ACYLATED CYANIDIN GLYCOSIDES IN THE ORANGE-RED FLOWERS OF SOPHRONITIS COCCINEA

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Key Word Index—Sophronitis coccinea; Orchidaceae; orange-red flower colour; acylated anthocyanins; cyanidin 3,7,3′-triglucoside; malonylglucoside; malonic, caffeic, p-coumaric and ferulic acids.

Abstract—Five acylated anthocyanins were isolated from the orange-red flowers of Sophronitis coccinea. Their structures were based on cyanidin 3,3′,7-triglucoside, acylated variously with malonic, p-coumaric, caffeic and ferulic acids. Three anthocyanins were fully determined to be cyanidin 3-O-[6-O-(malonyl)-β-D-glucopyranoside]-3′-O-[β-D-glucopyranoside]-7-O-[6-O-(trans-cafeoyl)-β-D-glucopyranoside] and its demalonyl derivative, and cyanidin 3-O-[6-O-(malonyl)-β-D-glucopyranoside]-3′-O-[β-D-glucopyranoside]-7-O-[6-O-(trans-feruloyl)-β-D-glucopyranoside]. Two other pigments were partly characterized as p-coumaroyl cyanidin 3-malonylglucoside-7,3′-diglucoside and feruloyl cyanidin 3,7,3′-triglucoside.

INTRODUCTION

Sophronitis plants with red, orange, pink and yellow colours are popular orchid ornamentals, and native to Brazil. Recently some species of Sophronitis have been hybridised with Cattleya with the purpose of producing the red or orange-red flower cultivars in the Cattleya alliance. There are two preliminary reports on the occurrence of anthocyanins in the flowers of Sophronitis [1, 2]. In continuing work on flower colour variation in orchids, we have already reported the occurrence of acylated cyanidin and peonidin glycosides in the flowers of Dendrobium (Pramot) [3], × Laeliocattleya “Mini Purple” [4, 5], Bletilla striata [6], Cymbidium hybrids [7] and Phalaenopsis hybrids [8]. In this paper we report the occurrence of new acylated cyanidin 3,7,3′-triglucosides in the orange-red flowers of Sophronitis coccinea and their structural elucidation.

RESULTS AND DISCUSSION

By HPLC analysis of the MAW (methanol-acetic acid-water, 4:1:15) extracts from the orange-red flowers of Sophronitis coccinea, we observed the presence of twenty anthocyanin peaks. Five anthocyanins (1-5) were isolated from this extracts, and purified using Diaion HP-20 column chromatography (C), paper C, HPLC and TLC. The relative concentrations were 1 (49.3%), 2 (16.4%), 3 (12.3%), 4 (6.7%) and 5 (6.3%). The Rf values, Rt (min) and spectral data of these five pigments are shown in Table 1.

Acid hydrolysis of these anthocyanins gave cyanidin, glucose and hydroxycinnamic acids. By alkaline hydrolysis these five pigments yielded only one deacylanthocyanin, identified as cyanidin 3,7,3′-triglucoside by direct comparison with an authentic sample from deacyl Dendrobium, × Laeliocattleya and Bletilla anthocyanins [3-6]. Also as acyl moieties, caffeic acid was detected in the hydrolysis products of 1 and 2, ferulic acid in 3 and 5, and p-coumaric acid in 4, and malonic acid in 1, 3 and 4, respectively, by acid and alkaline hydrolysis.

The measurements of FAB mass and 1H NMR spectra of these five acylated anthocyanins led to the determination of the molecular ratios of chemical composition (aglycone, sugar and acid) as shown in Table 2. Among these five pigments, the detailed structures of the three pigments (1–3) were successfully determined by spectral and chemical methods, but the other two pigments (4, 5) could not be determined because of the small amounts obtained.

