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A New Molecular Mechanism of Blue Color Development with Protocyanin, a Supramolecular Pigment from Cornflower, *Centaurea cyanus*

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Abstract: Protocyanin (**1**), a blue pigment of *Centaurea cyanus*, is composed of six molecules each of malonylflavone (**2**) and succinylcyanin (**3**), complexed with magnesium and ferric ions. The blue color of **1** is developed by an LMCT interaction between anthocyanin and ferric ion, rather than arising from the formation of a simple anhydrobase anion of the chromophore. The supramolecular structure of **1** was elucidated.

Protocyanin (**1**), an anthocyanin pigment from the blue petals of the cornflower,[1-3] *Centaurea cyanus*, is a stoichiometric self-assembled supramolecule consisting of succinylcyanin (**Sucy**, **2**), malonylflavone (**Mafl**, **3**), a ferric ion and a magnesium ion. The composition was revealed to be [**Sucy**$_6$**Mafl**$_6$Fe^{3+}Mg^{2+}],[4] similar to that of commelinin, a metalloanthocyanin, from blue petals of *Commelina communis*.[5] The blue color of commelinin is due to the keto-anion form[6] of the delphinidin nucleus while commelinin-like pigments reconstructed from **2** or malonylshisonin (cyanidin nucleus) only exhibited a violet color.[5, 7] However, the color of **1** was blue, and not dependent on the change in pH,[8] strongly suggesting that an unknown blueing mechanism exists other than that involving a simple metal complexation.[9] We report herein a new mechanism of blue-color development of **1** and the supramolecular structure of the pigment.

Reconstruction experiments of **1** using succinylcyanin methyl ester (**4**) and succinylpelargonin (**5**)[10] instead of **2** were performed. The former formed a protocyanin-like pigment, whereas the latter did not. Therefore, the metal ions coordinate with *ortho*-dihydroxy groups on the B-ring of **2** in **1** and its analogs.

We also prepared metal-ion replaced protocyanins for elucidation of the mechanism of the blue color development of **1**. ESI-MS and electrophoretical analysis showed that these analogs had the same composition as **1**, [**Sucy**$_6$**Mafl**$_6$M^{3+}M^{2+}]. The CD spectrum of these pigments showed the same large negative Cotton effect as **1**, indicating a structural similarity between **1** and its analogs. UV/VIS absorption spectra of the Mg^{2+}–replaced protocyanins [**Sucy**$_6$**Mafl**$_6$Fe^{3+}M^{2+}] (M^{2+} = Mn^{2+}(**6**), Zn^{2+}(**7**), Cd^{2+}(**8**)), which showed a blue color, were identical to that of **1** having five peaks in the secondary differential spectrum of the visible region (not shown). On the other hand, the Fe^{3+}–replaced protocyanins [**Sucy**$_6$**Mafl**$_6$M^{3+}Mg^{2+}] (M^{3+}=Al^{3+}(**9**), Ga^{3+}(**10**), In^{3+}(**11**)) gave only four peaks in the VIS absorption spectra, resulting in a violet color.[11] This difference in the color of Fe^{3+}–replaced protocyanins could be attributed to the lack of the peak at λ_{max} 676 nm. Interestingly, only Co,Mg–protocyanin (**12**), having a peak at λ_{max} 673 nm (Fig. 1), showed a blue color.[12] Thus, pigments containing transition metal ions (Fe^{3+} and Co^{3+}) had a peak around 675 nm, suggesting a charge transfer interaction occurs between **2**

