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MALVIDIN 3-(6-ACETYLGLUCOSIDE)-5-GLUCOSIDE AND OTHER ANTHOCYANINS FROM FLOWERS OF *GERANIUM SYLVATICUM*

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Key Word Index—*Geranium sylvaticum*; Geraniaceae; flower; anthocyanins; acetylation; malvidin 3-*O*-(6-*O*-acetyl-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside; homo- and heteronuclear NMR spectroscopy.

Abstract—From the flowers of *Geranium sylvaticum* five anthocyanin glycosides have been isolated by successive application of an ion-exchange resin, droplet counter-current chromatography and gel filtration. Even though the lability of the novel major (85%) anthocyanin, malvidin 3-*O*-(6-*O*-acetyl-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside, was high, it was possible by means of NMR spectroscopy, especially homo- and heteronuclear two-dimensional techniques, to assign all the proton and carbon atoms to give the full structure. The minor anthocyanins were identified as the 3,5-diglucosides of malvidin and cyanidin, and the 3-glucosides of cyanidin and delphinidin.

INTRODUCTION

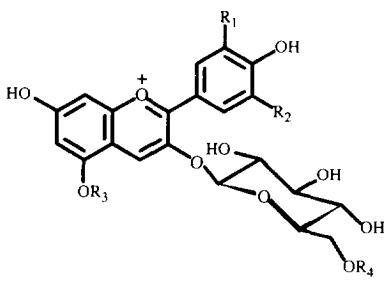
Geranium sylvaticum grows in most European countries and some parts of western Asia. There exists no report on the anthocyanin content of *G. sylvaticum*. Robinson and Robinson [1] reported malvidin 3,5-dimonosides in four species belonging to the Geraniaceae, while Meno *et al.* [2] identified malvidin 3,5-diglucoside in *G. eriostemon*.

The only anthocyanins reported to be acylated with acetic acid are to our knowledge: delphinidin 3,5-diacetylglucoside [3], acetylated 3,5-diglucoside of cyanidin, pelargonidin [3, 4] and malvidin [5], acetylated cyanidin 3-rutinoside [6], malvidin 3-coumarylglucoside-5-acetylxyloside [7] and acetylated 3-glucoside of the six common aglycones [8–9]. The acetylated anthocyanins are sensitive to acids, which may be one of the reasons that the positions of the acetyl group have not been properly assigned in many of the mentioned pigments. As opposed to anthocyanins acylated with aliphatic dicarboxylic or aromatic acids, the determination of acetylated anthocyanins is further complicated by no unusual electrophoretic mobility [10] and the lack of UV/Vis absorption, respectively.

In this paper we report the anthocyanin content of *G. sylvaticum* including a novel acetylated pigment. This is the first structure elucidation of a monoacetylated anthocyanidin 3,5-diglycoside with identical sugars, which prove the position of the acetyl group.

RESULTS AND DISCUSSION

Fractionation of the acidified methanolic extract of flowers of *Geranium sylvaticum* by a combination of ion-exchange chromatography (Amberlite XAD-7), gel filtration (Sephadex LH-20) and DCCC afforded five anthocyanins **1**–**5**. Compounds **3**–**5** were identified as cyanidin 3,5-diglucoside and the 3-glucosides of cyanidin and delphinidin based on co-chromatography (TLC and HPLC), acid hydrolysis and spectral data (Table 1).