Pigment 1 and 2

The FAB mass measurement of 1 gave a molecular ion [M]+ at 1021 m/z in good agreement with the mass
Table 1. Chromatographic and spectral properties of anthocyanins from flowers of *Sophronitis*

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<th>Anthocyanin</th>
<th>BAW Rf</th>
<th>BuHCl Rf</th>
<th>1% HCl Rf</th>
<th>AHW Rf</th>
<th>RI values (× 100)</th>
<th>Spectral data in 0.1% HCl-MeOH</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>( E_{1%} / E_{\text{max}} ) (%)</th>
<th>( E_{420} / E_{\text{max}} ) (%)</th>
<th>( A_{480} / A_{\text{max}} ) (%)</th>
<th>AlCl₃ (min)</th>
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<td>2 (demalonyl 1)</td>
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<td>41</td>
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<tr>
<td>Cy 3,3',3''-trigl.</td>
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Table 2. The estimated molecular formulae of acylated anthocyanins from *Sophronitis* and their molecular ratios of chemical composition based on FAB mass and ¹H NMR data.

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<td>1</td>
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Based on FAB-MS

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Based on ¹H NMR

Abbreviations: [M]+ and Mf = molecular ion mass values, and estimated molecular formulae as flavylum forms of anthocyanins isolated from *Sophronitis* based on FAB-mass data, respectively. Cy: Glc: pc: Caf: Fer: Mal = molecular numbers of their components; Cy = cyanidin, Glc = glucose, pc = p-coumaric acid, Caf = caffeic acid, Fer = ferulic acid and Mal = malonic acid.

Molecular numbers were based on the integrated intensities of proton signals such as cyanidin = H-4, glucose = H-1 and hydroxycinnamic acid = olefinic proton (H-x). Each integrated intensity of proton signal was normalized in such a way that cyanidin H-4 is 1, respectively.

Refs [4–6, 8].
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calculated for C_{45}H_{49}O_{27} (1021.246), indicating the presence of one molecule of cyanidin, three of glucose, one of caffeic acid and one of malonic acid (Tables 2 and 3). The 500 MHz proton NMR spectra of 1 were measured in DMSO-<sub>d6</sub> solvent containing 10% TFA. The proton signals were mainly assigned by analysis of its <sup>1</sup>H-'H COSY spectrum, and the sugar and acyl linkages were confirmed by the negative difference nuclear Overhauser effect (DIFNOE) spectra as described previously [9] (Fig. 1). The proton signals of the sugar moieties were observed in the region of δ 5.45–3.13. The signals of three anomeric protons appeared at δ 5.45 (<i>d</i>, <i>J</i> = 8.0 Hz, Glc A), δ 5.30 (<i>d</i>, <i>J</i> = 7.5 Hz, Glc B) and δ 4.88 (<i>d</i>, <i>J</i> = 7.5 Hz, Glc C), and the assigned sugar protons had the coupling constants with <i>J</i> = 7.5–12.0 Hz indicating all these glucose units to be β-D-glucopyranose forms. Four methylene protons, being shifted to a lower magnetic field, were

Table 3. <sup>1</sup>H NMR data of Sophronitis anthocyanins (CF<sub>3</sub>CO<sub>2</sub>D-DMSO-<sub>d6</sub>, 1:9, at 25°C)

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<td>8</td>
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<td>2'</td>
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<td>6'</td>
<td>8.63 brd (9.5)</td>
<td>8.63 brd (8.5)</td>
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<td>Hydroxycinnamyic acid* **</td>
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<td>6.81 brs</td>
<td>6.82 brs</td>
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<tr>
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<td>6.55 d (8.5)</td>
<td>6.55 d (7.5)</td>
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<td>6.68 brd (8.5)</td>
<td>6.71 brd (8.5)</td>
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<td>6.38 d (15.5)</td>
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<td>3.43</td>
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<td>3.27~3.40</td>
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<tr>
<td>5</td>
<td>3.87</td>
<td>3.10~3.92</td>
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<td>6b</td>
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<td>(B) 7 Gt</td>
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*Assigned by 'H-'H COSY.
**Assigned by DIFNOE

Coupling constants (J in Hz) in parentheses.
assigned to H-6a and 6b of Glc A (δ 4.18 and 4.50) and those of Glc B (δ 4.20 and 4.58) and also were correlated to their anomeric protons by analysis of the 'H-'H COSY spectrum. This result indicated that these two glucose units were acylated with acids at their OH-6 groups.

