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ANTHOCYANINS WITH AN UNUSUAL ACYLATION PATTERN FROM STEM OF *ALLIUM VICTORIALIS*

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Key Word Index—*Allium victorialis*; Liliaceae; stem; anthocyanins; 3-acylation; cyanidin 3-*O*-(3″-*O*-malonyl-β-glucopyranoside); cyanidin 3-*O*-(3″,6″-*O*-dimalonyl-β-glucopyranoside); homo- and hetero-nuclear NMR spectroscopy.

Abstract—Two novel anthocyanins have been isolated from the stem of *Allium victorialis*. By means of chemical degradation and spectroscopy, especially homo- and hetero-nuclear two-dimensional NMR techniques, the structures were determined to be cyanidin 3-*O*-(3″,6″-*O*-dimalonyl-β-glucopyranoside) (76.6%) and cyanidin 3-*O*-(3″,*O*-malonyl-β-glucopyranoside) (13.8%). This is the first report of acylation of the 3-position in the sugar moiety of any anthocyanin. The stability of malonyl substitution in the 3″-position on glucose is higher than the corresponding 6″-malonylation.

INTRODUCTION

In recent years, aliphatic acids have been found as acyl moieties of many anthocyanins. In addition to acetic acid, the dicarboxylic acids oxalic, malonic, succinic and malic have been identified [1]. The linkage between the aliphatic acyl moiety and the sugar is sensitive to acids, especially mineral acids, and the identification of the acyl moiety is further complicated by their lack of UV/Vis absorption. When determined, the substituting position of the acyl groups has in nearly all instances been at the 6″-position of the sugars [2]. The genus *Allium* in the Liliaceae includes several species of horticultural importance (e.g. onions, garlic). While flavonoids from *A. victorialis* have not been reported previously, much work has particularly been done on anthocyanins from the bulbs of red onion, *A. cepa* [3–5]. Here, we report on the anthocyanin content in the stem of *A. victorialis*. Both the two novel anthocyanins contain malonic acid connected to the 3″-position.

RESULTS AND DISCUSSION

The HPLC chromatogram of the crude anthocyanin extract from the stem of *A. victorialis* detected in the visible spectral region, showed two compounds, **1** (76.6%) and **2** (13.8%), in addition to two minor anthocyanins (together 9.6%). The extract was purified by partition against ethyl acetate and then chromatographed on an Amberlite XAD-7 column. The anthocyanins were separated by Sephadex LH-20 gel filtration and checked for homogeneity by HPLC and TLC before analysis. On acid hydrolysis of **1**, cyanidin and glucose were produced. Partial acid hydrolysis afforded two intermediates and cyanidin. One of these intermediates co-chromatographed (HPLC and TLC) with **2** (Table 1). The presence of aliphatic acyl groups, as indicated by the lability of **1** and **2** towards mineral acid, was supported by the similarities of the UV/Vis spectra of **1**, **2** and cyanidin 3-glucoside (**4**) (Table 1). The UV/Vis spectra of **1** and **2** taken during on-line HPLC showed

Table 1. Chromatographic and spectral data for the anthocyanins in stems of *Allium victorialis* (**1** and **2**), cyanidin 3-(6″-malonylglucoside) (**3**) and cyanidin 3-glucoside (**4**)

	TLC (R_f)		On-line HPLC		
Compound	FHW	BAW	Vis. max. (nm)	A_{440}/A_{max}(%)	R_t(min)
1	0.73	0.66	520	29.1	17.73
2	0.62	0.54	520	29.1	15.07
3	0.51	0.32	520	29.1	15.92
4	0.40	0.32	519	29.1	13.05

visible maxima at 520 nm with A_{440}/A_{520} of 29%, indicating the presence of cyanidin 3-glycosides [6].

In order to elucidate the structure of the acyl groups, the ring size and anomeric configuration of the sugar, to confirm the identities of the aglycone and the sugar and to determine the linkage points between the units comprising **1** and **2**, it was necessary to apply mass spectrometry (MS) data and homo- and hetero-nuclear NMR techniques in tandem. The chemical shifts of the protons and their corresponding aglycone carbons of cyanidin, the aglycone of both **1** and **2**, were assigned (Tables 2 and 3) using information regarding coupling constants, integration data (^{1}H NMR) and direct heteronuclear shift correlation (HSC). The remaining problem of assigning the quaternary carbon atoms of cyanidin was addressed by a heteronuclear coupling modulated spin echo (SEFT) experiment, which also confirmed the number of protons attached on each carbon [7].

