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Novel pigments and copigmentation in the blue marguerite daisy

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Abstract

The blue colour of the petals of the blue marguerite daisy, *Felicia amelloides*, has been found to arise from copigmentation between a novel malonylated delphinidin triglycoside, delphinidin 3-*O*-neohesperidoside 7-*O*-(6-*O*-malonyl-glucoside), and a new flavone *C*-glycoside, swertisin 2″-*O*-rhamnoside-4′-*O*-glucoside. Recombination, in vitro, of these two petal components at pH 6 recreates the blue petal colour.

Keywords: *Felicia amelloides*; Blue marguerite daisy; Asteraceae; Blue flower; Delphinidin 3-*O*-neohesperidoside 7-*O*-(6-*O*-malonylglucoside); Swertisin 2″-*O*-rhamnoside-4′-*O*-glucoside; Copigmentation

1. Introduction

The blue marguerite daisy, *Felicia amelloides* (Asteraceae), is a common ornamental plant valued for its display of blue flowers. Pigments in flowers of the Asteraceae family have been the subject of several studies and a wide variety of pigment structures have been determined, many of which are malonylated anthocyanins (Takeda, Harborne, & Self, 1986; Cheminat, Brouillard, Guerne, Bergmann, & Rether, 1989; Toki, Saito, & Honda, 1991). Blue flowers are of considerable interest as they invariably involve some sort of anthocyanin–copigment interaction and in several cases additional metallo-complexation (Goto & Kondo, 1991; Kondo et al., 1992; Bloor, 1997).

2. Results and discussion

2.1. Identification of the pigments

Freshly expressed juice from petals contained one major anthocyanin and one dominant flavone (by HPLC). However, the HPLC chromatograms of extracts or slightly aged pressed juice show that both of these components undergo partial degradation to give significant amounts of a less polar flavone and a more polar anthocyanin.

The native anthocyanin, **1**, was purified by column chromatography on cellulose and LH20. The anthocyanin showed mobility on cellulose acetate electrophoresis indicating a zwitterionic compound probably due to malonylation as this acyl group is common in pigments of the Asteraceae. Cursory analysis of the ^{1}H NMR spectra indicated a delphinidin with three sugars attached, one of which was a rhamnose. Connectivity information gained from C–H cosy experiments (HMBC, HMQC) showed the two other sugars were directly connected to the anthocyanidin at the 3- and 7-*O*-positions and the rhamnose was connected at the 2-position of the 3-linked sugar. Further inspection of the ^{13}C NMR shifts, the ^{1}H NMR anomeric signals and comparison with literature values (Andersen, 1988) confirmed the two hexose sugars to be β-D-glucosyl units, hence, the structure of **2**, the triglycoside, must be delphinidin 3-*O*-neohesperidoside 7-*O*-β-D-glucoside. The FAB(+) MS (MH^+ 860) showed a molecular species consistent with this structure substituted with a malonyl group. The downfield chemical shift of one set of glucose methylene protons was due to acylation by the malonyl group. These protons were shown to be part of the 7-*O*-glucoside by a TOCSY NMR experiment. A long-range coupling between these methylene protons and one of the malonyl carbonyls was also evident in the HMBC plot. Thus **1** is delphinidin 3-*O*-neohesperidoside 7-*O*-(6-*O*-malonyl-glucoside). The neohesperidoside disaccharide is relatively uncommon for anthocyanins, only reported previously in Podocarpaceae (Andersen, 1988).

