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An acetylated anthocyanin from the blue petals of *Salvia uliginosa*

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Abstract

The structure of a new anthocyanin from *Salvia uliginosa* petals has been identified as delphinidin 3-*O*-[6-*O*-(*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[4-*O*-acetyl-6-*O*-malonyl-β-D-glucopyranoside] using mass spectroscopy and NMR. This compound constitutes the anthocyanin component of the pigment responsible for blue flower colour in *S. uliginosa*. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Salvia uliginosa*; Lamiaceae; Petals; Blue pigment; Anthocyanins

1. Introduction

The pigments responsible for blue flower colours are complex and generally consist of several components, including a coloured anthocyanin, a colourless flavonoid copigment and one or more metal ions (Brouillard & Dangles, 1993; Kondo et al., 1992). A number of species in the genus *Salvia* L. (Lamiaceae) have intensely blue flowers and several years ago the anthocyanin component of the blue flower pigment of *S. patens* was identified as malonylawobanin (Takeda, Yanagisawa, Kifune, Kinoshita & Timberlake, 1994), a compound first isolated from *Commelina communis* (Goto, Kondo, Tamura & Takase, 1983; Hayashi, Abe & Mitsui, 1958). At present we are studying the pigment responsible for the sky-blue flowers of *S. uliginosa* and have already described the structures of several potential flavonoid copigments, including two unusual apigenin 7-*O*-cellobiosides (Veitch, Grayer, Irwin & Takeda, 1998). In this paper, the structural elucidation of the anthocyanin component of the blue pigment in *S. uliginosa* petals is reported.

2. Results and discussion

The major anthocyanin (**1**) of *S. uliginosa* petals was purified by semi-prep. HPLC. It was characterised by a UV–VIS spectrum (λ_{max} 1% MeOH–HCl: 281, 308 and 543 nm; $\varepsilon_{308\ nm}/\varepsilon_{543\ nm} = 0.57$) similar to that of malonylawobanin, an anthocyanin isolated from the deep blue petals of *S. patens* (Takeda et al., 1994). However, TLC comparison of **1** with an authentic sample of malonylawobanin (**2**) indicated that the two anthocyanins were different. Acid hydrolysis of **1** showed that this compound was based on delphinidin and glucose, while alkaline hydrolysis revealed the presence of three acyl groups, identified as *p*-coumaric acid, malonic acid and acetic acid. Delphinidin, glucose, *p*-coumaric acid and malonic acid are also components of **2**, but this anthocyanin does not contain an acetyl group. Hydrogen peroxide oxidation of **1**

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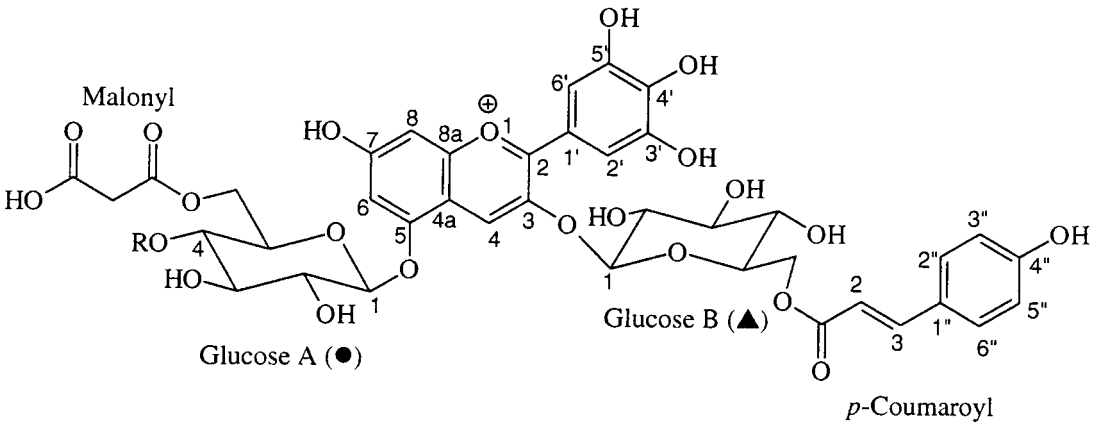