The anomeric carbon signal appears considerably downfield from the other sugar resonances, and thus the crosspeak at δ 5.47/103.39 in the HSC spectrum of **1** together with integration data, indicated one sugar ring. The spectral region between δ 80 and 60 in the SEFT spectrum showed five resonances which, together with the anomeric carbon resonance, were in agreement with those of substituted glucopyranose [8]. Starting from the anomeric protons we could, through the crosspeaks in the double quantum filtered ^{1}H–^{1}H shift correlated (DQF-COSY) spectrum, pursue the chain of coupled protons by a sequential 'walk' (Table 2). Thereafter we were able to assign the chemical shifts of the corresponding sugar carbons from the HSC experiment (Table 3). In the HSC spectrum, the carbon signal at δ 65.14 was correlated to the proton resonances at δ 4.66 (H-6″ A) and δ 4.41 (H-6″ B), and the signal at δ 78.61 to the proton resonance at δ 5.26 (H-3″). These outstanding shift values compared to similar values for cyanidin 3-*O*-β-glucopyranoside (Tables 2 and 3), together with the large ^{1}H–^{1}H coupling constants of the anomeric proton (7.7 Hz) and the sugar ring protons [7], agreed with all the axial protons in a β-linked D-glucopyranose with substitution in the 3″- and 6″-positions. In a similar manner the sugar moiety of **2** was determined to be a β-linked D-glucopyranose with substitution in the 3″-position.

The liquid chromatograph–MS analysis of **3** isolated from *A. cepa* gave its $[M + H]^+$ at 536.0 *m/z*, corresponding to the mass calculated for $C_{24}O_{14}H_{23}$ (535.44 *m/z*). FAB-MS showed the *m/z* 535.0 and the fragment ions at *m/z* 449.0 and 286.9 in accordance with cyanidin malonylglucoside, cyanidin glucoside and cyanidin, respectively. The confirmation of malonic acid as the acyl group of **3** came from the two signals at δ 168.64 and 170.19 in the SEFT spectrum and the 2H singlet at δ 3.44 in the ^{1}H NMR spectrum, attributable to the two carbonyl C and the CH_2 of the malonate moiety, respectively. Similarly, two and one malonate moieties were observed in **1** and **2**, respectively (Tables 2 and 3). Thus, the structures of **1** and **2** were elucidated to be cyanidin 3-*O*-(3″,6″-*O*-dimalonyl-β-glucopyranoside) and cyanidin 3-*O*-(3″-*O*-malonyl-β-glucopyranoside).

Table 2. ^{1}H NMR spectral data for cyanidin-3-(3″,6″-dimalonylglucoside) (**1**), cyanidin-3-(3″-malonylglucoside) (**2**), cyanidin-3-(6″-malonylglucoside) (**3**) and cyanidin-3-glucoside (**4**) in CD_3OD–CF_3COOD (95:5) at 25°

	1 δ (ppm)	2 δ (ppm)	3 δ (ppm)	4 [7] δ (ppm)
Cyanidin				
4	8.99	9.09	8.99	9.10
6	6.76	6.72	6.74	6.76
8	6.95	6.95	6.95	6.98
2′	8.02	8.04	8.07	8.14
5′	7.05	7.05	7.08	7.11
6′	8.28	8.26	8.33	8.31
3-*O*-β-Glucopyranoside				
1″	5.47	5.49	5.36	5.38
2″	3.95	3.95	3.79	3.78
3″	5.26	5.26	3.58	3.65
4″	3.72	3.76	3.43	3.56
5″	4.02	3.78	3.88	3.67
6″ A	4.66	4.0	4.65	4.02
6″ B	4.41	3.8	4.37	3.82
3″-Malonyl				
CH_2	3.44	3.44		
6″-Malonyl				
CH_2	3.44		3.44	

