment from Blue Flowers of *Salvia patens***

Tadao Kondo,* Kin-ichi Oyama, and Kumi Yoshida

Most blue flower color is the result of a metalloanthocyanin,[1, 2] a stoichiometric self-assembled supramolecular pigment consisting of six molecules of an anthocyanin, six molecules of a flavone, and two metal atoms.[3, 4] We have isolated various metalloanthocyanins from the petals of blue flowers and elucidated their structures. We have clarified the mechanisms of the development of blue color by chemical reconstruction of some supramolecules and structural analysis by NMR spectroscopy and X-ray crystallography.[1, 3] Very fine and strict molecular recognition,[5] including chiral molecular recognition, occurs during reconstruction. The aromatic chromophores in commelinin and protocyanin stack in a chiral manner, with anticlockwise self-association between two molecules of the anthocyanin or two molecules of the flavone, and clockwise stacking of anthocyanidin and flavone nuclei (co-pigmentation).[3] These phenomena must arise from the chirality of the sugars attached to the anthocyanidin and flavone units, although, no evidence has hitherto been provided in support of this conclusion.

Herein we describe the chiral molecular recognition on formation of the metalloanthocyanin protodelphin (**1**).[6] Elucidation of the gross structure of **1**, a genuine pigment from blue petals of *Salvia patens* (Figure 1), demonstrated

Figure 1. *Salvia patens.*

that it consists of the anthocyanin malonylawobanin (**2**),[7] the flavone apigenin 7,4′-di-*O*-*β*-D-glucoside (**3a**),[6, 8] and Mg^{2+} ions (Scheme 1). General and effective synthetic procedures for anthocyanins and/or flavones containing the optically antipodal glycosides are necessary for chiral recognition studies. We have developed a reliable glycosylation method for less-reactive phenols in flavonoids utilizing a new Lewis acid/base promoted glycosylation[9] with the peracetylglucosyl fluoride (**4**),[10] and succeeded in preparing natural apigenin 7,4′-di-*O*-*β*-glucoside **3a** and unnatural derivatives (partly) substituted with L-glucose (**3b**–**d**). Employment of these synthetic apigenin derivatives and natural **2** in the presence of Mg^{2+} ions allowed an examination of the chiral molecular recognition that occurs during formation of the blue complex pigment.

For the structural determination,[11] **1** was synthesized from the components according to our reconstruction method.[3] Compounds **2** and **3a** and Mg^{2+} ions were mixed in a weakly alkaline solution, then the mixture was purified by gel-permeation chromatography/liquid chromatography (GPC-LC)[12] to give pure protodelphin (**1**) in 61% yield. The UV/Vis spectra of the product were completely identical with those of the naturally occurring form[6] (Figure 2). Electrospray ionization mass spectrometry (ESI-MS)[13] of **1** gave a multiply charged molecular ion at m/z: 1751.8 ($[M-5H]^{5-}$) corresponding to the constitution $C_{396}H_{408}O_{222}Mg_2$ (average molecular weight: 8767.95, calcd 1751.37 $[M-5H]^{5-}$). The

