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Cyanosalvianin, a supramolecular blue metalloanthocyanin, from petals of *Salvia uliginosa*

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ABSTRACT

A metalloanthocyanin, cyanosalvianin, was found in blue petals of *Salvia uliginosa*. Cyanosalvianin consisted of 3-*O*-(6-*O*-*p*-coumaroylglucopyranosyl)-5-*O*-(4-*O*-acetyl-6-*O*-malonylglucopyranosyl) delphinidin, 7,4′-di-*O*-glucopyranosylapigenin and magnesium ion. We reproduced the same blue color as the petals by mixing the three components together. An ESI-MS measurement gave a molecular weight of 9014 indicating the composition of cyanosalvianin to be six molecules of the anthocyanin component, six molecules of the flavone component and two magnesium ions. The special arrangement of the organic components in cyanosalvianin was analyzed by CD and 2D-NMR spectroscopy. It was clarified that cyanosalvianin has a similar structure to that of commelinin, a metalloanthocyanin isolated from blue dayflower, *Commelina communis*.

1. Introduction

Metalloanthocyanin was first named by Hayashi et al. (1958) for the blue pigment found in *Commelina communis*. The term metalloanthocyanin is now understood as a stoichiometric self-assembled metal complex-pigment, a natural supramolecule, composed of six molecules of anthocyanin, six molecules of flavone and two metal ions (Goto et al., 1986; Goto and Kondo, 1991). Metalloanthocyanins play a very important role in true blue flower color development by a very sophisticated mechanism. In such a supramolecular pigment, anthocyanins are in the anhydrobase anion form by metal complexation with chiral molecular-stackings (self-association of anthocyanins, self-association of flavones and co-pigmentation between anthocyanin and flavone) that stabilize the blue-colored form by preventing hydration followed by decoloration (Goto and Kondo, 1991; Kondo et al., 1992). When all components are mixed in a suitable buffered aqueous solution, chiral stackings of each component and metal complexation to anthocyanins occur spontaneously to form a blue-colored supramolecule (Goto and Kondo, 1991; Kondo et al., 1992). Furthermore, in the formation of metalloanthocyanin, undesired co-pigment component is strictly excluded (Kondo et al., 2001).

Until now only three metalloanthocyanins have been found in blue flowers, blue dayflower, *C. communis* (Hayashi et al., 1958; Takeda and Hayashi, 1977; Kondo et al., 1992), blue cornflower, *Centaurea cyanus* (Kondo et al., 1994a, 1998; Shiono et al., 2005), and blue salvia, *Salvia patens* (Takeda et al., 1994; Kondo et al., 2001). However, we think that metalloanthocyanins may exist more widely in nature. Therefore, we examined many blue petals to investigate, whereas a new metalloanthocyanins were present. In the blue petals of *Salvia uliginosa*, anthocyanin (Ishikawa et al., 1999) and flavone (Veitch et al., 1998) were already isolated and they had much structural similarities to the components of other metalloanthocyanins. Therefore, we focused on the blue petal color of *S. uliginosa* and found a new one, cyanosalvianin (**1**). In this report we describe the components and composition of **1**, and discuss the chiral molecular-stacking properties observed and the mechanism of its blue color development.

2. Results and discussion

2.1. Blue petal color of S. uliginosa

We searched for possible metalloanthocyanins in blue petals by first measuring the reflection spectrum of the petals and by spectroscopic analysis of the pressed juice of the petals. The blue petals of *S. uliginosa* (Fig. 1a) showed two λ_{max} at 585 and 640 nm in the reflection spectrum (Fig. 1b), which was similar to that of the blue dayflower. The pressed juice of the frozen petals also showed a blue color, and the VIS absorption spectrum and circular dichroism

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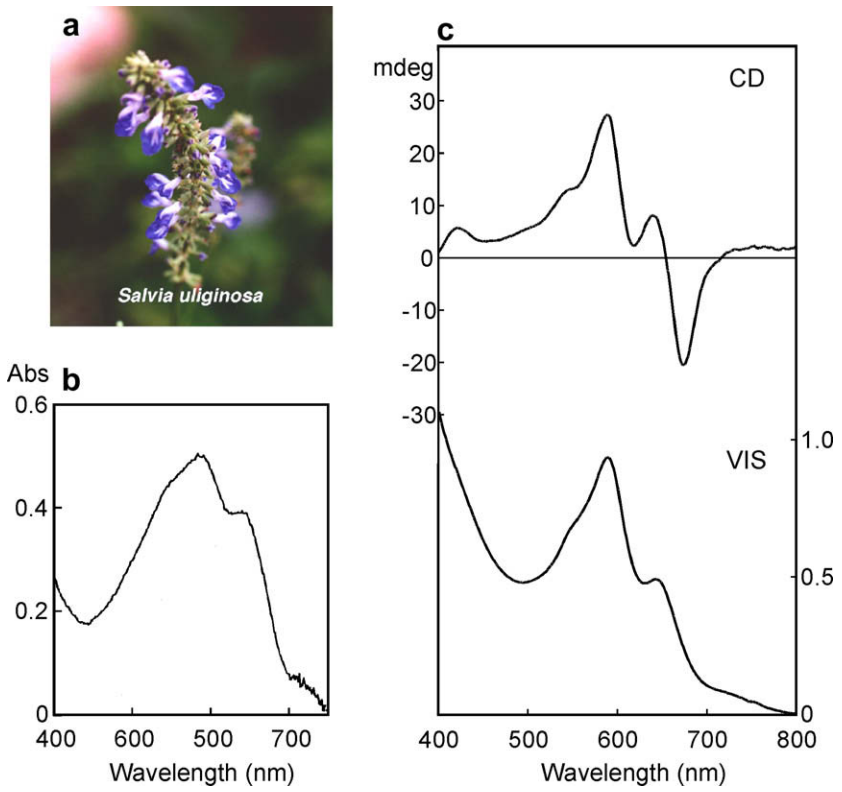