(1) R_1,R_2=OMe; R_3=Glc; R_4=Acetyl

(2) R_1,R_2=OMe; R_3=Glc; R_4=H

(3) R_1=OH; R_2,R_4=H; R_3=Glc

(4) R_1=OH; R_2,R_3,R_4=H

(5) R_1,R_2=OH; R_3,R_4=H

Acid hydrolysis of **1**, the major (84.6% of total anthocyanin content) pigment, produced malvidin and glucose. The peak corresponding to **1** recorded during HPLC gradually turned into the peak corresponding to **2** after short storage (hrs) in the extraction solvent containing methanol acidified with small amounts of conc. HCl. The presence of an aliphatic acyl group as indicated by the lability of **1** towards mineral acid, was supported by the similarities of the UV/Vis spectra of **1** and **2**. When the mineral acid was replaced by acetic acid in the extrac-

Table 1. Spectral and chromatographic data and relative proportions of the anthocyanins in flowers of *Geranium sylvaticum*

Compound*	TLC ($R_f \times 100$) FHW	BAW	Vis. max. (nm)	On-line HPLC A_{440}/A_{max} (%)	R_t(min)	Area (%)
Mv-3-acglc-5-glc (**1**)	0.71	0.42	526	13	14.93	84.6
Mv-3,5-diglc (**2**)	0.67	0.27	523	14	11.63	†
Cy-3,5-diglc (**3**)	0.47	0.14	512	16	9.13	1.5
Cy-3-glc (**4**)	0.24	0.30	516	32	11.23	†
Dp-3-glc (**5**)	0.13	0.11	524	30	9.94	3.4

*Mv = malvidin; Cy = cyanidin; Dp = delphinidin; ac = acetyl; glc = glucose.
†10.5% together.

tion solvent, the partial hydrolysis of **1** to **2** was reduced. The UV/Vis spectrum of **1** taken during on-line HPLC showed a visible maximum at 526 nm with A_{440}/A_{526} of 13.3% indicating the presence of a 3,5-diglycoside [11].

In order to elucidate the structure of the acyl group, the ring size and anomeric configuration of the sugars, and to confirm the identities of the aglycone and the sugar comprising **1**, it was necessary to apply several NMR techniques in tandem. Because the acyl group is positioned on one of two identical glucose units, the point of attachment was based on full assignment of the carbon and proton atoms in the NMR spectra of **1**. The chemical shifts of the protons and their corresponding aglycone carbons of malvidin, the aglycone of **1** and **2**, were completely assigned (Tables 2 and 3) using information regarding coupling constants, integration data (^{1}H NMR) and direct heteronuclear shift correlation (HSC, Fig. 2). The remaining problem of assigning the quaternary carbon atoms was addressed by a heteronuclear multiple bond correlation (COLOC) experiment (Fig. 1) optimized for $^2J_{(CH)}$ and $^3J_{(CH)}$ couplings, and a heteronuclear coupling modulated spin echo (SEFT) experiment which also confirmed the number of protons attached on each carbon [12].

The anomeric carbon signals appear considerably downfield of the other sugar resonances [13], and thus the two cross-peaks at δ5.4/104.1 and δ5.2/102.7 in the HSC spectrum of **1** (Fig. 2) together with integration data indicated two sugar rings. The spectral region between δ80 and δ60 in the SEFT spectrum showed 10 resonances which, together with the two anomeric carbon resonances, were in agreement with two hexoses. Starting from the anomeric protons, we could through the cross-peaks in the double quantum filtered ^{1}H–^{1}H shift correlated (DQF–COSY) spectrum follow the chain of coupled protons in each of the two rings by a sequential 'walk' (Table 2). Thereafter we were able to assign the chemical shifts of the corresponding sugar carbons from the HSC experiment (Fig. 2). The chemical shifts (Table 2) and the large ^{1}H–^{1}H coupling constants of the anomeric protons (7.7 Hz) and the sugar ring protons (around 9 Hz) agreed with all the protons axial in two β-linked D-glucopyranoses [14].