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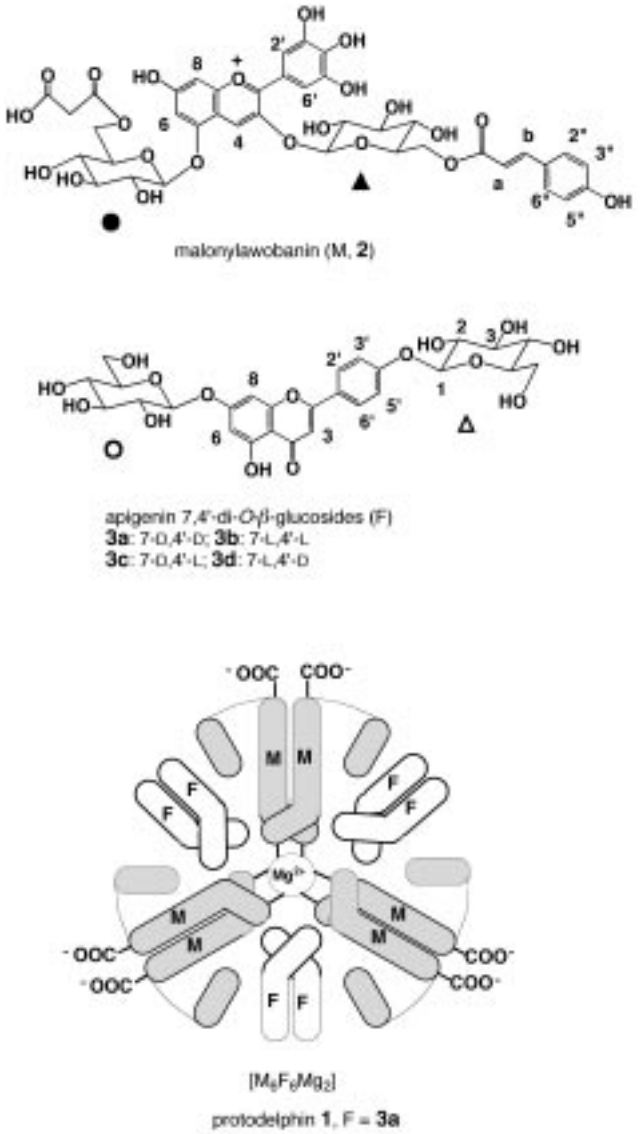


Scheme 1. The structure of components (top and middle) and the entire (bottom) protodelphin. M (**2**) as a bidentate ligand is coordinated to Mg^{2+} ions, and F (**3a**) is intercalated.

CD spectrum (Figure 2) showed a strong negative exciton-type Cotton effect around 620 nm, which indicates that the anthocyanidin nuclei must be closely stacked in an anticlockwise manner, and the ^{1}H NMR spectrum showed simple signals attributable to one anthocyanin and one flavone residue. Analysis of long-range NOEs between protons of **2** and those of **3a** suggested a cross-parallel arrangement of nuclei. These data indicate that the arrangement of the components in the gross structure of **1** is very similar to that of commelinin. Thus, protodelphin is a stoichiometric supramolecular metal complex pigment, that is, a metalloanthocyanin, similar to commelinin and protocyanin (Scheme 1).

Although glycosylflavones are widely distributed in plants and have attracted attention because of their important biological and medical aspects, only a few syntheses of poly-*O*-glycosylflavones with satisfactory yield have, surprisingly, been reported. The nucleophilicity of the 4′-hydroxy group on the B-ring of the flavone nucleus is very poor, therefore, there are very few examples of direct 4′-O-glycosylation.[14]