Fig. 1. Flowers of *S. uliginosa* (a), the reflection spectrum of the blue petals (b) and VIS absorption spectrum and CD of the pressed juice of the petals of *S. uliginosa* (c).

(CD) showed typical spectra to that of metalloanthocyanins; in the VIS absorption spectrum, a sharp peak with λ_{max} at 585 and a shoulder peak at 640 nm and a strong exiton-type negative Cotton effect around the area at the λ_{vismax} (Fig. 1c). An HPLC chromatogram of the pressed juice showed two major peaks; one corresponded to anthocyanin (**3**) and the other corresponded to flavone (**2**) by photo-diode array detection (Fig. 2). These results indicated that the blue petal color of *S. uliginosa* should be developed by a metalloanthocyanin.

2.2. Isolation and structural identification of petal components

HPLC chromatograms of extracts of *S. uliginosa* with CH_3CN:H_2O (1:1, v/v) containing 1% trifluoroacetic acid (TFA) exhibited one major anthocyanin component (**3**). However, when petals were extracted with 1% HCl–MeOH, the chromatogram showed two more peaks, **4** and **5**, during storage at rt for 24 h. These results indicated that some labile acyl residue such as malonyl and/or acetyl moieties might exist in the petal anthocyanin (Goto et al., 1983; Kondo et al., 1989; Yoshida et al., 1997). In fact, malonylawobanin and acetylated malonylawobanin have been already isolated in the same blue flower (Ishikawa et al., 1999). Therefore, we used acidic CH_3CN for large-scale extraction. The frozen petals (190 g) were extracted by CH_3CN:H_2O (1:1, v/v) containing 1% TFA, and the obtained extract purified by Amberlite XAD-7 chromatography followed by ODS-LC and ODS-HPLC gave **2** (9.5 mg) and **3** (7.9 mg). During the extraction and isolation procedure, decomposed anthocyanins (**4**, **5**) were also detected.

The structures of **4** and **5** were identified as malonylawobanin and awobanin, respectively, by comparison with authentic samples (Goto et al., 1983). The structures of **2** and **3** were also determined by MS and NMR spectroscopic analyses (Table 1; Fig. 3). Structure **2** was identified as 7,4′-di-*O*-glucopyranosylapigenin (Veitch et al., 1998; Kondo et al., 2001). From the MS (m/z = 901) an NMR (acetyl signal: 2.12 ppm, H-4 of glc2: 4.91 ppm) analysis of **3** suggested it to be an acetylated anthocyanin. Treatment of **3** in HCl–MeOH gave

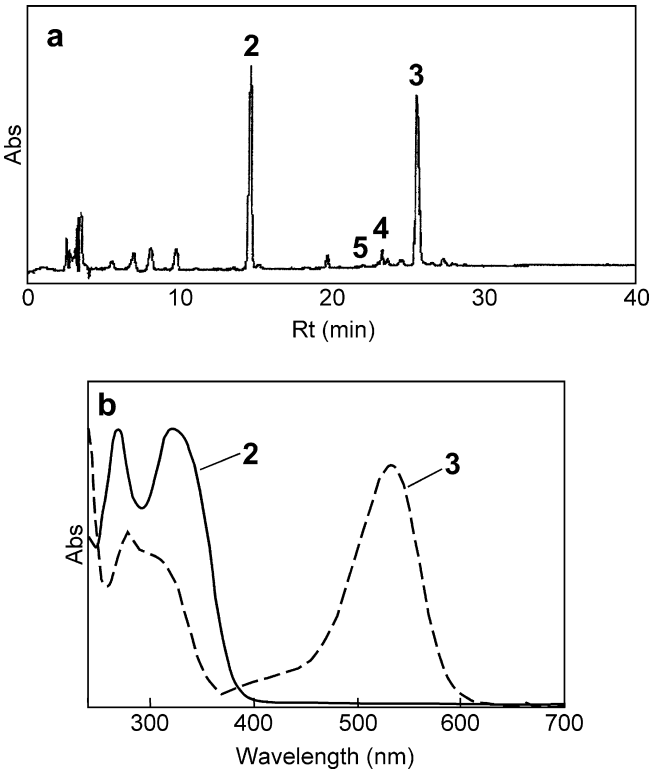

Fig. 2. HPLC chromatogram of the pressed juice of *S. uliginosa* (a) and the obtained spectra of peaks **2** and **3** (b).