Table 2. ^{1}H NMR spectral data for anthocyanins isolated from flowers of *Geranium sylvaticum* (in CD_3OD–CF_3CO_2D, 9:1, at 25°)

	1	**2**
Malvidin		
4	9.15	9.24
6	7.05	7.17
8	7.16	7.26
2′,6′	8.01	8.12
OMe	3.87	4.09
3-*O*-β-D-glucopyranosyl		
1″	5.44	5.45
2″	3.76	3.75
3″	3.69	3.65
4″	3.57	3.51
5″	3.88	3.61
6A″	4.55	4.03
6B″	4.40	3.79
5-*O*-β-D-glucopyranosyl		
1‴	5.24	5.26
2‴	3.81	3.73
3‴	3.66	3.59
4‴	3.50	3.44
5‴	3.76	3.69
6A‴	4.09	4.01
6B‴	3.83	3.82
Acetyl		
Me	2.08	

The acyl group was identified as acetic acid by the signals at δ20.5 and 172.9 in the SEFT spectrum and the 3H singlet at δ2.1 in the ^{1}H NMR spectrum (Tables 2 and 3). These signals did not occur in the corresponding spectra of **2**, the hydrolysed form of **1**, and their assignments were confirmed by the presence of cross-peaks at δ2.1/20.5 and 2.1/172.9 in the HSC and COLOC spectra of **1**, respectively.

In the HSC spectrum of **1**, a signal due to the hydroxymethyl carbon of one of the glucose rings appeared at lower field than the corresponding carbon signals of

Table 3. ^{13}C NMR spectral data for anthocyanins isolated from flowers of *Geranium sylvaticum* (in CD_3OD–CF_3CO_2D, 9:1, at 25°)

	1	2
Malvidin		
2	164.0	164.5
3	146.8	146.8
4	135.9	136.2
5	156.6	157.4
6	106.1	105.9
7	169.7	169.8
8	97.5	97.6
9	157.1	156.9
10	113.5	113.6
1′	119.5	119.7
2′	110.9	111.1
3′	149.8	149.9
4′	147.2	147.1
5′	149.8	149.9
6′	110.9	111.1
OMe	57.3	57.4
3-*O*-*β*-D-glucopyranosyl		
1″	104.1	102.7
2″	74.4	74.5
3″	78.5	78.7
4″	71.5	71.4
5″	76.7	77.7
6″	64.6	62.5
5-*O*-*β*-D-glucopyranosyl		
1‴	102.7	104.4
2‴	75.3	74.9
3‴	77.7	78.5
4‴	71.4	71.1
5‴	79.1	79.0
6‴	62.6	62.4
Acetyl		
Me	20.5	
C=O	172.9	

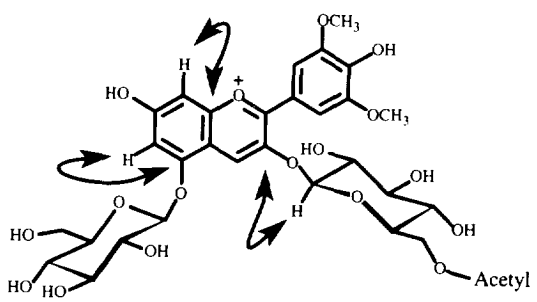


Fig. 1. The most important correlations found in the long-range heteronuclear shift correlation NMR spectrum of malvidin 3-(6-acetylglucoside)-5-glucoside, **1**. The correlation between H-1″ and C-3 connects the acetylated sugar to the 3-position on the aglycone. The H-6/C-5 and H-8/C-9 correlations are used for unambiguous carbon assignments.

the other glucose ring and of pigment **2** (Fig. 2). This signal was correlated to the proton resonances at δ4.6 and 4.4 which were assigned to H-6A and H-6B by means of the DQF–COSY spectrum. These protons were found to be on the same glucose units as the anomeric proton at δ5.4 (DQF–COSY). When a cross-peak between this anomeric proton and C-3 on the aglycone was observed in the COLOC spectrum (Fig. 1), it was found that the acyl group was linked to the 6-hydroxymethyl of the glucose in 3-position on the aglycone. Thus the structure of **1** was elucidated to be malvidin 3-*O*-(6-*O*-acetyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside.