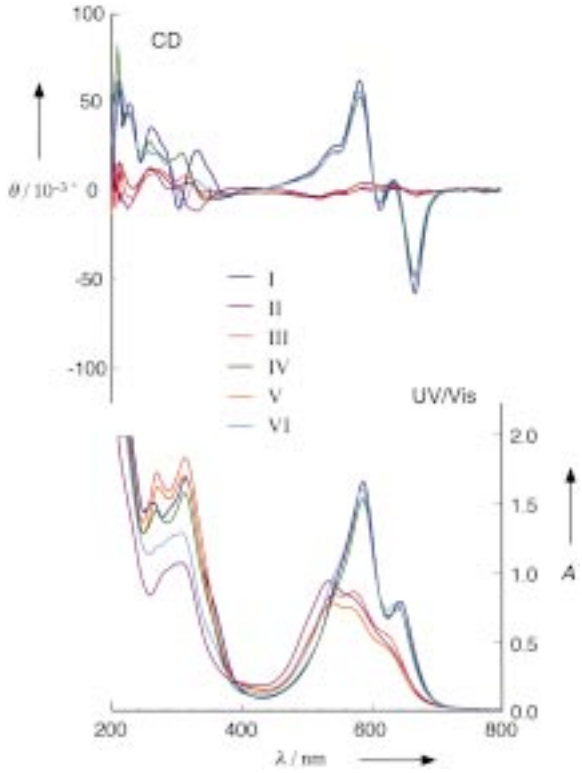
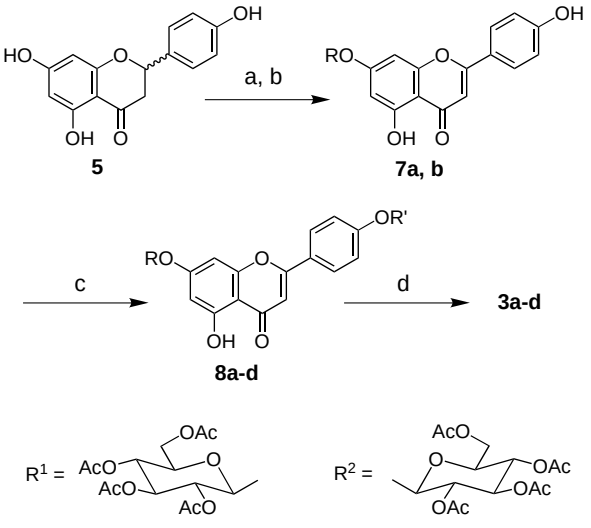


Figure 2. UV/Vis and CD spectra of reconstructed mixtures of **2** (5×10^{-4} M) and **3**, **9**, or **10a** (1 equiv) in the presence of Mg^{2+} ions (5 equiv) in phosphate buffer (pH 6.0). I = **2** + **3a** + Mg^{2+}, II = **2** + **3b** + Mg^{2+}, III = **2** + **3c** + Mg^{2+}, IV = **2** + **3d** + Mg^{2+}, V = **2** + **9** + Mg^{2+}, VI = **2** + **10a** + Mg^{2+}.

We achieved the first synthesis of apigenin 7,4′-di-*O*-*β*-D-glucoside (**3a**) by direct glycosylation of the flavone nucleus (Scheme 2). Condensation of naringenin (**5**) with peracetyl-D-glucosyl bromide (**6a**) and consequent oxidization with DDQ



Scheme 2. Synthesis of apigenin 7,4′-di-*O*-*β*-glucosides **3**: a) peracetylglucosyl bromide (**6a** or **6b**), Ag_2CO_3, quinoline; b) DDQ, 1,4-dioxane, **7a**: R = R^1 (66%), **7b**: R = R^2 (66%); c) peracetylglucosyl fluoride (**4a** or **4b**), $BF_3\cdot Et_2O$, DTBMP/TMG (4/1), CH_2Cl_2/PhCl (1/6), **8a**: R = R′ = R^1 (70%), **8b**: R = R′ = R^2 (72%), **8c**: R = R^1, R′ = R^2 (66%), **8d**: R = R^2, R′ = R^1 (68%); d) 1. NaOMe, MeOH/$CHCl_3$ (2/1); 2. Dowex 50W-8X (H^+), **3a** (92%), **3b** (88%), **3c** (92%), **3d** (94%). **4a**, **6a**: D-glucosyl; **4b**, **6b**: L-glucosyl. DDQ = 2,3-dichloro-5,6-dicyanobenzoquinone; DTBMP = 2,6-di-*tert*-butyl-4-methylpyridine; TMG = 1,1,3,3-tetramethylguanidine.

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gave the apigenin 7-*O*-peracetyl-β-D-glucoside (**7a**). Treatment of **7a** with peracetyl-D-glucosyl fluoride (**4a**) in the presence of $BF_3 \cdot OEt_2$, DTBMP, and TMG afforded apigenin 7,4′-*O*-di-β-D-glucoside as an acetate[14b] (**8a**) in 70% yield, which was deprotected to yield **3a**. A combination of DTBMP and TMG was essential for the glycosylation of the 4′-OH group of **7a**. By using the same procedure we could prepare a series of unnatural apigenin 7,4′-di-*O*-β-glucosides (**3b**–**d**) derived from L-glucose: the 7,4′-di-L-glucoside **3b**, 7-D-glucoside-4′-L-glucoside **3c**, and 7-L-glucoside-4′-D-glucoside **3d**.