1 R = Ac
2 R = H

yielded 6-*O*-*p*-coumaroylglucose, which suggested that the acetyl group was not attached to the same glucose moiety as the *p*-coumaroyl group. Positive ion FAB-MS applied to **1** and **2** gave molecular ions of *m*/*z* 901 and *m*/*z* 859, respectively. This difference of 42 mass units is consistent with an additional C_2H_2O (acetyl) group attached to **1**. The MS analysis was extended by means of high energy CAD spectrometry as shown in Fig. 1, where the assignments of fragment ions for **1** and **2** are indicated. These data support the conclusion that the additional acetyl group in **1** is attached to the glucose moiety which bears the malonyl, rather than the *p*-coumaroyl, group. However, it was not possible to determine the linkage position of the acetyl group to glucose A of **1** on the basis of MS data alone.

Analysis of the ^{1}H NMR spectra of **1** and **2** confirmed the presence of an acetyl group in **1** (δ 2.12, 3H, s), and indicated that other differences were confined to the spectral region between 3.00 and 5.50 ppm corresponding to resonances of the two glucose moieties (labelled A and B, see Table 1). The DQF-COSY spectrum of **1** (Fig. 2) was used to obtain the complete assignment of both glucose A and B using the anomeric proton resonances at δ 5.23 (d, J = 7.7 Hz) and 5.43 (d, J = 7.7 Hz) as starting points. 1D-ROESY spectra were also acquired in order to assign the location of these two glucose moieties. Irradiation of the anomeric proton resonances at δ 5.23 and 5.43 gave ROE connectivities to H-6 (but not H-8) and H-4, respectively. This indicated that the glucose moiety (A) with an anomeric proton resonance at δ 5.23 must be attached at the 5-OH position, while glucose B, with an anomeric proton resonance at δ 5.43, must be attached at the 3-OH position. The magnitudes of the $^3J_{1,2}$ coupling constants for these anomeric proton resonances indicate that glucose is only present in the β-D configuration (Agrawal, 1992). The chemical shift values for the 6-CH_2 protons of glucose A and B are shifted downfield (Table 1), indicating acylation at the C-6 position in both cases. It should be noted that the ^{1}H chemical shift values for glucose B in **1** are identical within experimental error to the corresponding values for **2**, where glucose is acylated at the C-6 position by *p*-coumaric acid. The assignments for the proton resonances of glucose A in **1** are of particular interest (Table 1). Here the most striking feature is the large downfield shift experienced by H-4, which, at δ 4.92 represents a perturbation of +1.48 ppm compared to H-4 of glucose A in **2**. This indicates that C-4 is an additional site of acylation on glucose A of **1**. A number of the resonances of neighbouring atoms on glucose A, including H-3, H-5 and one of the 6-CH_2 protons, also experience smaller chemical shift perturbations due to C-4 acylation as expected. Analysis of the HMBC spectrum of **1** uncovered long-range $^3J(^1$H, ^{13}C) connectivities between the 6-CH_2 proton of glucose A at δ 4.13 and the carbonyl carbon of the malonyl group, the 6-CH_2 proton of glucose B at δ 4.42 and the carbonyl carbon of the *p*-coumaroyl group and between H-4 of glucose A at δ 4.92 and the carbonyl carbon of the acetyl group. The spectroscopic results indicate that anthocyanins **1** and **2** are identical apart from C-4 acetylation of glucose A in **1**. Compound **1** is therefore delphinidin 3-*O*-[6-*O*-(*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[4-*O*-acetyl-6-*O*-malonyl-β-D-glucopyranoside], a new anthocyanin which can be referred to by the trivial name acetylmalonylawobanin. It is interesting to note that both this anthocyanin and the two apigenin 7-*O*-cellobiosides isolated previously from the petals of *S. uliginosa* (Veitch et al.,