EXPERIMENTAL

Extraction and purification. Flowers of *Geranium sylvaticum* were picked at Indre Arna, Bergen, Norway. A voucher specimen has been deposited in BG, Norway. Petals were extracted (×3) with MAW (MeOH–HOAc–H_2O; 10:1:10). The filtered extracts were first combined and concd under red. pres. and then purified by partition against EtOAc before application on an Amberlite XAD-7 column [15]. Pigments 1 and 2 were purified on a Sephadex LH-20 column. (100 × 1.6 cm, Pharmacia) using MAW as an eluent.

Hydrolysis. Acid hydrolysis and partial acid hydrolysis were carried out according to previously published procedures [16].

Chromatography. Analyt. TLC was carried out on microcrystalline cellulose (F1440, Schleicher & Schüll) with the solvents BAW (*n*-BuOH–HOAc–H_2O; 4:1:5, upper phase) and FHW (HCO_2H–conc. HCl–H_2O; 1:1:2). HPLC was carried out using a slurry packed ODS-Hypersil column (20 × 0.5 cm, 5 μm). Two solvents were used for elution: HCO_2H–H_2O (1:9) (A) and HCO_2H–H_2O–MeOH (1:4:5) (B). The elution profile was composed of isocratic elution (90% A, 10% B) over 4 min, linear gradient from 10% B to 100% B over the next 17 min, followed by linear gradient from 100% B to 10% B over 1 min. The flow rate was 1.5 ml min^{-1}, and aliquots of 10 μl were injected. UV/Vis absorption spectra were recorded using a photodiode array detector (HP 1050, Hewlett-Packard), and spectral measurements were made over the wavelength range 210–600 nm in steps of 2 nm. The quantitative data were based on the average values of the absorptions on every second nm between 500 and 540 nm, and do not consider different molar absorption coefficients of the pigments.

NMR spectroscopy. The NMR experiments were obtained at 400.13 MHz and 100.62 MHz for ^1H and ^{13}C, respectively, on a Bruker AM-400 instrument at 20°. The residual ^1H signal of the solvent (CF_3COOD:CD_3OD; 1:9) and the ^{13}C signal were used as secondary reference (δ3.4 and δ49.0 from TMS, respectively). The 1D ^1H and 2D homonuclear correlation experiments were performed on a 5 mm inverse probe. The 1D spin echo Fourier transform (SEFT) and the 2D one-bond (HSC) and the long-range (COLOC) heteronuclear shift correlation experiments were performed on a 5 mm ^1H–^{13}C dual probe. The data in the 2D experiments were col-