Table 1
Assignment of the ^{1}H NMR spectra of **1**, **2** and **3**

		flavone **2**			**2** in **1**			anthocyanin **3**			**3** in **1**
		^{1}H δ (ppm)		J (Hz)	^{1}H δ (ppm)			^{1}H δ (ppm)		J (Hz)	^{1}H δ (ppm)
3		6.89	s		5.71	4		8.85	s		6.88
6		6.85	d	2.0	6.15	6		6.97	d	2.0	5.79
8		7.11	d	2.0	5.48	8		6.93	d	2.0	4.97
2'		7.81	d	8.5	7.65, 6.88	2'		7.75	s		8.28
6'		7.81	d	8.5		6'		7.75	s		7.97
3'		7.38	d	8.5	7.65, 6.88						
5'		7.38	d	8.5							
						pC	α	6.25	d	15.5	6.05
							β	7.36	d	15.5	6.92
							2, 6	7.21	d	9.0	6.53
							3, 5	6.70	d	9.0	6.61
							$COCH_3$	2.12	s		
glcA	1	5.88	d	7.5	5.13	glcl	1	5.45	d	8.0	5.02
	2	4.35	dd	9.0, 7.5			2	3.80	dd	9.0, 8.0	
	3	4.42	t	9.0			3	3.63	t	9.0	
	4	4.35	t	9.0			4	3.49	dd	9.5, 9.0	
	5	4.24	ddd	9.0, 5.0, 2.0			5	3.96	ddd	9.5, 8.0, 3.0	
	6a	4.40	m				6a	4.43	dd	12.0, 8.0	
	6b	4.59	dd	13.0, 2.0			6b	4.53	dd	12.0, 3.0	
glcB	1	5.76	d	7.5	5.33	glc2	1	5.23	d	8.0	4.75
	2	4.37	dd	9.0, 7.5			2	3.86	dd	9.0, 8.0	
	3	4.41	t	9.0			3	3.77	dd	9.5, 9.0	
	4	4.34	t	9.0			4	4.91	t	9.5	
	5	4.22	ddd	9.0, 5.0, 2.0			5	4.00	ddd	9.5, 6.0, 2.0	
	6a	4.40	m				6a	4.13	dd	12.0, 6.0	
	6b	4.61	dd	13.0, 2.0			6b	4.23	dd	12.0, 2.0	

(600 MHz; **1**: 22 °C, D_2O, **2**: 24 °C, C_5D_5N; **3**: 21 °C, 10% TFA-*d*-CD_3OD).
In cyanosalvianin, **1**, strong NOEs were observed between the signals as follows: H-6 of **2** and H-1 of glcA of **2**, H-4 of **3** and H-1 of glc1 of **3**, H-6 of **3** and H-1 of glc2 of **3**, H-8 of 3 and H-2′ of **3**.

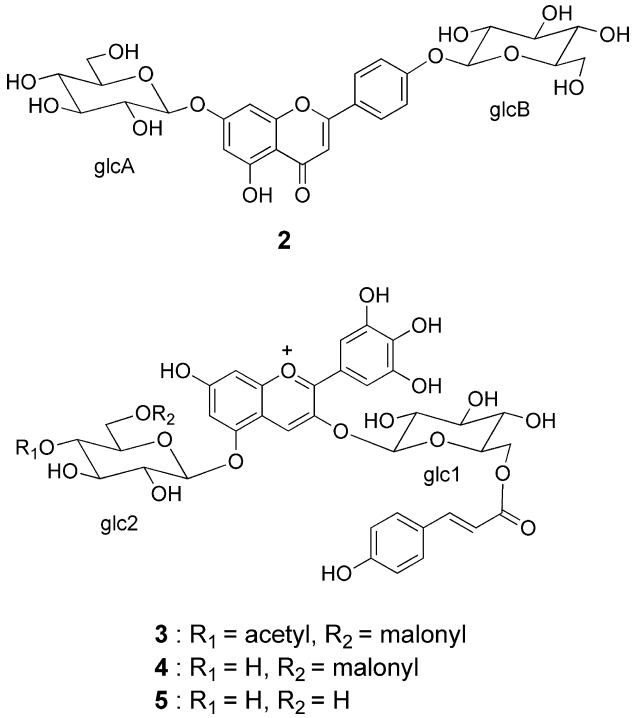

Fig. 3. Structure of components in blue petals of *S. uliginosa*.

4. Thus, structure **3** was identified as 3-*O*-(6-*O*-*p*-coumaroylglucopyranosyl)-5-*O*-(4-*O*-acetyl-6-*O*-malonylglucopyranosyl) delphinidin (Ishikawa et al., 1999).

