Structure of Malonylshisonin, a Genuine Pigment in Purple Leaves of *Perilla ocimoides* L. var. *crispa* Benth

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The structure of malonylshisonin, a genuine pigment in purple *Perilla* leaves, was determined to be 3-*O*-(6-*O*-(*E*)-*p*-coumaryl- β -D-glucopyranosyl)-5-*O*-(6-*O*-malonyl- β -D-glucopyranosyl)-cyanidin.

Purple leaves of *Perilla ocimoides* (Japanese name shiso) are widely used for coloring traditional Japanese plum pickles. In 1935, Kuroda isolated from the purple leaves a pigment and named it shisonin.¹⁾ About 30 years later, Takeda and Hayashi,²⁾ and Watanabe *et al.*³⁾ determined its structure to be cyanidin 3-(6-*p*-coumaryl-D-glucosido)-5-glucoside, whose complete structure was established by us.⁴⁾ We have recently reported that shisonin is actually an artifact; the genuine anthocyanin⁵⁾ is a malonylated shisonin named malonylshisonin, whose complete structure and stereochemistry is 3-*O*-(6-*O*-(*E*)-*p*-coumaryl- β -D-glucopyranosyl)-5-*O*-(6-*O*-malonyl- β -D-glucopyranosyl)-cyanidin. Takeda *et al.*⁶⁾ also assumed it to be malonylated shisonin from its FABMS analysis. This paper is a detailed report on the isolation and structural determination of malonylshisonin.

Fresh leaves of *Perilla ocimoides* were extracted with 3% aqueous trifluoroacetic acid (TFA), chromatographed on an Amberlite XAD-7 column and purified by means of a preparative ODS-HPLC to give a pigment, malonylshisonin (1), as a dark red amorphous TFA salt.

By acid hydrolysis, 1 was converted to shisonin (2). FABMS of 2 gave its molecular peak at m/z 757. Its ^1H NMR spectrum (500 MHz) showed the presence of a cyanidin nucleus, a (*E*)-*p*-coumaryl moiety ($J_{\alpha,\beta}$ = 16 Hz) and two hexopyranosides, in which all of the vicinal methine coupling constants were 7.5~9.0 Hz, indicating that both of the hexosides were β -D-glucopyranosides. The position of attachment of the sugars to the anthocyanidin nucleus was shown by means of low-temperature NOE difference spectra. Irradiation of the anomeric protons, $\blacktriangle$ -1 (5.49 ppm) and $\bullet$ -1 (5.21 ppm), at -20°C in CD_3OD containing

Fig. 1. ^1H - ^1H COSY Spectrum of the Sugar Moieties of **1** in 3% TFA- d - CD_3OD at 25°C .

3% TFA- d decreased the intensity of the peaks of H-4 (−11%) and H-6 (−6%), respectively, indicating that the ▲- and ●-glucose moieties were attached at the 3 and 5 positions of the cyanidin nucleus, respectively.⁷⁾ The assignments of all protons of the sugar moieties were made by ^1H - ^1H spin decoupling experiments. The methylene protons of ▲-glucose appeared in about 0.5 ppm lower field than that of ●-glucose, indicating that the 6-position of ▲-glucose was acylated. Thus, **2** must have been 3-*O*-(6-*O*-(*E*)-*p*-coumaryl)- β -D-glucopyranosyl)-5-*O*-(β -D-glucopyranosyl)cyanidin.

The FABMS of **1** (m/z 843) suggested the existence of a malonyl group on **2**, which was confirmed by the isolation of dimethyl malonate (38% yield) from an acid methanolysis of **1** with 4% methanolic hydrochloric acid at 100°C . All signals in the ^1H NMR spectrum of **1** could be assigned by means of ^1H - ^1H COSY (Fig. 1) and low-temperature NOE difference experiments (Fig. 2). The methylene signals (4.54 and 4.23 ppm) of ●-glucose were shifted to lower fields than those of **2**, indicating the attachment of the malonyl moiety at this position. Therefore, the structure of **1** was de-

Fig. 2. NOE Difference Spectra of the Sugar Moieties of **1** at 0°C, Irradiating at $\blacktriangle$ -1 (i), and $\bullet$ -1 (ii).

terminated as that shown before.