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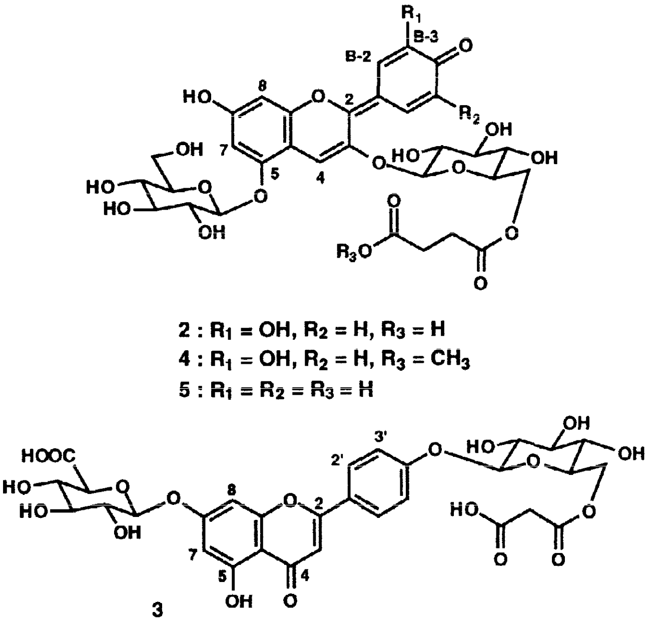
and trivalent transition metal ions. The MCD[13] (magnetic circular dichroism) spectrum of paramagnetic **1** showed two peaks at λ_{max} = 676 and 599 nm, but diamagnetic **9** showed no such peak (Fig. 1). Therefore, the peak at 676 nm must be attributed to an LMCT (ligand–to–metal charge transfer) absorption band.[14] Thus, **1** develops its blue color by an LMCT interaction between the cyanidin nucleus of **2** and the high–spin Fe^{3+} ion (Fig. 2).[15] The existence of an LMCT interaction could also be verified by the resonanced Laser Raman spectrum.[16]

Since the ^{1}H NMR spectrum of **1**, a paramagnetic Fe^{3+}–complex, gave unanalyzable broad signals, we used the diamagnetic Al^{3+}–complex, **9**, which gave a analyzable spectrum, for the structural analysis.[17] Signals corresponding to two anthocyanidins, **2**, and two flavones, **3**, were found to exist in ratios of 1 : 1 : 1 : 1, indicating that their components are three-fold symmetrically arranged. Each **2** is coordinated with either an Al^{3+} or a Mg^{2+} ion since the signals in the aromatic region of one of the anthocyanins, **Sucy1**, were shifted to a lower field than that of the others, **Sucy2**. Therefore, **Sucy1** was assigned to coordinate with the Al^{3+} ion, and **Sucy2** with the Mg^{2+} ion.[18] Signals corresponding to **3**, **Mafl1** and **Mafl2**, showed the same behavior. In particular, signals of 2', 3', 5' and 6' of the B ring of **Mafl1** were observed separately, suggesting that free rotation around the pivot bond is hindered by the strong intermolecular stacking. Thus, **9** is a binuclear complex arising from two different metal ions and a C_3 axis exists in the molecule.



2 : R$_1$ = OH, R$_2$ = H, R$_3$ = H
4 : R$_1$ = OH, R$_2$ = H, R$_3$ = CH$_3$
5 : R$_1$ = R$_2$ = R$_3$ = H

3

Scheme

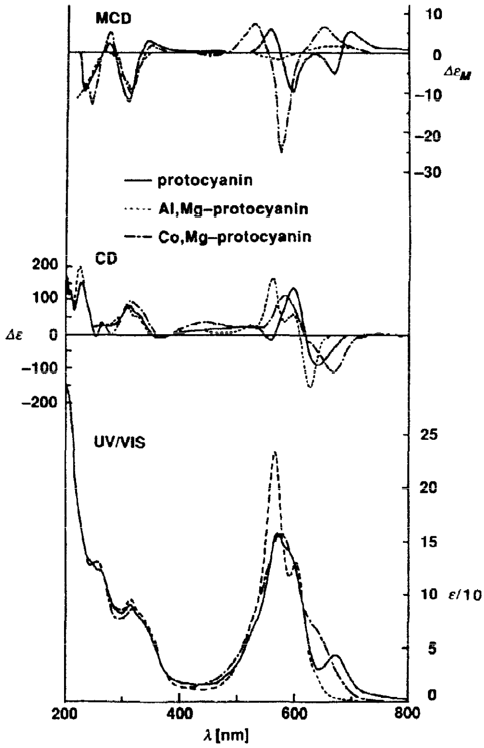
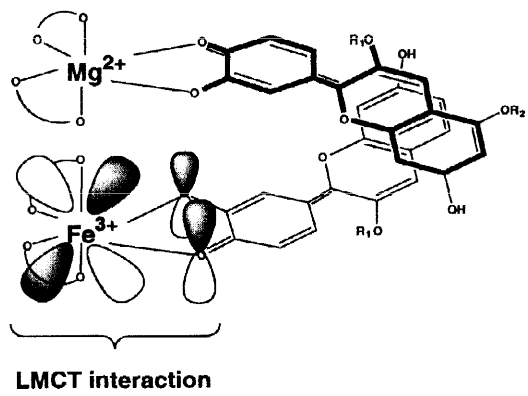


Figure 1

Figure 1. UV/VIS, CD and MCD spectrum of protocyanin (**1**), Al,Mg-protocyanin (**9**) and Co,Mg-protocyanin (**10**) in 0.1 M acetate buffer solution at pH 5.0.