To clarify the role of the flavone part in the chiral recognition on formation of the metalloanthocyanin, we mixed **2** with **3b**–**d** in the presence of Mg^{2+} ions. Among the three unnatural apigenin diglucosides used, only **3d** gave a blue solution (Figure 3, IV); **3b** and **3c** gave purple solutions (II and III, respectively). Solution IV showed almost the same UV/Vis and CD spectra as protodelphin (Figure 2), which indicates that a protodelphin-like metalloanthocyanin was produced from **3d**. The stability of the colors of those mixtures was recorded and the results are shown in Figure 3. The color of **1** in a neutral aqueous solution (I) was very stable, while the blue color of IV was less so. The purple color of II and III rapidly faded. To reveal the role of the glucosides at the 7- and 4′-OH groups of apigenin, we prepared apigenin

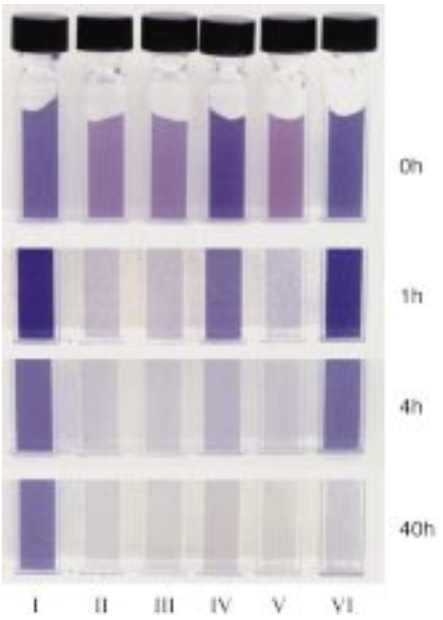

Figure 3. Color stabilities of mixtures of **2** (5×10^{-4} M) and **3**, **9**, or **10a** (1 equiv) in the presence of Mg^{2+} ions (5 equiv) in phosphate buffer (pH 6.0). I = **2** + **3a** + Mg^{2+}, II = **2** + **3b** + Mg^{2+}, III = **2** + **3c** + Mg^{2+}, IV = **2** + **3d** + Mg^{2+}, V = **2** + **9** + Mg^{2+}, VI = **2** + **10a** + Mg^{2+}.

7-*O*-β-D-glucoside[15] (**9**) and 4′-*O*-β-D-glucoside[16] (**10a**), respectively, and examined whether a protodelphin-like supramolecule was formed. Compound **10a** gave the blue pigment with **2** and Mg^{2+} ions, but **9** did not (Figure 2, 3). Surprisingly, the blue color of VI, constructed from **10a**, was more stable than that of IV produced from **3d**. Furthermore, we checked its enantiomer, apigenin 4′-*O*-β-L-glucoside (**10b**), for complex formation but no metalloanthocyanin was generated. These phenomena indicate that the D-glucosyl residue at the 4′-OH position is indispensable for formation of a metal complex pigment. The D-glucose residue at the 7-OH position could stabilize the molecular association, while an L-glucose would destabilize the complex by steric hindrance.

To estimate the enantioselectivity of the molecular recognition on formation of the supramolecule, we attempted a reconstruction experiment from **2** and a 1:1 mixture of **3a** and **3b** with Mg^{2+} ions. The blue mixture was purified by GPC-LC, and a blue-black amorphous mass was obtained in 85% yield. The ratio of **3a** to **3b** in the supramolecule was analyzed by HPLC on a chiral stationary phase.[17] No peak corresponding to **3b** was observed in the chromatogram, thus the ratio of **3b** to **3a** is less than 2:98.[18] This result indicated that L,L-diglucoside **3b** was completely excluded from the metal complex. The natural anthocyanin molecule chose only the D-series of glycosylflavones as partners to form metalloanthocyanin.