Experimental

General procedures. UV-VIS data were recorded on a Hitachi UV-228 spectrometer. ^1H NMR and ^1H - ^1H COSY spectra were obtained on a Bruker WM 360 and a JEOL GX500 spectrometer in a 5 mm tube at variable temperatures. Chemical shifts are reported as parts per million (ppm) downfield from internal tetramethylsilane (TMS) as a standard. FABMS data were recorded on a JEOL DX-300/DA5000 system, using a 1 N HCl-glycerol matrix. For GC, a Shimadzu GC 9A equipped with a glass column and a FID detector was used. HPLC was carried out using a JASCO Trirotar III with a UVDEC 100-III detector. Analytical HPLC was carried out by using an ODS-column (Develosil ODS-5, Nomura Chemical), which was eluted with a 40% A solution (diluted with

H_2O ; A was a mixture of AcOH - CH_3CN - H_2O =20:25:55) containing 3% H_3PO_4 . For preparative HPLC, an ODS glass column (Develosil ODS-lop) was used, which was eluted with a 35% A solution containing 3% TFA. For replacing H_3PO_4 with a volatile acid, the eluate was diluted by four times the volume of H_2O , absorbed on the ODS-column, and eluted with solution A containing 1% TFA.

Isolation of malonylshisonin (1). Fresh purple leaves (500 g) of *Perilla ocimoides* were extracted three times with a 3% aqueous trifluoroacetic acid (TFA) solution (500 ml) at ambient temperature. The combined red extract was filtered and poured into an Amberlite XAD-7 column, and the column was eluted stepwisely from H_2O to CH_3CN containing 1% TFA. The 20% CH_3CN fraction contained mainly a pigment, which was purified by preparative ODS-HPLC, using AcOH - CH_3CN - H_2O (7:9:84) containing 3% H_3PO_4 as an eluate. After replacing the counter anion,

the pure fraction was dried to give malonylshisonin (**1**) as a dark-red amorphous solid of the TFA salt (yield 650 mg); UV-VIS $\lambda_{\max}$ (0.01% HCl-MeOH) nm (ϵ): 517 (27,700), 315 (17,000), 280 (22,200); $E_{315\text{nm}}/E_{517\text{nm}}=0.613$; ^1H NMR (3% TFA-*d* in CD_3OD at 25°C) δ : 8.89 (1H, s, H-4), 8.23 (1H, dd, $J=1.5$ and 9.5 Hz, H-6'), 7.98 (1H, d, $J=1.5$ Hz, H-2'), 7.37 (1H, d, $J=16$ Hz, H- β), 7.21 (2H, d, $J=8.5$ Hz, H-2'' and 6''), 6.99 (1H, d, $J=8.5$ Hz, H-5'), 6.98 (1H, d, $J=1.8$ Hz, H-6), 6.92 (1H, br. s, H-8), 6.71 (2H, d, $J=8.5$ Hz, H-3'' and H-5''), 6.24 (1H, d, $J=16$ Hz, H- α), 5.45 (1H, d, $J=7.5$ Hz, $\blacktriangle$ -1), 5.19 (1H, d, $J=7.5$ Hz, $\bullet$ -1), 4.54 (1H, dd, $J=2.5$ and 12 Hz, $\bullet$ -6a), 4.52 (1H, dd, $J=3.0$ and 12 Hz, $\blacktriangle$ -6a), 4.43 (1H, dd, $J=8$ and 12 Hz, $\blacktriangle$ -6b), 4.23 (1H, dd, $J=6.5$ and 12 Hz, $\bullet$ -6b), 3.95 (1H, ddd, $J=3, 8$ and 9 Hz, $\blacktriangle$ -5), 3.80 (1H, ddd, $J=2.5, 6.5$ and 9 Hz, $\bullet$ -5), 3.78 (1H, dd, $J=7.5$ and 9 Hz, $\bullet$ -2), 3.76 (1H, dd, $J=7.5$ and 9 Hz, $\blacktriangle$ -2), 3.62 (1H, t, $J=9$ Hz, $\blacktriangle$ -3), 3.58 (1H, t, $J=9$ Hz, $\bullet$ -3), 3.50 (1H, t, $J=9$ Hz, $\blacktriangle$ -4), 3.46 (1H, t, $J=9$ Hz, $\bullet$ -4); a negative NOE at 0°C , H-4 (irrad.) $\rightarrow$ $\blacktriangle$ -1 (-14%), H-6 $\rightarrow$ $\bullet$ -1 (-10%).