In order to determine the linkages and/or positions of the attachments between the glucose and acyl units in this pigment molecule, DIFNOE spectra of 1 were measured (Fig. 1). Observed DIFNOE spectra between H-1 of Glc A and H-4 of cyanidin indicates that Glc A is attached to OH-3 of cyanidin through a glycosidic bond by irradiations of H-1 of Glc A and H-4 of cyanidin. Glc B was determined to be attached to the OH-7 of cyanidin through a glycosidic bond, because of the presence of NOES between H-6 and H-8 of cyanidin and H-1 of Glc B. Similarly, Glc C was determined to be glycosylated at O1-3' of cyanidin, because of the presence of NOES between H-2' of cyanidin and H-1 of Glc C. Moreover, irradiation at H-6b of Glc B gave a DIFNOE spectrum in which NOES to protons of H-CY and j of caffeic acid were observed. Thus, caffeic acid is confirmed to be attached to OH-6 of Glc B. By H2O2 degradation of 1 malonylgucose was detected, indicating that malonic acid is attached to 6-OH of Glc A [10]. Therefore, 1 is cyanidin 3-O-[6-O-(malonyl)-β-D-glucopyranoside]-7-O-[6-O-(trans-cafeoyl)-β-D-glucopyranoside]-3'-O-[β-D-glucopyranoside], which is a new anthocyanin [11, 12].

**Fig. 1. Anthocyanins from Sophronitis coccinea. R 1: OH 2: OH 3: OCH3.** Observed NOEs are indicated by arrows.

Pigment 3, 4 and 5

The FAB mass spectra of 3, 4 and 5 gave their molecular ions 1035 m/z (3), 1005 m/z (4) and 949 m/z (5) as shown in Tables 1 and 2. These values are in good agreement with the mass calculated for their theoretical molecules (Table 2), respectively, which are composed of cyanidin 3,7,3'-triglucoside with each of p-coumaric or ferulic acid. The pigments 3 and 4 have malonic acid as an additional acid. Their Rf values, Rt (min) and spectral properties are summarized in Table 1. By H2O2 degradation of 3 and 4, malonylgucose was detected, however similar treatment of 5 afforded glucose without any acyl residue, indicating that the 3-glucose residues of 3 and 4 are substituted with malonic acid and that of 5 is free from malonic acid. On alkaline hydrolysis 3-5 gave cyanidin 3,7,3'-triglucoside as their deacylanthocyanin, where ferulic acid was obtained in 3 and 5, and p-coumaric acid was detected in 4. Based on these findings, the molecular ratios of cyanidin:glucose:acyls (acids) in 3,4 and 5 were determined by the analysis of FAB mass data as shown in Table 2. Furthermore, the structure of 3 was confirmed by analysis of their 'H NMR spectra including 'H-'H COSY spectra. The 'H NMR spectrum of 3 was superimposed on that of 1 except for the signals of methoxyl group in ferulic acid moieties (Table 3). Also, four methylene proton signals of Glc A and B were shifted to the lower magnetic field (δ 4.14, 4.49 and δ 4.18, 4.59). Thus, 3 is cyanidin 3-O-[6-O-(malonyl)-β-D-glucopyranoside]-7-O-[6-O-(trans-feruloyl)-β-D-glucopyranoside]-3'-O-[β-D-glucopyranoside], which is a new pigment [11, 12].
In the 1H NMR spectrum of 4, the chemical shifts of cyanidin and p-coumaric acid moieties were determined, but other proton chemical shifts except three anomic and four methylene protons were not detected due to the heavy overlapping of proton signals. Also the linkages between glucose units and a p-coumaric acid unit could not be determined because of a small amount of 4 obtained. Thus, 4 was tentatively assigned as trans-p-coumaroyl cyanidin 3-malonylglucoside-7,3'-diglucoside. The further structure study of 5 was not carried out because of its low yield. Consequently the structure of 5 was assumed to be feruloyl cyanidin 3,7,3'-triglucoside.