The CD spectra of **1** and metal ion–replaced protocyanins showed a strong negative exciton–coupling type Cotton curve in the visible region (Fig. 1.), indicating the chromophores of **Sucy1** and **Sucy2** (self-association) to be arranged in a left–handed screw manner, this conclusion also being supported by an NOE between **Sucy1** B-2 and **Sucy2** B-2, in ^{1}H NMR signals on the B-ring of **9**. NOE relations between the aromatic proton signals of **Sucys** and **Mafls**, *e.g.*, **Sucy1** B-2 – **Mafl1** 5'. **Sucy2** B-2 – **Mafl2** 5' or 6', **Sucy1** B-6 – **Mafl1** 8, **Sucy2** B-6 – **Mafl2** 8, **Sucy1** B-5 – **Mafl1** 8, **Sucy2** B-5 – **Mafl2** 8 and **Mafl1** 5' – **Mafl2** 2' were observed. Therefore, the **Mafls** are arranged together in a left-handed screw manner, and **Sucy** and **Mafl** (copigmentation) in a right-handed screw manner, and the stacking of each component is face-to-face and in a chirally cross-parallel arrangement as well as **Sucys**.[19] Therefore, the gross structure of **9** should be cyclo–[(**Sucy1**–**Sucy2**–**Mafl2**–**Mafl1**–)3] around the *C3* axis through ferric and magnesium ions (Fig.3).[20, 21]



R_1 = 6-*O*-succinyl-β-D-Glc*p*
R_2 = β-D-Glc*p*
R_3 = 6-*O*-malonyl-β-D-Glc*p*
R_4 = β-D-Glc*p*A

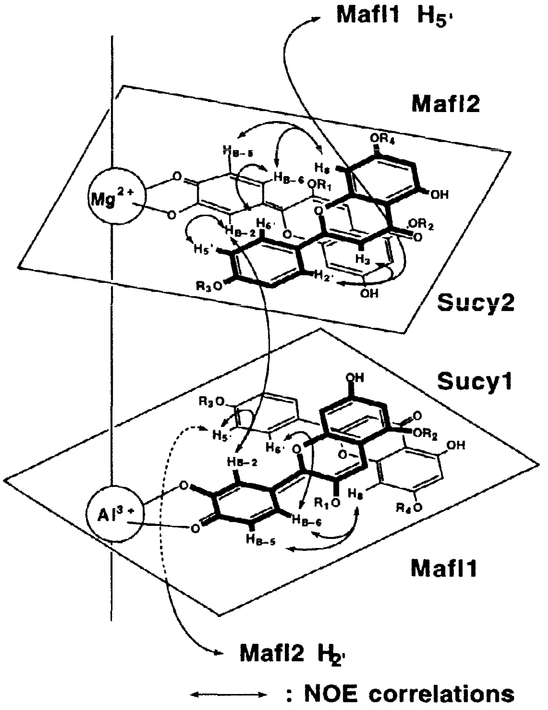


Figure 2

Figure 3

Figure 2. Schematic representation of the LMCT interaction between 2 and the Fe^{3+} ion in protocyanin.
Figure 3. The arrangement of components in Al,Mg–protocyanin (**9**). The orientation between A ring and B ring in **Sucy** was determined according to the NOE between $H_{B\text{-}5'}$ and sugar moiety.

The supramolecular structure of **1** is very similar to that of commelinin. The molecular stacking interaction between the organic components prevents hydration of the anthocyanidin nucleus and brings about the color stabilization. The LMCT interaction between **Sucy** and the Fe^{3+} ion realizes the blue color development of **1**. This new mechanism is greatly different from that known to operate for commelinin. The LMCT based mechanism is a new concept for the blue color development of anthocyanin extending beyond the metal–complex theory.[1, 2, 5] We have here obtained the answer to an intriguing question, "what is the molecular basis for the color of blue cornflower?", asked from the early part of this century.