The major flavone in fresh pressed juice, **3**, had an apigenin-like on-line UV spectrum and rapidly degraded to a partial hydrolysis product, **4**. The NMR spectra of **3** and **4** in DMSO were unusual in that a doubling of many of the signals was apparent, this doubling could not be collapsed by an increase in temperature or change

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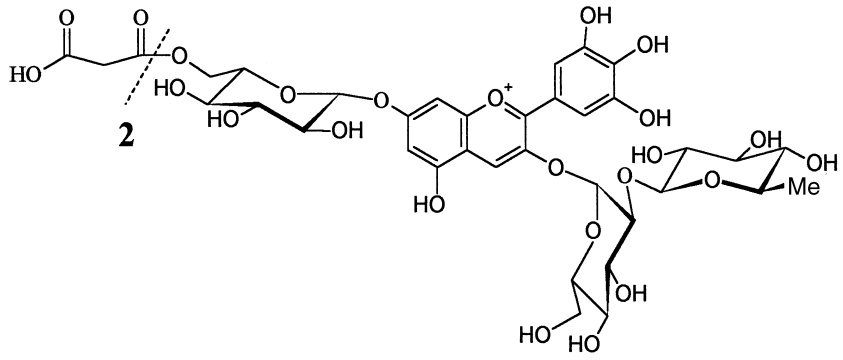
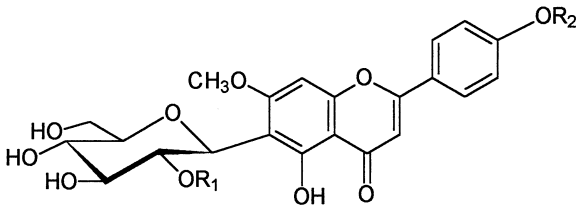


Felicia anthocyanin, **1**



3 R_1 = Glc, R_2 = H
4 R_1 = Rha, R_2 = Glc

in solvent. However, the NMR spectra clearly showed the presence of a methoxy group and a rhamnose sugar as well as the familiar signals of an apigenin flavone unit. The ^{13}C NMR signals of **4** were consistent with an apigenin *C*-glucoside substituted with a rhamnosyl and a methyl group. Compound **3** had an additional glucosyl group. The downfield shift of the *C*-glucosyl C-2 proton and carbon signals showed the rhamnose to be connected at this position. These data suggested swertisin 2″-*O*-rhamnoside (7-*O*-methylapigenin 6-*C*-(2″-*O*-rhamnosyl-glucoside)) as the likely structure for **4**. This was confirmed by cochromatography of the acid hydrolysis product from **4** with a standard sample of swertisin (7-*O*-methylapigenin 6-*C*-glucoside). Compound **4** has been reported previously but no mention made of the signal doubling in the NMR spectrum (Hilsenbeck & Mabry, 1990). This doubling must be due to a restricted rotation of the diglycoside substituent at C-6, as the doubling is most pronounced in the signals arising from this region of the molecule. The extra glucose in **3** is readily placed at the 4′-position and the chemical shift differences between **3** and **4** in the B-ring are consistent with this assignment i.e. **3** is swertisin 2″-*O*-rhamnoside-4′-*O*-glucoside. Since the 4′-*O*-glucose is remote from the site of hindered rotation almost no signal doubling is seen for the ^{13}C NMR signals for this part of the molecule. The ready hydrolysis of the 4′-*O*-glucoside must be due to enzymic activity in the freshly pressed juice.

2.2. Colour reconstruction

The violet blue colour of *Felicia* petals is closest to 96 B or C in the RHS colour chart, while freshly pressed petal sap is slightly more purple (93 A) and has a pH of 5.8. The visible spectrum of an upper epidermal peel shows the characteristic triple maxima shape of many violet or blue flowers with specific absorption maxima at 550, 585 and 632 nm (Fig. 1). It is now well accepted that copigmentation can be an important factor in petal colouration involving anthocyanins and *Felicia* provides a further example. Here, the flavones described above are present at high concentration in the petal — the molar ratio of flavone to anthocyanin is estimated to be at least 18:1 and the anthocyanin concentration in petal sap is ca. 1.8 mM. Recombination of purified flavones with the anthocyanin (1 mM) in McIlvaine buffer at pH 6 regenerated the petal spectrum Fig. 1. The copigmentation effect observed here is an initial significant bathochromic shift with addition of 1–2 equivalents of copigment followed by a steady increase in absorption intensity as more copigment was added. This absorbance enhancement was greater for flavone **3** than for **4**, once again demonstrating a specific copigmentation arrangement.