Six papers have so far been published on the occurrence of anthocyanidin 3-malonylglucosides in the flowers of Dendrobium, Laelia, Cattleya, Bletilla, Cymbidium, and Phalaenopsis [3-8, 14]. Thus, this is the seventh report of occurrence of malonylated anthocyanin in orchid flowers. Our previous studies showed that the orchids with red-purple flowers, such as Cattleya, Bletilla contained di- or tri-(hydroxycinnamoyl) cyanidin 3,7,3'-triglucosides as their major pigments [3-6, 8]. However, Sophronitis coccinea contained mono-(hydroxycinnamoyl) cyanidin 3,7,3'-triglucosides with carotenoid pigments [2]. A simple acylation in Sophronitis flowers might be thought to play an important role in producing orange-red flowers [16]. According to Manuel et al., this colour of flowers attracts hummingbirds as a pollinator [15, 17] as contrasted with the red-purple flowered orchids with di- or tri-aromatic acid-acylation which are known to attract insects for pollination [18].

**EXPERIMENTAL**

**Plant material**

The flowers of Sophronitis coccinea were obtained from the cultivators of Utsunomiya Ranyuyaki (Utsunomiya Orchid Club, Tochigi, Japan), Mr. M. Abou (Gifu, Japan) and Mr. T. Yamamoto (Cc-Orchid Ltd., Shizuoka, Japan). The fresh orange-red flowers were collected in spring of 1994 and 1995.

**Isolation of Sophronitis anthocyanins**

Dried orange-red flowers (100 g) of S. coccinea were extracted with MAW (11, MeOH-HOAc-H2O, 4:1:15). The extract was purified by Diaion HP-20, CC, PC, TLC and HPLC by the previous procedures [3, 4, 5, 6, 7, 8]. Solvents used were 15% HOAc, BAW (n-BuOH-HOAc-H2O, 4:1:2), 5% HOAc-MeOH and MAW for CC, PC and TLC. HPLC was performed on LC-6A system (Shimadzu). Prep. HPLC was run on a Waters C18 (4.6x50mm) column at 40°C with a flow rate of 1 ml min⁻¹ monitoring at 530 nm for anthocyanins. A solvent system used was as follows: a linear gradient elution for 40 min from 25 to 85% solvent B in solvent A.

**FAB mass and NMR measurements**

FAB mass spectra were recorded in positive mode using the magic bullet and in negative mode in glycerol. NMR spectra were recorded at 500 MHz for 1H spectra in DMSO-d6,CF3COOD (9:1). Chemical shifts are reported relative to a TMS int. standard (δ) and coupling constants are reported in Hz.

**1H NMR spectral data**

**Pigment 4:** cyanidin δ 8.87 (1H, s, H-4), 6.82 (1H, brs, H-6), 7.51 (1H, brs, H-8), 8.17 (1H, brs, H-2'), 7.08 (1H, d, J = 7.5, H-5'), 8.64 (1H, brd, J = 7.5, H-6'), p-coumaric acid 7.18 (2H, d, J = 8.5, H-2,6), 6.54 (2H, d, J = 8.5, H-2,6), 6.28 (1H, d, J = 15.0, H-α), 7.42 (1H, d, J = 15.0, H-β), Sugars 5.47 (1H, d, J = 7.3, Glc A-1), 5.31 (1H, d, J = 7.0, Glc B-1), 4.86 (1H, d, J = 7.6, Glc C-1), 4.20 (1H, m, Glc A-6a), 4.40 (1H, brd, J = 11.0, Glc A-6b), 4.23 (1H, m, Glc B-6a), 4.59 (1H, brd, J = 11.5, Glc B 6b) [14].
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