Acknowledgments

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6. The keto-anion form was stabilized with metal coordination and hydrophobic stacking among the chromophores. Goto, T. and Kondo, T. *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 17.
7. Commelinin having delphinidin as a chromophore showed a blue color, while a commlinin-like pigment, constructed from malonylshisonin, flavocommelin and two magnesium ions and not having ferric ion, demonstrated a violet color. See 5.
8. The blue color of the solution did not change on variation of the pH between 4 and 6.5.
9. Hayashi and coworkers proposed that the ferric ion is essential for the blue color development of **1**. However, they did not clarify its exact role.; Saito, N.; Hayashi, K. *Sci. Rept. Tokyo Kyoiku Daigaku* **1965**, *B 12*, 39.
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11. The Fe^{3+}–replaced protocyanins have almost the same stability in 0.1 M acetate buffer solution (pH 5.0), while Mg^{2+}–replaced species have varied stability in the order of Mg^{2+}>Zn^{2+}>Mn^{2+}> Cd^{2+}.
12. Co,Mg–protocyanin (**12**) also shows peaks of MCD spectrum. CD: $\lambda max(\Delta\varepsilon)$: 668(-106), 584(+112) nm, MCD: $\lambda max(\Delta\varepsilon)$: 650(+6.57), 578(–26.3), 530(+6.60) nm. Atomic analysis: Atomic analysis: Found; Mg, 0.20%; Co, 0.44% for reconstructed protocyanin: Calcd.; Mg, 0.29%; Co, 0.69% for $C_{366}H_{384}O_{228}CoMg$.The error might arise from incorporation of water in the pigment.
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17. protocyanin (**1**): MCD: $\lambda max(\Delta\varepsilon)$: 703(+5.72), 677(–5.30), 599(–9.75), 568(+6.36) nm

    Al,Mg–protocyanin (**5**): ^{1}H NMR of aromatic region (500 MHz, 0.5%CD_3COOD–D_2O): δ= 4.64(s,1H; **Sucy1** or **2** A–6), 4.90(s,1H; **Sucy1** or **2** A–6), 5.57(s,1H; **Mafl1** 8), 5.67(s,1H; **Mafl2** 8), 5.91(s,1H; **Sucy1** or **2** A–8), 5.93(s,1H; **Sucy1** or **2** A–8), 6.16(s,1H; **Mafl2** 3), 6.17(s,1H; **Mafl1** 3), 6.40(s,1H; **Mafl1** 6), 6.42(s,1H; **Mafl2** 6), 6.74(d, J = 10 Hz, 2H; **Sucy1&2** B–5), 7.05(d, J = 10 Hz, 1H; **Mafl2** 3'), 7.10(d, J = 10 Hz, 1H; **Mafl1** 3'), 7.17(d, J = 10 Hz, 1H; **Mafl2** 5' or 6'), 7.17(s, 1H; **Sucy 1** or **2** A–4), 7.26(d, J = 10 Hz, 1H; **Mafl2** 2'), 7.29(d, J =10 Hz, 1H; **Mafl2** 5' or 6'), 7.33 to 7.31 (m, 2H; **Mafl1** 2'&3'), 7.33(s, 1H; **Sucy1** or **2** A–4), 7.58(d, J = 10 Hz, 1H; **Mafl1** 5'), 8.16(s, 1H; **Sucy2** B–2), 8.26(s, 1H; **Sucy1** B–2), 8.62(d, J = 10 Hz, 1H; **Sucy2** B–6), 8.73(d, J = 10 Hz, 1H; **Sucy1** B–6) ppm. CD: $\lambda max(\Delta\varepsilon)$ = 628 (–150), 597 (+54.4), 562 (+160) nm. MCD: no signal.

    Atomic analysis: Found; Mg, 0.23%· Al, 0.34% for reconstructed protocyanin: Calcd.; Mg, 0.29%· Al, 0.32% for $C_{366}H_{384}O_{228}AlMg$. ESI-MS (negative) m/z 8486 : averaged MW 8482.
18. The Al^{3+} ion has stronger acidity than the Mg^{2+} ion, suggesting that **Sucy** coordinating to an Al^{3+} ion is more deshielded than that to Mg^{2+} for chemical shift of the NMR spectrum.
19. This is the same stacking pattern as observed for commelinin.; see 5
20. Also, Ga,Mg–protocyanin (**10**) had completely the same structure as Al,Mg–protocyanin (**9**).; Diamagnetic Ga^{3+} is structurally very close to paramagnetic Fe^{3+} in physical and chemical properties.; Shannon, R. D. *Acta Crysatllogr. Sect A.* **1976**, *A32*, 751. Tor, Y.; Libman, J.; Shanzer, A.; Felder, C. E.; Lifson, S. *J. Am. Chem. Soc.* **1992**, *114*, 6661.
21. No other orientation of the molecules in **9** can exist from the NOE data and the CD spectrum.