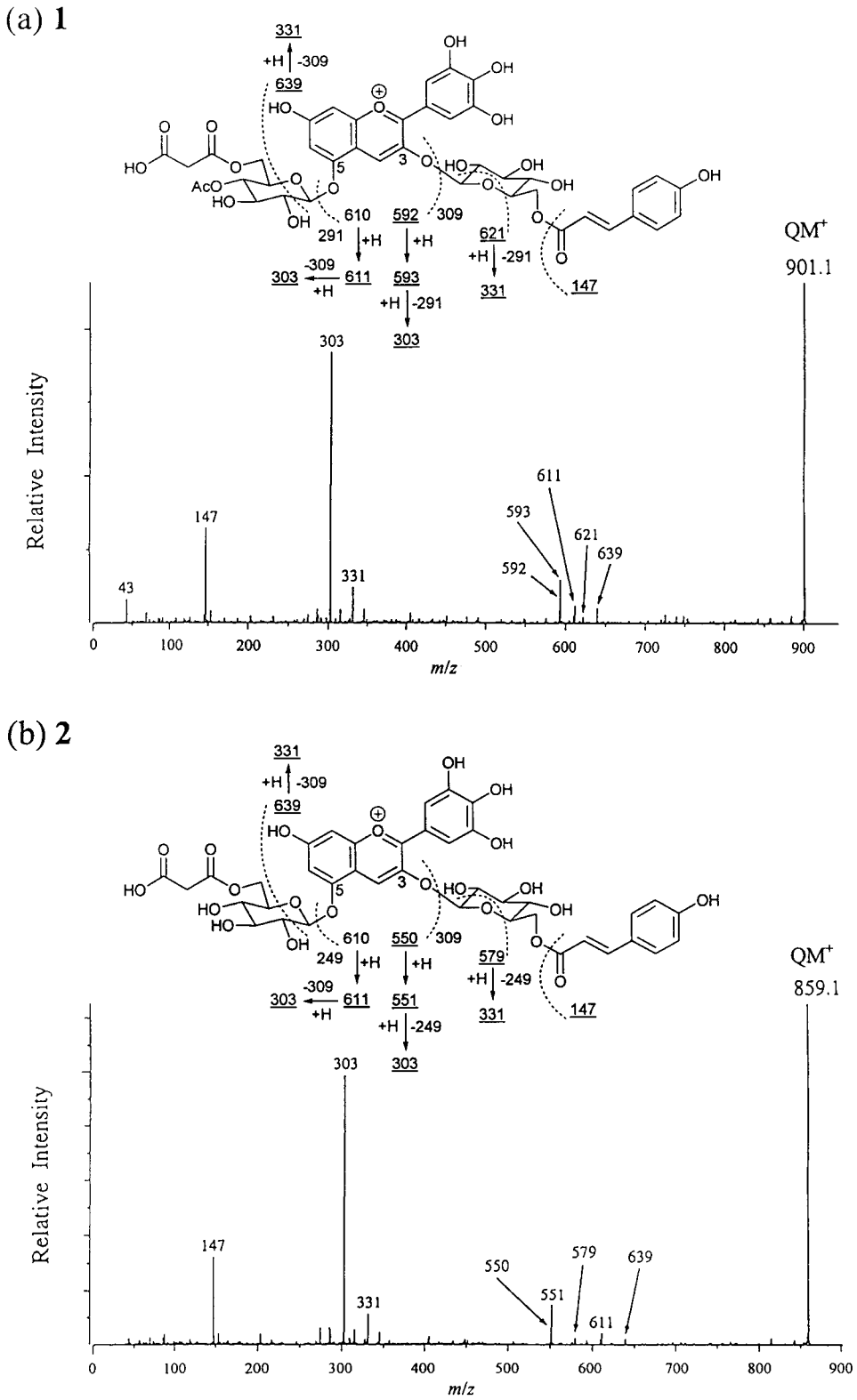

Fig. 1. High-energy CAD spectra obtained from quasi-molecular ions (QM+) of (a) *m/z* 901 and (b) *m/z* 859, corresponding to anthocyanins **1** and **2** respectively.