The combination of delphinidin anthocyanins and apigenin glycosides appears to be one of the more favoured copigmentation arrangements in blue flowers and is seen in *Commelina* (a malonylated delphinidin and flavocommelin) (Kondo et al., 1992) and *Salvia* (malonyl awobanin and apigenin 7,4′-diglucoside) (Takeda,

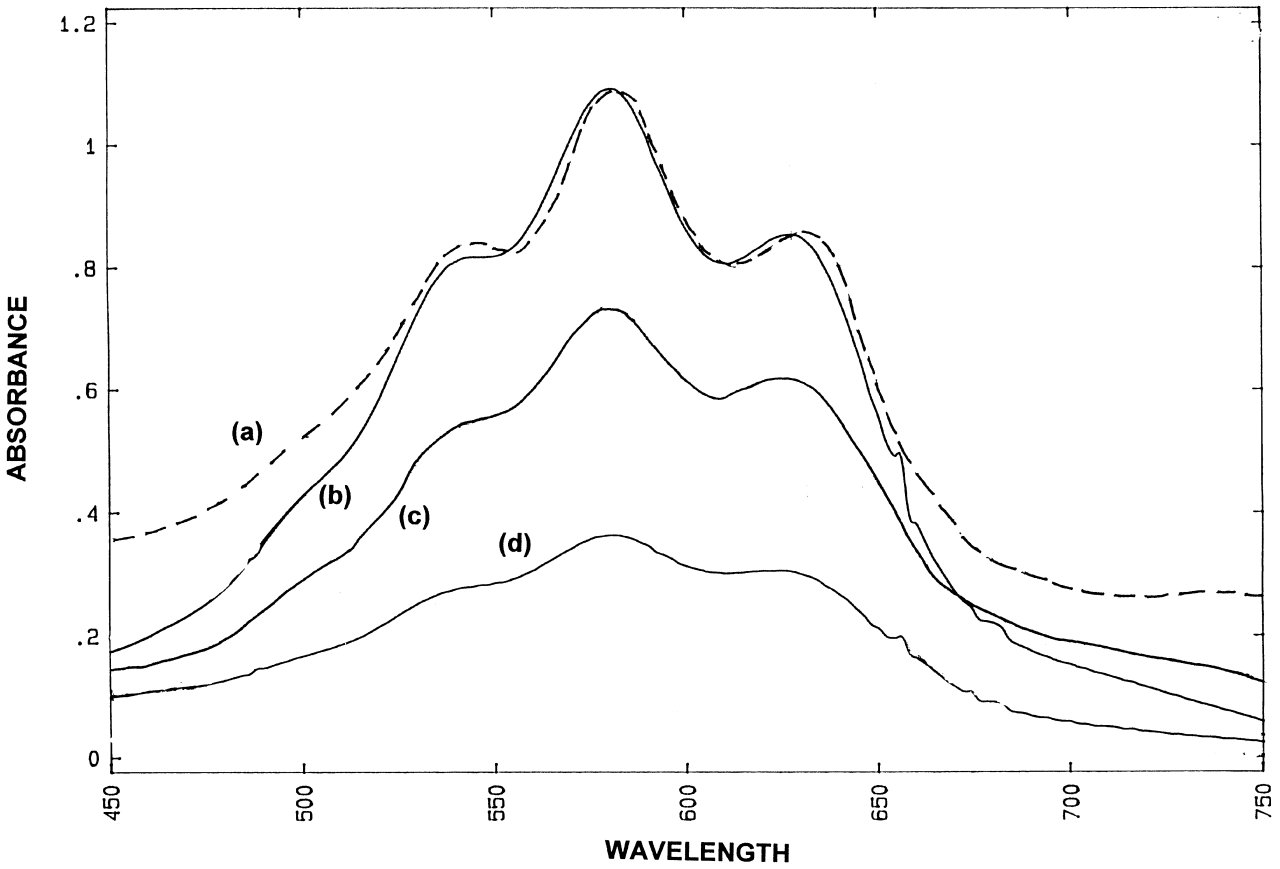


Fig. 1. Visible spectra of (a) — upper epidermal peel of petal, (b) anthocyanin (**1**, 1 mM)+8 equivalents of flavone **3**, (c) **1**+8 equivalents of flavone **4**, (d) **1**+2 equivalents of flavone **3**, in pH 6 buffer.