2.3. Analysis of petal components

To assess the amount of metal ions that may be involved in blue coloration, the amount of **3** and metals were quantified. Anthocyanin was extracted from the blue part of the petals with 2% HCl–MeOH and the combined extracts were analyzed by HPLC. During extraction, some amount of **3** was decomposed to **4** and **5.** Therefore, the total anthocyanin content was quantified using three authentic samples. The metal content was quantified by ICP-AES using ashed blue petals. As shown in Table 2, the contents of anthocyanin in the blue part of the petals were 0.40 μmol/g FW. Mg, Al and Fe were also detected in the blue petal, with the Mg content being than 5 equal to total anthocyanin, whereas the Al and Fe content was very low. These data indicate that Mg^{2+} might be complexed to the anthocyanins, and Al and Fe may not perform as complexation metal ions.

2.4. Reconstruction of cyanosalvianin

To confirm whether the blue color of *S. uliginosa* is developed by a metalloanthocyanin and which components are essential for the

Table 2
Contents of anthocyanin and metal elements in blue petals of *S. uliginosa*

	Anthocyanin (**3** + **4** + **5**)	Mg	Al	Fe
(μmol/1g FW petals)	0.40	2.2 ± 0.001	0.21 ± 0.004	0.094 ± 0.001
(Equal to total anthocyanin)	1	5.4	0.52	0.23

Data were shown as average ± SD (n = 3).

blue pigment, the petal components were mixed together in a buffered solution and the UV–vis spectrum and CD were recorded. Compound **3** was mixed with compound **2** with/without Mg^{2+} in a buffered solution (pH 7.0), then the mixture was diluted with a buffer (pH 5.0) (Kondo et al., 1991). The mixture of **2**, **3** and Mg^{2+} gave the same blue color as that of the petals (Fig. 4), but without Mg^{2+} the solution showed purple color and the hue was unstable. When the mixture of **2**, **3** and Mg^{2+} was diluted with a buffered solution at pH of 7.0, the solution was a little bit greenish in color. Furthermore, the pH of the pressed juice of petals was around 5, therefore, the vacuolar pH of colored petal cells may be weakly acidic, around 5. By gel filtration chromatography, blue pigment could be purified and the obtained blue pigment showed a typical UV–vis spectrum and CD for metalloanthocyanins. In the VIS spectrum, a sharp peak at 588 nm was observed with a small peak at 645 nm and in the CD, a strong exciton-type negative Cotton effect was observed. These findings are very similar to those of commelinin (Goto et al., 1986; Tamura et al., 1986; Kondo et al., 1992). Thus, the metalloanthocyanin in the blue petals of *S. uliginosa* was clarified to be composed of **2**, **3** and Mg^{2+} and named cyanosalvianin (**1**).

The molecular weight of the reconstructed **1** was recorded by ESI-MS. **1** gave multiple-charged peaks at $m/z = 1949.1$ and 1801.8 corresponding to $[M\text{-}6H]^{6-}$ and $[M\text{-}5H]^{5-}$, respectively (Supplementary Data-1). By deconvolution of the Fenn method, as we reported previously (Mann et al., 1989; Kondo et al., 1994b), the molecular weight was measured to be 9014.0. Therefore, the composition of **1** was determined to be six molecules each of **2** and **3**, and two Mg^{2+} ions; the calculated average molecular weight for **1** as $[C_{408}H_{414}O_{228}Mg_2]$-6H was 9008.0.

Using the same procedure, a different metalloanthocyanin was obtained by mixing **2**, **4** and Mg^{2+} and the pigment (protodelphin, Takeda et al., 1994; Kondo et al., 2001) showed a very similar property, UV–vis spectra and CD as that of **1** (Fig. 3). The stability of the blue color (0.05 mM in aqueous buffer solution at pH 5.0) was also measured (Fig. 5). Both reconstructed metalloanthocyanins, cyanosalvianin (**1**) and protodelphin, showed high stability and more than 90% of the color density was retained after 2 days.

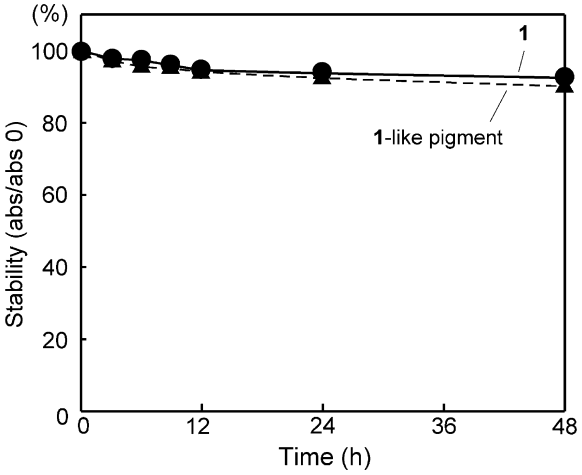

Fig. 5. Stability of cyanosalvianin (**1**) and **1**-like pigment (protodelphin, **2** + **4** + Mg^{2+}) in an aqueous solution (0.05 mM, pH 5, 20 °C, path length: 1 mm).

The absence of the acetyl group at glc2 in **3** did not affect either blue coloration or stability.