Table 1
^{1}H NMR chemical shift assignment and coupling constant data for anthocyanins **1** and **2** (δ in CD_3OD at 25°C)

Assignment	H	**1** (δ (^{1}H))	**2** (δ (^{1}H))
Aglycone	4	8.86 s	8.85 s
	6	6.98 br s	6.98 br s
	8	6.94 br s	6.90 br s
	2′,6′	7.78 s	7.74 s
Glucose A (●)	1	5.23 d (7.7)	5.18 d (7.8)
	2	3.84 m	3.76 m
	3	3.75 m	3.55 m
	4	4.92 m	3.44 m
	5	4.01 m	3.80 m
	6	4.22 m, 4.13 m	4.53 m, 4.20 m
Glucose B (▲)	1	5.43 d (7.7)	5.45 d (7.8)
	2	3.78 m	3.78 m
	3	3.60 m	3.61 m
	4	3.48 m	3.48 m
	5	3.95 m	3.96 m
	6	4.52 m, 4.42 m	4.51 m, 4.43 m
E-Coumaroyl	2″,6″	7.22 d (8.6)	7.22 d (8.6)
	3″,5″	6.71 d (8.6)	6.70 d (8.6)
	2	6.25 d (15.9)	6.25 d (15.9)
	3	7.38 d (15.9)	7.36 d (15.9)
Acetyl		2.12 s	
Malonyl		3.38 s	3.38 s

1998) share the less common feature of C-4 substitution of glucose.

3. Experimental

3.1. General

^{1}H NMR spectra were recorded at either 500 or 600 MHz using JEOL A500 and Bruker DMX600 instruments, respectively. Samples were dissolved in CD_3OD containing 0.1% DCl and referenced to the residual HOD resonance at 3.30 ppm. A temp. of 25°C was used for all NMR experiments with the exception of HMBC, for which a temp. of 10°C was used. FAB MS and high-energy collision-activated dissociation (CAD) MS were obtained using a JMS-SX/SX 102A four sector tandem mass spectrometer (BEBE geometry; JEOL, Tokyo, Japan). Xenon (6 keV) was used to facilitate FAB ionization. The mass spectrometer was operated at an accelerating voltage of 10 kV and in the positive ion mode. Argon was employed as a collision gas for activating ions in CAD and its pressure was increased until the intensity of the selected beam was reduced by 80%. The collision cell in the third field free region was floated at 5 kV.

3.2. Plant material

Salvia uliginosa was cultivated outside at the Royal Botanic Gardens (RBG) Kew, during the summers of 1996 and 1997 (accession number: 1987-524). The plants were harvested during the month of October and the sky-blue petals carefully removed and freeze-dried. A voucher specimen of the plant has been deposited in the Herbarium, RBG Kew.

3.3. Anthocyanin isolation and purification

The anthocyanin **1** was extracted from freeze-dried petals of *S. uliginosa* using HCO_2H–MeOH–H_2O (5:50:45, FMW). Filtered extracts were evapd in vacuo to a small volume before purification by semi-prep. HPLC with an ODS column of dimension 10.0 mm (i.d.) × 250 mm and a solvent system based on FMW (5:30:65 to 5:40:55). The HPLC step was repeated once more using HCO_2H–MeCN–H_2O (5:16:79) to achieve final purification.

3.4. Preliminary characterisation of (*1*) by chemical methods

The properties of the purified anthocyanin were examined by procedures described previously (Takeda, Harborne & Self, 1986). Acid hydrolysis gave only delphinidin and glucose, identified by TLC on cellulose, while alkaline hydrolysis yielded deacylated anthocyanin and organic acids. The R_f values (×100) of the deacylated anthocyanin (TLC in three solvent systems) were identical to those of delphin (delphinidin 3,5-*O*,*O*-diglucoside), namely 16 (HOAc–HCl–H_2O, 15:3:82), 2 (*n*-BuOH–2N HCl, 1:1) and 3 (1% HCl). Malonic acid, acetic acid and *p*-coumaric acid were detected on TLC using *n*-BuOH–HOAc–H_2O (4:1:2) and EtOAc–HOAc–H_2O (3:1:1) as solvents with visualisation by the aniline xylose reagent and UV light for aliphatic and phenolic acids, respectively. H_2O_2 oxidation of the purified anthocyanin gave 6-*O*-*p*-coumaroylglucose, the identity of which was confirmed by TLC in *n*-BuOH–HOAc–H_2O (4:1:2), *n*-BuOH–toluene–pyridine–H_2O (5:1:3:3) and *n*-BuOH–EtOH–H_2O (4:1:2.2). On electrophoresis, **1** moved 1.0 cm towards the anode in 0.1 M acetate buffer, pH 4.4, Toyo No. 51B paper (malonylawobanin, 1.0 cm).