Yanagisawa, Kifune, Kinoshita, & Timberlake, 1994). These examples also involve the participation of a metal ion. However, there is no evidence to suggest metallocomplexation in *Felicia*. Flavone **3** is, in fact, flavocommelinin rhamnoside, and *C*-glycosides such as swertisin have been shown to be excellent copigments in some situations (Asen, Stewart, & Norris, 1972). The additional colour intensity of the copigmentation involving flavone **3**, compared with flavone **4**, and the fact that similar glycosylation patterns are seen in *Commelina*, *Salvia* and others such as *Centaurea cyanus* (Kondo et al., 1994), indicates flavones with glycosylation at both 'ends' of the molecule may be superior copigments.

3. Experimental

3.1. General

NMR experiments were run at 500 or 300 MHz (75 MHz for ^{13}C). Anthocyanin samples were dissolved in 1% CF_3COOD in CD_3OD. Flavones were run in DMSO-d_6 (RT and 90°C). MS were obtained using a VG 70-250S (FAB) or VG Platform II (electrospray) instrument. RP HPLC analyses were performed using a Waters 600 solvent delivery system coupled to a Waters 994 PDA detector.

3.2. Isolation of anthocyanin and flavones

Flower petals collected from the grounds of this institute were extracted immediately after collection by grinding in 5% aqueous formic acid. The pigments were absorbed onto a Diaion HP20 column and eluted with MeOH:HOAc:H_2O (10:1:9, MAW) and freeze dried. Pigments and copigments were purified by cellulose CC (t-BuOH:HOAc:H_2O (3:1:1), followed by HOAc:H_2O (1:9)) and LH20 (MAW).

3.3. Delphinidin 3-O-neohesperidoside, 7-O-(6-O-malonyl-β-D-glucopyranoside) (**1**)

UV $\lambda_{max}^{0.1\,N\,HCl}$ nm: 279, 525; FAB+MS 860.224 (MH^+, $C_{36}H_{44}O_{24}$ requires 860.222); ^1H NMR δ 8.85 (1H, s, H-4 of del), 7.81 (2H, s, H-2′,6′ of del), 7.21 (1H, d, J=2 Hz, H-8 of del), 6.79 (2H, d, J=2 Hz, H-6 of del), 5.37 (1H, d, J=8 Hz, H-1 of glc), 5.33 (1H, d, J=8 Hz, H-1

Table 1
^{13}C NMR data for **1**, **3**[a] and **4**[a]

	1			**3**	**4**
2	166.0	aglycone	2	165.2	163.3
3	148.1		3	105	104.8
4	135.1		4	182.4	182.3
5	159.2		5	160.3	160.3
6	105.0		6	110	109.9
7	167.8		7	163.5	163.7
8	96.8		8	90.5	90.3
9	157.6		9	157	156.8
10	114.7		10	105	102.7
1′	121.0		1′	123.7	120.9
2′,6′	114.4		2′,6′	128.3	128.5
3′,5′	148.7		3′,5′	116.7	116
4′	147.3		4′	163.1	161.4
1″	101.2	6-*C*-glc	1″	71.6	71.6
2″	80.0		2″	74.7	74.6
3″	78.9		3″	79.8	79.9
4″	72.4		4″	70.5	70.4
5″	79.8		5″	81.5	81.5
6″	63.6		6″	61.7	61.7
1	103.6	2″-*O*-rha	1	100.3	100.3
2	73.2		2	70.3	70
3	73.2		3	70.6	70
4	75.0		4	70.9	70
5	71.0		5	68.3	68.2
6	19.0		6	17.6	17.5
1	104.1	4′-*O*-glc	1	101.3	
2	75.6		2	73.3	
3	79.6		3	76.1	
4	72.3		4	69.8	
5	77.0		5	77.2	
6	66.5		6	60.7	
	169.6	MeO		56.7	56.6
	42 (broad)[b]				