2.5. Spatial arrangement of *2* and *3* in cyanosalvianin

The spatial arrangement of organic components, **2** and **3**, in cyanosalvianin (**1**) was studied by CD and NMR spectroscopy. The anti-clockwise self-association of two molecules of **3** in **1** was confirmed from the strong exciton-type negative Cotton effect observed at the same absorption maximum wavelength in the VIS spectrum (Fig. 4) as that of commelinin and other metalloanthocyanins (Kondo et al., 1992, 1994a, 2001; Ellestad, 2006).

To determine the stacking manner between **2** and **3**, various NMR spectroscopic experiments were carried out. The ^1H NMR spectra of **1** in D_2O did not give six-times the signals of components, although **1** consisted of six molecules each of **2** and **3**, but relatively simple spectrum (Fig. 6) indicated their symmetrical arrangement of them as was observed in the case of com-

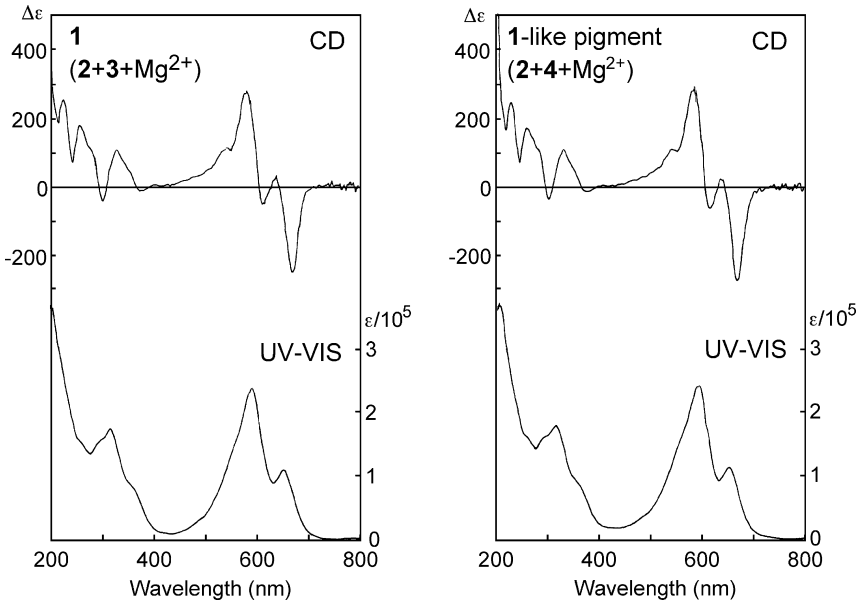

Fig. 4. UV–vis spectra and CD of reproduced cyanosalvianin (**1**) and **1**-like pigment composed of **2**, **4** and Mg^{2+}.

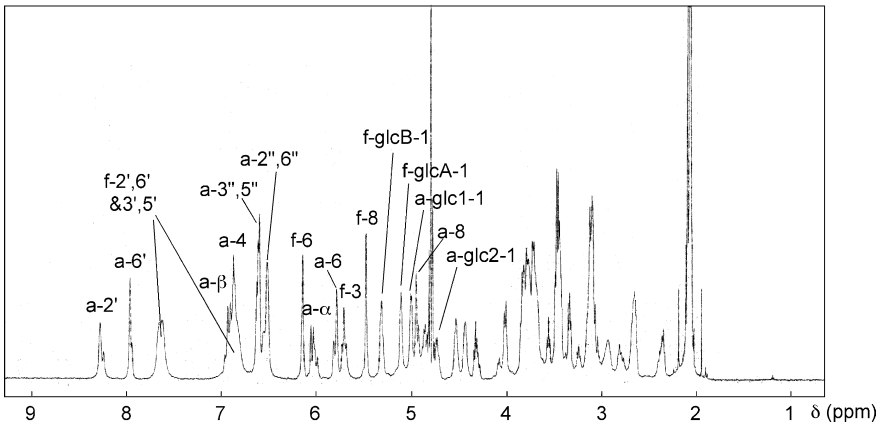

Fig. 6. ^{1}H NMR spectra of cyanosalvianin (**1**) in D_2O. The signals with "a"– indicate those of anthocyanin component (**3**) in **1** and the signals with "f" – indicate those of flavone component (**2**).

melinin (Kondo et al., 1992) and metal-substituted protocyanin (Kondo et al., 1998). Therefore, to assign the signals, the same procedure for the previously reported metalloanthocyanins (Kondo et al., 1992, 1998) was employed. Where the NOE correlations between the anomeric proton of sugars and the signals of chromophores of **2** and **3** (Table 1) were used as the starting point, all the signals of aromatic protons were determined (Table 1, Figs. 6 and 7). The signals of H-2′ and H-6′ of **3** were observed separately, indicating that the bond between the C-ring and the B-ring could not rotate freely. The same phenomenon was observed as that in commelinin (Kondo et al., 1992). Therefore, the existence of strong molecular-stacking among components and anhydrobase anion form of anthocyanidin nucleus were confirmed. The high field signals corresponding to H-6 and H-8 of **3** were also observed at 5.79 ppm and 4.97 ppm, respectively, compared with that of the monomeric flavilium ion form (Table 1). This was caused by the strong anisotropic effect of aromatic rings, indicating the existence of chromophore–chromophore stacking. Long-range NOEs were observed between H-6′ of **3** and H-8 of **2**, the signals of the *p*-coumaroyl residue of **3** and H-3 of **2**, H-2′ of **3** and H-3 of **2**, H-5, 6 of glc1 in **3** and H-3 of **2**. Because of overlapping of signals, NOEs between the protons of 2′, 3′, 5′ and 6′ of **2** and signals of **3** could not be determined. Combining these data, we propose an anti-clockwise chiral self-association of **3**s and a clockwise chiral co-pigmentation of **2** and **3** (Fig. 8).