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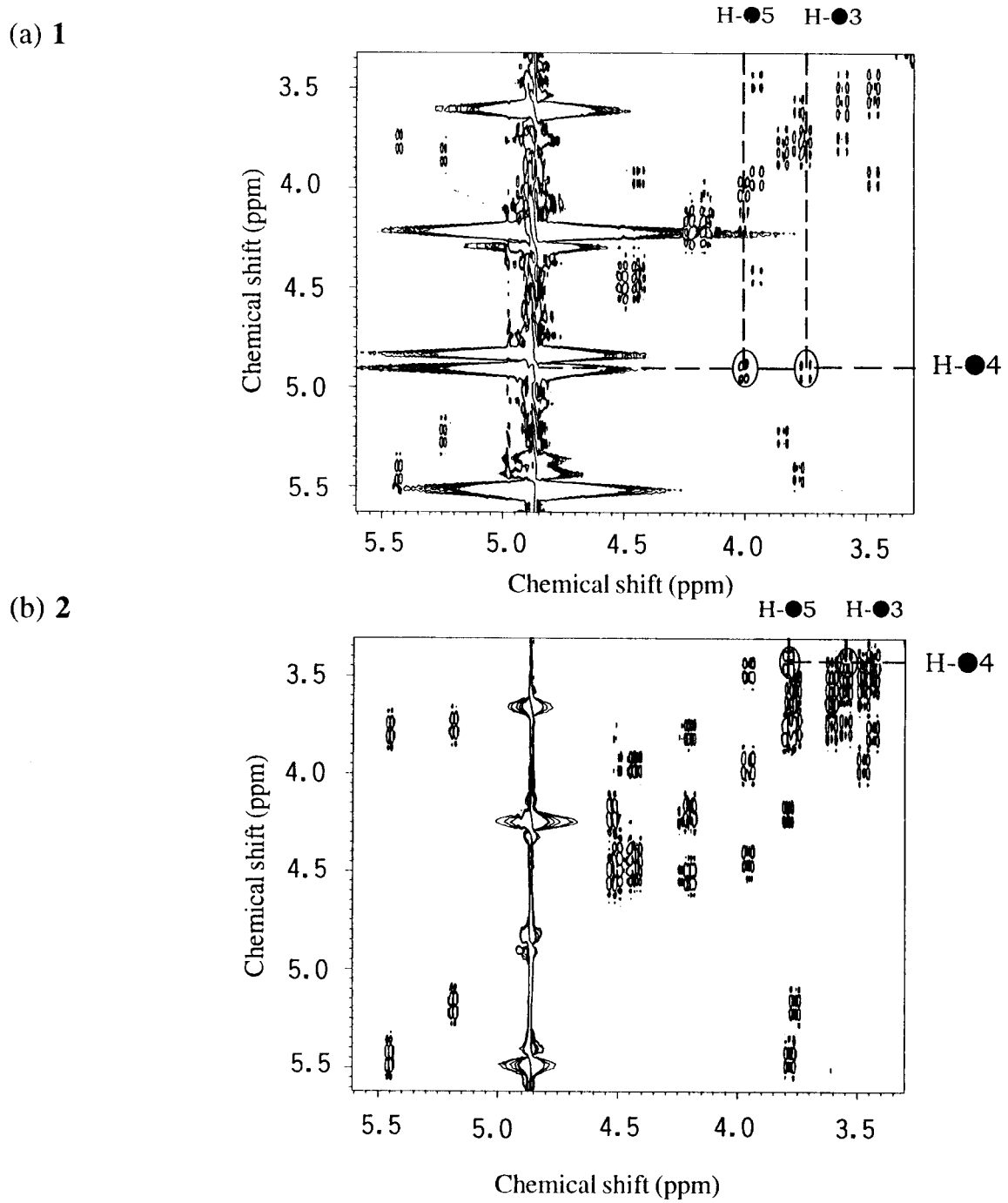

Fig. 2. 500 MHz DQF-COSY spectra of (a) **1** and (b) **2**, showing sequential ^{1}H-^{1}H connectivities associated with resonances of glucose A (●). Note the substantial downfield shift of the H-●4 resonance of **1** compared to that of **2**.

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