The high enantioselectivity observed during formation of the supramolecule could be caused by the chiral stacking arrangement of components in **1** as a consequence of the presence of the sugar moiety. Three flavone molecules[19] in **1** associate to form a *M* (minus) helical structure, similar to a propeller with three blades. They are bound at the pivot point by a strong hydrogen-bonding network among the hydroxyl groups at C-2 and C-3 of the 4′-*O*-β-D-glucopyranosides (Figure 4). The two sets of *M*-helical flavone associates fit closely into the vacant space formed from the metal complex of six molecules of **2** and two Mg^{2+} ions. Replacement of D- by L-glucose at the 4′-OH position of apigenin could invert the helicity to obtain the *P* (plus) form, with the consequence that the associates of the flavones **3b**, **3c**, and **10b** do not fit into the vacant space. Thus, the *M* helicity formed by the three molecules containing the D-glucosyl residue at the 4′-OH site plays a key role in the formation of metalloanthocyanin.

In conclusion, malonylawobanin (**2**) chooses only the D chirality of the 4′-*O*-glucosyl residue in apigenin 7,4′-diglucoside to form the stoichiometric supramolecular metal complex pigment protodelphin. This restricted chiral and structural recognition controls the entire self-assembly of the metalloanthocyanin and is responsible for the beautiful blue color of the flowers.

Experimental Section

Reconstruction of protodelphin (**1**): *Caution*: the reaction requires concentrations of at least 10^{-2}–10^{-3} M. A solution of **3a** (20 mg) in water (1 mL) and 0.5 M $Mg(OAc)_2$ (0.2 mL) was added under stirring at RT to a solution of **2** (34 mg, 35 μmol) in water (0.6 mL) which was neutralized with 1.3% aqueous ammonia. The resulting blue solution was separated by GPC-LC (Cellurofine GC-15-m) to give **1** as a blue-black mass (34 mg, 61%). ^{1}H NMR (600 MHz, D_2O; for ●, ▲, ○, and △ see Scheme 1): δ = 4.53 (br d, *J* = 7.5 Hz, 1H; ●-1), 4.77 (br s, 1H; M-8), 4.87 (br d, *J* = 7.5 Hz, 1H; ▲-1), 4.94 (br d, *J* = 7.5 Hz, 1H; ○-1), 5.13 (br d, *J* = 7.5 Hz, 1H; △-1), 5.31 (br s, 1H; F-8), 5.52 (br s, 1H; F-3), 5.62 (br s, 1H; M-6), 5.82 (d, *J* = 16 Hz, 1H; M-a), 5.98 (br s, 1H; F-6), 6.44 (3H; M-3″, 5″, F-2′), 6.62 (br d, 2H; F-2′, 3′), 6.73 (1H; M-4), 6.35 (d, *J* = 9 Hz, 2H; M-2″,6″), 6.75 (d, *J* = 16 Hz, 1H; M-b), 5.62 (s, 1H; F-3), 7.46 (br s, 2H; F-5′,6′), 7.79 (br s, 1H; M-6′), 8.10 (br s, 1H; M-2′); MS (ESI): *m*/*z*: 1751.8 $[M-5H]^{5-}$.