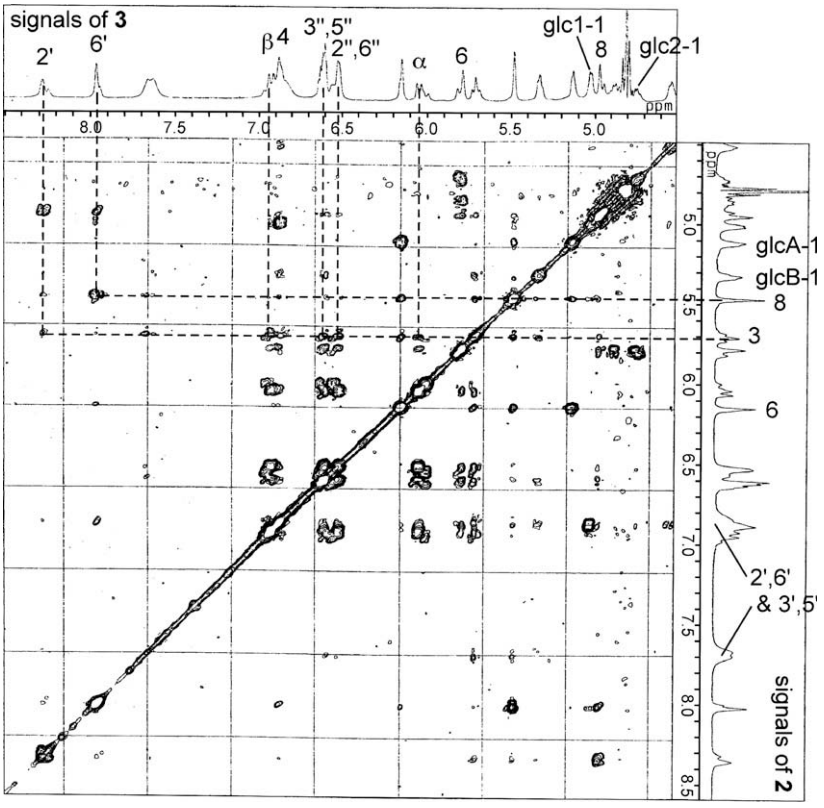

Fig. 7. NOESY spectrum of cyanosalvianin (**1**) in D_2O. Long-range correlations between signals of **2** and those of **3** are indicated as dotted lines. The assigned signals in the horizontal 1D-spectrum indicate those of component **3**, and the assigned signals in the perpendicular 1D-spectrum indicate those of component **2**.

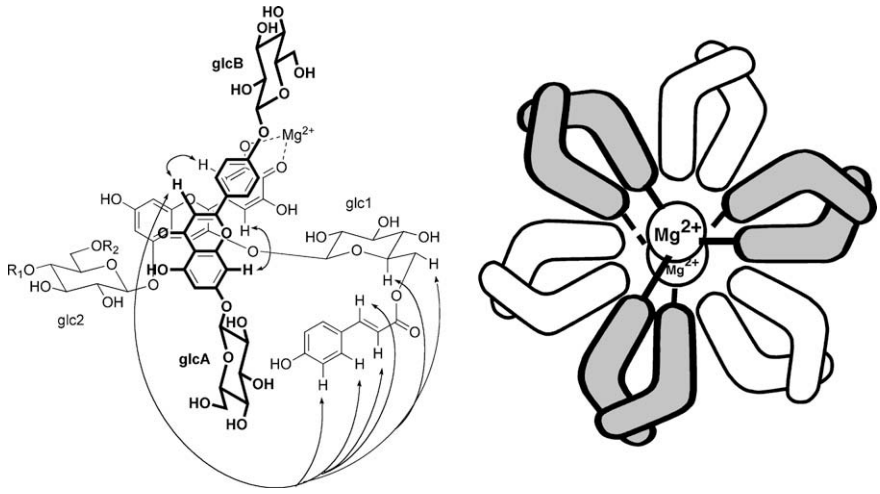

Fig. 8. Chiral stacking of **2** and **3** in cyanosalvianin (**1**) as proposed by long-range NOEs (left), and a schematic model of **1** (right). The left: the molecule drawn with thick lines at the front side indicates **2** and the rear molecule with thin lines is **3**. Arrows indicate long-range NOEs. The right: surrounding the two Mg^{2+} ions that is located at the center of the molecule (**1**), self-associated anthocyanins (**3**, gray colored components) and self-associated flavones (**2**, white colored components with black lines) were alternatively arranged. Three molecules of **3** cheleted to each Mg^{2+}.