Analysis of malonic acid. **1** (1.5 mg) was heated at 100°C in 4% methanolic HCl (1 ml) for 16 min. The solution was diluted with ether, washed with H_2O , and evaporated to give dimethyl malonate in a 38% yield, which was identified by comparing with an authentic sample by GC analysis using a column (1 m) of 5% Carbowax 20M on Chromosorb W at 70°C (t_R 8.8 min, the yield was calculated from the calibration curves).

Hydrolysis of 1 to shisonin (2). **1** (15.1 mg) was allowed to stand for 12 hr in a 1% HCl-MeOH solution (2 ml) at 40°C , and the reaction mixture was condensed *in vacuo*. The residue was dissolved in 1% aq. TFA and the solution was charged to a preparative ODS column. Elution of the column with 35% A solution containing 3% TFA gave shisonin TFA salt (**2**) (7.6 mg, 55% yield) as a dark-red mass; UV-VIS $\lambda_{\max}$ (0.01% HCl-MeOH) nm (ϵ) 516 (22,400), 314 (14,800), 279 (19,700); $E_{314\text{nm}}/E_{516\text{nm}}=0.667$; ^1H NMR (3% TFA-*d* in CD_3OD at 25°C) δ : 8.94 (1H, s, H-4), 8.25 (1H, dd, $J=2.5$ and 8.5 Hz, H-6'), 8.00

(1H, d, $J=2.5$ Hz, H-2'), 7.37 (1H, d, $J=16$ Hz, H- β), 7.28 (2H, d, $J=8.5$, H-2'' and 6''), 7.01 (1H, d, $J=8.5$ Hz, H-5'), 7.00 (1H, br. s, H-6), 6.93 (1H, br. s, H-8), 6.76 (2H, d, $J=8.5$ Hz, H-3'' and 5''), 6.22 (1H, d, $J=16$ Hz, H- α), 5.43 (1H, d, $J=7.5$ Hz, $\blacktriangle$ -1), 5.17 (1H, d, $J=7.5$ Hz, $\bullet$ -1), 4.50 (1H, dd, $J=3$ and 12 Hz, $\blacktriangle$ -6a), 4.47 (1H, dd, $J=6.5$ and 12 Hz, $\blacktriangle$ -6b), 3.98 (1H, dd, $J=2$ and 12 Hz, $\bullet$ -6a), 3.91 (1H, ddd, $J=3, 6.5$, and 9 Hz, $\blacktriangle$ -5), 3.76 (1H, dd, $J=7.5$ and 9 Hz, $\blacktriangle$ -2), 3.75 (1H, $J=7.5$ and 9 Hz, $\bullet$ -2), 3.74 (1H, dd, $J=6$ and 12 Hz, $\bullet$ -6b), 3.62 (1H, ddd, $J=2, 6$ and 9 Hz, $\bullet$ -5), 3.62 (1H, t, $J=8.5$ Hz, $\blacktriangle$ -3), 3.58 (1H, t, $J=9$ Hz, $\bullet$ -3), 3.53 (1H, t, $J=9$ Hz, $\blacktriangle$ -4), 3.45 (1H, t, $J=9$ Hz, $\bullet$ -4).

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