A comparison of chromatographic data (Table 1) reveals that cyanidin 3-*O*-(3″-*O*-malonyl-β-glucopyranoside) is easily separated from cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) by TLC on cellulose using acidified aqueous and acidified alcoholic solvents and by the use of reversed-phase HPLC. It is interesting to note that a malonyl group in the 6″-position (as in pigments **1** and **3**) causes a significant upfield shift effect (0.10 ppm) on H-4 (aglycone) compared to the corresponding signals of **2** and **4** (Table 2). This indicates that, in acidified methanolic solutions, malonylation in the 6″-position influences the aglycone more than malonylation in the 3″-position. When **1** was subjected to partial acid hydrolysis at 90°, cyanidin 3-*O*-(3″-*O*-malonyl-β-glucopyranoside) (**2**) was observed after 10 min. Thereafter, different proportions of **2**, cyanidin 3-*O*-β-glucopyranoside (**4**) and the aglycone cyanidin were observed for the next 30 min. There was no sign of cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**3**), showing that the acyl linkage to the 6″-position of the sugar is more labile than the corresponding linkage to the 3″-position.

EXPERIMENTAL

Extraction and sepn. Stems of *Allium victorialis* L. were collected in September 1994 from the Botanical Garden of the University of Bergen. The identity was verified by D. O. Øvstedal, and a voucher specimen has been deposited in the Botanical Garden. Bulbs of red onion (*A. cepa*) were purchased from the nearest grocery store. The stems and bulbs were cut with a pair of scissors and extracted

Table 3. ^{13}C NMR spectral data for cyanidin-3-(3″,6″-dimalonylglucoside) (**1**), cyanidin-3-(3″-malonylglucoside) (**2**), cyanidin-3-(6″-malonylglucoside) (**3**) and cyanidin-3-glucoside (**4**) in $CD_3OD–CF_3COOD$ (95:5) at 25°

	1 δ (ppm)	**2** δ (ppm)	**3** δ (ppm)	**4** [7] δ (ppm)	SEFT*
Cyanidin					
2	164.12	164.11	164.30	164.36	↑
3	145.31	145.31	145.55	145.64	↑
4	136.63	136.63	136.69	137.03	↓
5	159.11†	159.11†	159.70†	159.55†	↑
6	103.52	103.52	103.43	103.50	↓
7	170.60	170.60	170.49	170.56	↑
8	95.37	95.37	95.26	95.19	↓
9	157.66†	157.65†	157.71†	157.75†	↑
10	113.20	113.20	113.23	113.45	↑
1′	121.07	121.07	121.18	121.31	↑
2′	118.31	118.31	118.37	118.56	↓
3′	147.38	147.38	147.44	147.41	↑
4′	155.81	155.81	155.86	155.78	↑
5′	117.39	117.39	117.39	117.48	↓
6′	128.47	128.29	128.47	128.22	↓
3-*O*-β-Glucopyranoside					
1″	103.39	103.39	103.58	103.79	↓
2″	72.80	72.93	74.64	74.80	↓
3″	78.61	78.95	77.90	78.13	↓
4″	69.52	69.18	71.31	71.11	↓
5″	75.76	78.5	75.93	78.79	↓
6″	65.14	62.02	65.47	62.39	↑
3″-Malonyl§					
COOH	171.80‡	171.94‡			↑
COOR	172.31‡	172.40‡			↑
6″-Malonyl§					
COOH	168.57		168.64		↑
COOR	170.15		170.19		↑

*SEFT = coupling modulated spin echo NMR experiment: C_q and CH_2, ↑; CH and CH_3, ↓.

†, ‡ Assignments with the same superscript may be reversed.

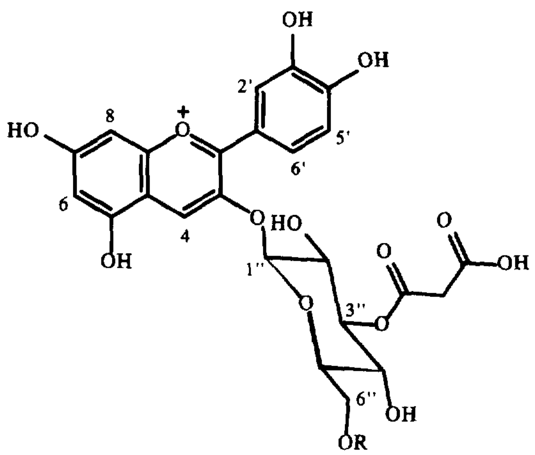
§ Due to deuterium exchange of the protons of the malonyl CH_2, the carbons of the malonyl CH_2 are not detected.