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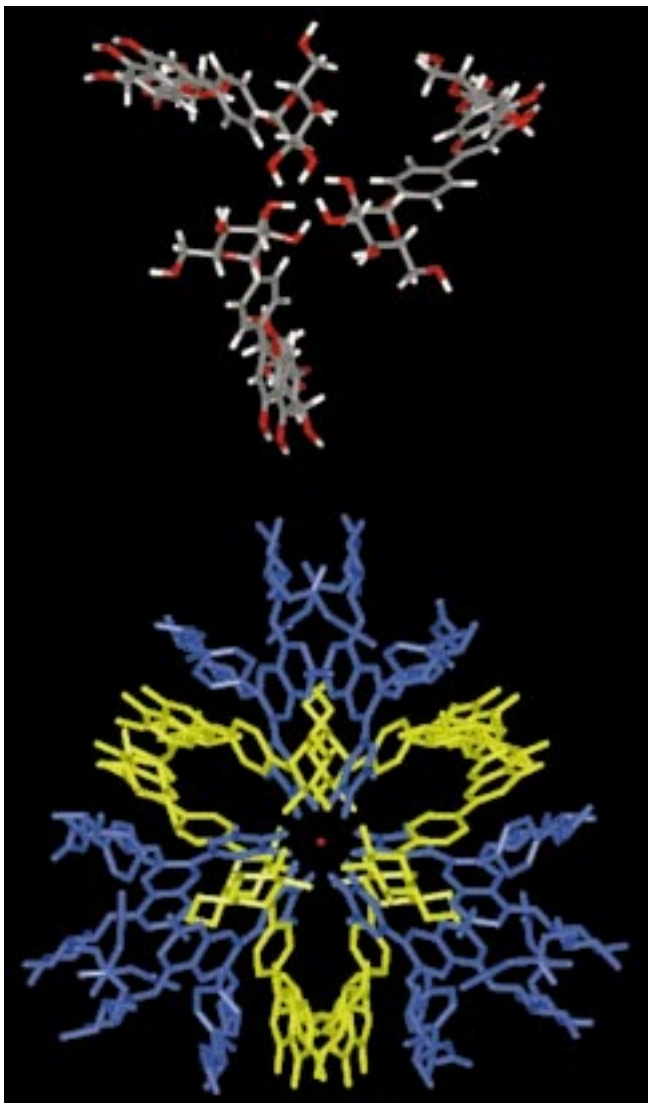

Figure 4. The optimized *M*-helical conformer of three **3a** molecules, similar to a propeller with three blades (top), and the gross structure of **1** (bottom). One set of three F molecules is located at the upper side of the pigment and one at the lower side. The F molecules are intercalated in a face-to-face manner. Mg^{2+} ions exist at both the upper and lower part of the superstructure. The hydrogen atoms are omitted in **1** for clarity. Color scheme: anthocyanin **2**: blue, flavone **3a**: yellow, and Mg^{2+}: red.

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[11] The composition and gross structure of natural **1** could not be clarified yet because of difficulty in purification. Complex **1** is soluble only in aqueous media and upon dilution quickly dissociates and becomes decolorized.

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[16] Apigenin 4′-*O*-*β*-D- and -L-glucoside (**10a**,**b**) were prepared according to our procedure. Naringenin (**5**) was selectively silylated at the 7-OH position with *tert*-butyldimethylsilyl chloride/imidazole in DMF. The resulting 7-*O*-silylnaringenin was oxidized, glycosylated with peracetyl-D- or L-glucosyl fluoride, and deprotected with tetrabutylammonium fluoride (TBAF) and MeONa/MeOH to provide **10a**,**b**.

[17] Aqueous trifluoroacetic acid (TFA) was added to the produced pigment, then the solution was analyzed by HPLC using a CHIRALCEL OD-R column (DAICEL CHEMICAL) eluted with TFA/CH_3CN/water 0.3/13/86.7 at RT.

[18] A mixture of **3a** and **3b** (ratio 98:2) gave two peaks completely separated in HPLC.

[19] The starting structure of **1** was built by changing the flavocommelin molecule to **3a** on the basis of the crystallographic structure of commelinin.[3] The local optimization of this structure was performed by QUANTA-97-CHARMm (Ver. 23.2) software.

Synthesis of an Array Comprising 837 Variants of the hYAP WW Protein Domain**

Florian Toepert,* José R. Pires, Christiane Landgraf, Hartmut Oschkinat, and Jens Schneider-Mergener*

The elucidation of structure – function relationships of proteins contributes to a better understanding of how they work and also provides clues for the synthesis of agonists and antagonists. Today, variants of the investigated protein required for structure – function analyses are produced almost

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