[a] Signals of major isomer only.
[b] This signal and that of the distal carbonyl were not seen clearly.

of 7-glc), 5.31 (1H, d, $J=1$ Hz, H-1 of rha), 4.56 (1H, dd, $J=12$, 2 Hz, H-6a of 7-glc), 4.28 (1H, dd, $J=12$, 7 Hz, H-6b of 7-glc), 1.34 (3H, d, $J=6$ Hz, H-6 of rha). ^{13}C NMR data, see Table 1.

3.4. Apigenin 6-C-(2″-O-rhamnosylglucoside)-7-O-methyl-4′-O-glucoside (3)

UV λ_{max} (MeOH) 276, 326; +NaOMe 298; +$AlCl_3$ 304, 322, 362; +$AlCl_3$–HCl 304, 324, 362; +NaOAc 276, 326. ESMS 755.5 ($C_{34}H_{42}O_{19}=754.2$). ^{1}H NMR (peaks of major isomer only) δ 8.09 (2H, d, $J=8.8$, H-2′,6′) 7.21 (2H, d, $J=8.8$, H-3′,5′), 6.96 (1H, s, H-3), 6.89 (1H, s, H-8), 5.08 (1H, s, H-1 of rha), 5.03 (1H, d, $J=7$, H-1 of 4′-glc), 4.66 (1H, d, J = 10 Hz, H-1 of C-glc), 4.33 (1H, dd, $J=10$, 8 Hz, H-2 of C-glc), 0.48 (3H, d, J = 6, H-6 of rha). ^{13}C NMR data see Table 1.

3.5. Apigenin 6-C-(2″-O-rhamnosylglucoside)-7-O-methyl (4)

UV λ_{max} (MeOH) 274, 336; +NaOMe 392; +$AlCl_3$ 306, 320, 362; +$AlCl_3$–HCl 306, 320, 362; +NaOAc 274, 340. ^{1}H NMR (peaks of major isomer only) δ 7.9 (2H, d, $J=8.7$, H-2′,6′) 6.94 (2H, d, $J=8.7$, H-3′,5′), 6.87 (1H, s, H-3), 6.85 (1H, s, H-8), 5.07 (1H, s, H-1 of rha), 4.65 (1H, d, $J=10$ Hz, H-1 of glc), 4.35 (1H, dd, $J=10$, 8 Hz, H-2 of glc), 3.90 (3H, s, 7-OCH_3), 3.71 (1H, d, $J=11$ Hz, H-2 of glc), 3.59 (1H, s, H-2 of rha), 2.16 (1H, dq, $J=9$, 6 Hz, H-5 of rha), 0.48 (3H, d, $J=6$, H-6 of rha). ^{13}C NMR data see Table 1.

3.6. Petal colour reconstruction experiments

All experiments were in pH 6.0 McIlvaine buffer. The concentration of flavones was calculated from HPLC

peak integration data at 352 nm vs. standard samples of apigenin 7-*O*-neohesperidoside. Anthocyanin concentrations were determined using $\varepsilon = 30{,}000$ from the visible absorption maxima in 0.1 N HCl. For petal colour reconstruction experiments the flavones were dissolved in 200 µl buffer, which was then added to the freeze-dried anthocyanin, mixed and transferred to a 1 mm pathlength cuvette for spectroscopic analysis. Spectra presented in the figure were measured about 20 min after mixing. The concentration of anthocyanin used in these experiments was 1.0 mM.

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