(×2) with 5% HOAc in MeOH. The filtered extracts were combined, concd under red. pres., purified by partition against EtOAc and applied to an Amberlite XAD-7 column [9]. The anthocyanins were sepd on a Sephadex G-15 column (100 × 1.6 cm, Pharmacia) using $MeOH–H_2O–HOAc$ (19:19:2) as eluent.

Hydrolysis. Acid hydrolysis and partial acid hydrolysis were carried out according to previously published procedures [6].

Chromatography. Analyt. TLC was carried out on microcrystalline cellulose (F1440, Schleicher & Schüll) with the solvents BAW ($1-BuOH–HOAc–H_2O$, 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). 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 consisted of isocratic elution (90% A, 10% B) in 4 min, linear gradient from 10 to 100% B during the next 17 min, isocratic elution (100% B) in 4 min followed by linear gradient from 100 to 10% B over 1 min. The flow rate was 1.2 ml min^{-1}, and 10 μl aliquots were injected. UV/Vis absorption spectra were recorded using a photodiode array detector (HP 1050, Hewlett-Packard), and spectral measurements were made over the range 210–600 nm. The quantitative data were based on the average values of the absorptions on every second nm between 500 and 540 nm without taking into account the different molar absorption coefficients of the pigments.

MS systems. The samples were dissolved in 5% TFA (in MeOH). LC-MS was performed on a VG Platform LC-MS system with electrospray as interface and $MeCN–H_2O$ as mobile phase. FAB-MS were obtained on a TRIO-2 mass spectrometer using a Xe beam. The sample was mixed with glycerol.



	R
1	malonyl
2	H

NMR spectroscopy. The NMR experiments were performed at 400.13 and 100.62 MHz for ^1H and ^{13}C, respectively, on a Bruker AM-400 instrument at 25°. The residual ^1H signal of the solvent (CF_3CO_2D–CD_3OD, 1:19) and the ^{13}C signal were used as secondary references (δ 3.4 and 49.0 from TMS, respectively). The 1D ^1H, the SEFT and the 2D HSC experiments were performed on a 5 mm ^1H–^{13}C dual probe. The 2D homonuclear correlation experiment (DQF-COSY) was performed on a 5 mm inverse probe. The data in the 2D experiments were collected with 2K complex data points. In DQF-COSY a sweepwidth of 3247 Hz and 680 t_1 increments were used. For the HSC experiment, a sweepwidth of 3721 and 18 111 Hz were used for ^1H and ^{13}C, respectively, with 72 t_1 increments. Note that the exchange with deuterium of the CH_2 protons of the malonate moieties in the acylated anthocyanins is observable after a few hours of storage in acidified CD_3OD.

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REFERENCES

1. Harborne, J. B. and Grayer, R. J. (1988) in *The Flavonoids: Advances in Research* (Harborne, J. B., ed.), p. 539. Chapman and Hall, London.
2. Strack, D and Wray, V. (1994) in *The Flavonoids: Advances in Research since* 1986 (Harborne, J. B., ed.), p. 1. Chapman and Hall, London.
3. Fuleki, T. (1971) *J. Food Sci.* **36**, 101.
4. Moore, A. B., Francis, F. J. and Jason, M. E. (1982) *J. Food Prot.* **45**, 590.
5. Terahara, N., Yamaguchi, M.-A. and Honda, T. (1994) *Biosci. Biotech. Biochem.* **58**, 1324.
6. Andersen, Ø. M. (1987) *J. Food Sci.* **52**, 665.
7. Andersen, Ø. M., Aksnes, D. W., Nerdal, W. and Johansen, O.-P. (1991) *Phytochem. Anal.* **2**, 175.
8. Andersen, Ø. M., Opheim, S., Aksnes, D. W. and Frøystein, N. Å. (1991) *Phytochem. Anal.* **2**, 230.
9. Andersen, Ø. M. (1988) *Acta Chem. Scand.* **42**, 462.