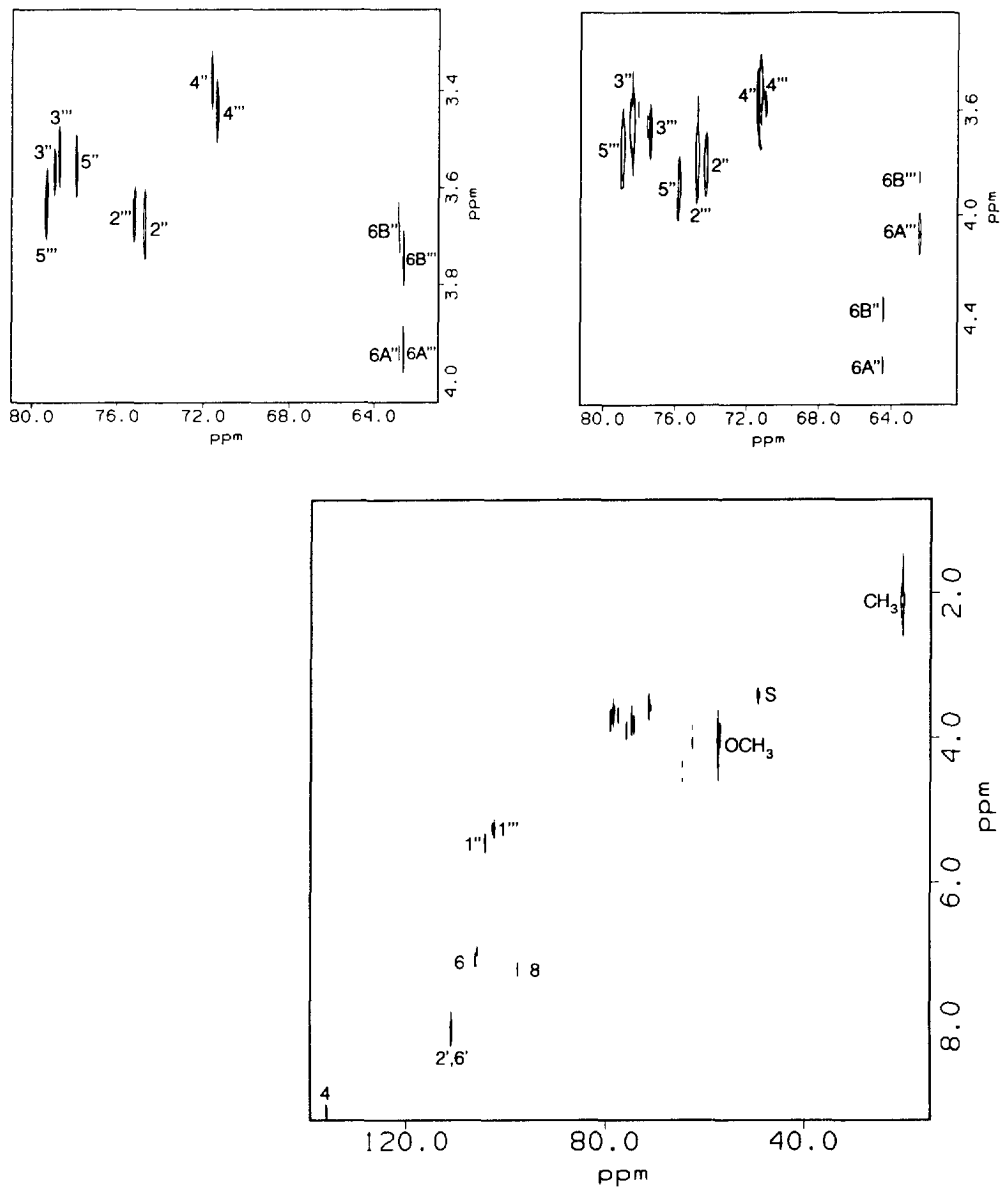


Fig. 2. Bottom: contour plot of the one-bond heteronuclear shift correlation NMR spectrum of malvidin 3-(6-acetylglucoside)-5-glucoside, **1**. Top left: expanded sugar region except the anomeric resonances of malvidin 3-glucoside-5-glucoside, **2**. Top right: expanded sugar region except the anomeric resonances of **1**. The downfield shifts of the two proton-carbon correlations (H-6A″/C-6″ and H-6B″/C-6″) show the position of the acetyl group of **1**.

lected with 2K complex data points. In COSY a sweepwidth of 3441 Hz and 656 t_1 increments were used. For the HSC experiment, sweepwidths of 3441 Hz and 12 500 Hz were used for ^{1}H and ^{13}C, respectively, with 116 t_1 increments. In the COLOC experiment, sweepwidths of 3441 Hz and 16 129 Hz were used for ^{1}H and ^{13}C, respectively, with 128 t_1 increments. The double quantum filtrated correlation spectroscopy (DQF–COSY) spectra were processed using a skewed squared sinebell function shifted 70 degrees, a skewness of 0.7, and dropped to zero at the last data point in both dimensions. The heteronuclear spectra were processed using a sinebell function shifted 90 degrees and dropped to zero at the last data point in both dimensions.

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