3. Concluding remarks

In conclusion, we have discovered a new metalloanthocyanin, cyanosalvianin (**1**) from the blue petals of *S. uliginosa*. The anthocyanin component of **1** was an acetylated derivative of malonylawobanin (**3**) and the flavone component was 7,4′-diglucosylapigenin (**2**). A reconstruction experiment and ESI-MS analysis determined the composition of **1** to be six molecules of **2**, six molecules of **3** and two Mg^{2+} ions, all of which were essential for the blue coloration. Malonylawobanin (**4**) is the anthocyanin component of the first observed metalloanthocyanin, commelinin, and 7,4′-diglucosylapigenin (**2**) is the same flavone component as that of protodelphin from *S. patens*. The whole molecular structure of **1** may be similar to that of commelinin.

All known anthocyanin components in metalloanthocaynins contained a 3,5-diglucosylanthocyanidin structure with at least one acyl moiety, an optimally cinnamoyl derivative, at the 6-OH of the 3-*O*-sugar residue. On the co-pigment components, all are diglycosylflavones, in which one sugar is attached to the 6 or 7-positions of the A-ring and the other sugar is attached to be 4′-OH group of the B-ring. Furthermore, only one sugar molecule's deficit brings a loss of blue petal color (Kondo et al., 1991). Thus suggest diversity of flavonoid biosynthesis being under some genetic control the strongly affects floral color changes. The reason for the small structural diversities of the anthocyanin and flavone component in metalloanthocyanins and their combination to grow the supramolecule is an interesting problem from the viewpoint of the evolution of blue flowers.

4. Experimental

4.1. General experimental procedures

UV–vis spectra and reflection spectra were recorded on a JASCO U best-55 spectrometer with TIS-417 integral sphere apparatus. Circular dichroism (CD) was measured with a JASCO J-720 spectrometer. NMR spectra were obtained with JNM-A600 spectrometer (^{1}H: 600 MHz, ^{13}C: 150 MHz) in a Ø 5 mm tube at variable temperature using 5–10% TFA*d*-CD_3OD, pyridine-d_5 or D_2O as a solvent. Chemical shifts were recorded as parts per million (ppm) with the CD_2HOD, C_5ND_4H or CD_2HCN resonance as a standard. In the NOESY experiments, mixing time were set at 500 ms using a standard pulse sequence of JEOL. FABMS data were recorded on a JEOL JMS-600 and JMS-700 spectrometer using glycerol–HCl as a matrix. Negative mode ESI-MS data were obtained with a PE-Sciex API-III triple-quadropole mass spectrometer using a solution of EtOH–H_2O (1:1, v/v). Elemental analysis was conducted with Seiko Electric SPS1200A apparatus. Analytical HPLC was conducted according to our procedure (Yoshida et al., 1992, 1996, 1997) using an ODS-column (Develosil ODS-HG-5 Ø 4.6 mm× 250 mm, Nomura Chemical). HPLC was performed at 40 °C with 30 min linear gradient elution from CH_3CN–H_2O containing 0.5% CF_3CO_2H (TFA) (1:9, v/v) to CH_3CN–H_2O containing 0.5% TFA (3:7, v/v), or by isocratic elution with various concentrations of CH_3CN aqueous solution containing 0.5% TFA. Preparative ODS-LC was conducted according to our procedure (Yoshida et al., 1992, 1996, 1997) using an ODS-column (Develosil lop ODS Ø 24 mm× 250 mm and ODS-10 Ø 20 mm× 250 mm, Nomura Chemical). Preparative ODS-HPLC was carried out using a column (Develosil ODS HG-5 Ø 20 mm× 250 mm, Nomura Chemical) (Yoshida et al., 2003; Mori et al., 2006).

4.2. Plant materials

Seeds of *S. uliginosa* were purchased from Sakata Seed Co. (Yokohama, Japan) and cultivated at Nagoya University Farm. For isolation of pigments, the blue parts of the petals were collected and stored at −30 °C until use.

4.3. Reflection spectrum of petals

The reflection spectrum of the petals was recorded according to our previously reported procedure (Yoshida et al., 2003).

4.4. Analysis of pressed juice of blue petals

Blue parts of the petals (30 g) were collected and frozen with liquid N_2 and pulverized. The powder was warmed to rt, with H_2O (5 mL) added and the mixture pressed. A blue-colored juice was obtained by filtration with gauze. The UV–vis spectrum and CD of the obtained pressed juice were recorded in a quartz cuvette (path length: 2 mm).

4.5. Isolation of components

4.5.1. Isolation of **2** and **3**

Blue petals (190 g) of *S. uliginosa* were extracted with CH_3CN:H_2O containing 1% TFA (600 mL, 1:1, v/v) and filtered. The residue was re-extracted two times more as above, with the combined extract evaporated under reduced pressure to approximately half volume. The condensed extract was purified using an Amberlite XAD-7 column (Ø 20 mm × 200 mm), with the column eluted with a stepwise gradient elution from 0.5% TFA–H_2O to CH_3CN: 0.5% TFA–H_2O (1:1, v/v). The fraction eluted with CH_3CN: 0.5% TFA–H_2O (5:95 → 15:85, v/v) was evaporated to give a crude flavone. The fraction eluted with CH_3CN: 0.5% TFA–H_2O (20:80 → 25:75, v/v) was evaporated to give a crude anthocyanin and the fraction (CH_3CN: 0.5% TFA–H_2O, 30:70, v/v) contained crude rosmarinic acid. Further purification was conducted by a preparative ODS-LC eluted with AcOH–CH_3CN–H_2O solution containing 0.5% TFA. **2** was eluted at 4% AcOH–5% CH_3CN fraction and the fraction was dried *in vacuo* to give pure **2** (10 mg). **3** was eluted at 8% AcOH–10% CH_3CN fraction to give pure **3** (8 mg) as a TFA salt.

4.5.2. 7,4′-Di-O-glucopyranosylapigenin (**2**)

Amorphous powder: λ_{max} 0.1% HCl–MeOH (logε): 317 (4.15), 269 nm (4.18).

4.5.3. 3-O-(6-O-p-coumaroylglucopyranosyl)-5-O-(4-O-acetyl-6-O-malonylglucopyranosyl) delphinidin (**3**)

Amorphous powder: λ_{max} 0.1% HCl–MeOH (logε): 539 (4.42), 310 nm (4.23), 280 nm (4.21).

4.6. Quantification of anthocyanin content and metals in blue petals

Fresh petals (500 mg) were extracted with 2% HCl–MeOH (1 mL) for 2 h. The extract was separated by decantation, and then 2% HCl–MeOH (1 mL) was added to the residue and which was extracted for 2 h at room temperature. This procedure was repeated twice and the combined extract was analyzed by HPLC. The total amount of anthocyanins (**3**–**5**) was calculated directly by a calibration curve obtained by HPLC analyses of the authentic samples. Other blue petals (514 mg) were degraded in a crucible and the obtained ash was dissolved in 1% (w/v) aqueous HCl. Metal elements were analyzed by ICP-AES under standard operating conditions.

4.7. Reconstruction of cyanosalvianin (**1**)

4.7.1. Small-scale reconstruction

Each stock solution of **2** and **3** (75 mM) in MeOH and 0.5 M aqueous $Mg(OAc)_2$ was added to 0.1 M phosphate buffer (pH 7.0) and mixed together. The mixture was diluted with an acetate buffer (pH 5.0) with final concentrations of **2** and **3** being 0.5 mM and Mg^{2+} to be 0.2 mM; the UV–vis spectrum and CD of the obtained mixture were recorded in a cuvette (path length: 1.0 mm).

4.7.2. Large-scale reconstruction

TFA salt of **3** (21 mg, 21 μmol) and **2** (18 mg, 31 μmol) was dissolved in H_2O (200 μL) and neutralized with aqueous NH_4OH, then dried up *in vacuo*. To the residue was added H_2O (200 μL), 0.5 M aqueous $Mg(OAc)_2$ (100 μL) and 1 M KOAc (100 μL) with the mixture allowed to stand at rt for 18 h. To the solution was added EtOH (5 mL) and the mixture was stored at −45 °C for 1 h. The precipitate was gathered by centrifugation (10,000 rpm, 10 min) and evaporated to dryness *in vacuo*. The obtained dark blue residue was dissolved in H_2O (100 μL) and applied to a gel-filtration column (Cellulofine GC-15m, Ø 12 mm × 100 mm), eluted with H_2O. The blue-colored fraction was combined and evaporated to dryness *in vacuo* to give pure cyanosalvianin (**1**) (7.1 mg, yield, 23%, based on **3**). Using a TFA salt of **4** with the same procedure, cyanosalvianin-like pigment (**2** + **4** + Mg^{2+}) was obtained (yield, 28%, based on **4**).

4.7.3. Cyanosalvianin (**1**)

Amorphous blue powder: CD $\delta\varepsilon_{570}$ +382, $\delta\varepsilon_{606}$ −50, $\delta\varepsilon_{626}$ +26, $\delta\varepsilon_{660}$ −243 (acetate buffer, pH 5.0; *c* 0.045); λ_{max} acetate buffer, pH 5.0 nm (logε): 312 (5.22), 588 (5.38), 645 (5.06). For ^{1}H NMR spectroscopic data, see Table 1. ESI-MS *m*/*z* 1801.8 $[M\text{-}5H]^{5-}$ ($C_{408}H_{414}O_{228}Mg_2$/5 calc. as 1802.8), 1494.1 $[M\text{-}6H]^{6-}$ ($C_{408}H_{414}O_{228}Mg_2$/6 calc. as 1502.3).

4.8. Stability of cyanosalvianin and cyanosalvianin-like pigment

The obtained metalloanthocyanin was dissolved at a concentration of 50 mM in 0.1 M acetate buffer (pH 5.0) and the absorption spectrum was recorded in a cuvette (path length, 10 mm). The stability was measured as the decrease of the absorbance at the λ_{vismax} at 20 °C.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.03.015.

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