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ACYLATED ANTHOCYANINS FROM LEAVES OF THE WATER LILY, *NYMPHAÉA × MARLIACEA*

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Key Word Index—*Nymphaéa* × *marliacea*; Nymphaéaceae; leaves; anthocyanins; diacylation; delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside); delphinidin 3-*O*-(6″-*O*-acetyl-β-galactopyranoside); ^{13}C NMR.

Abstract—Three anthocyanins have been isolated from the leaves of the water lily, *Nymphaéa × marliacea*. Even though the lability of the two major anthocyanins was high, especially the linkage between the acetic acid and the galactose moieties, it was possible by means of homo- and heteronuclear two-dimensional NMR techniques and electrospray mass spectrometry to give the full structures of the novel pigments; delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside) (69%) and delphinidin 3-*O*-(6″-*O*-acetyl-β-galactopyranoside) (24%). The third anthocyanin (1.5%) was identified as delphinidin 3-*O*-β-galactopyranoside. Diacylation including gallic acid as one acyl moiety has previously not been reported for any flavonoid, and this is the first report of a 2″,6″-diacylated anthocyanin involving an aliphatic acid. © 1997 Elsevier Science Ltd

INTRODUCTION

Even though acylation of anthocyanins with aromatic acids of the cinnamoyl type has widespread occurrence, similar acylation with benzoic acid derivatives is much more restricted [1]. Gallic acid (3,4,5-trihydroxybenzoic acid) has for instance been identified only as cyanidin 3-(6″-galloylglucoside) in *Dipteronia sinensis* and *Acer* taxa, as cyanidin 3-(6″-galloylrutinoside) in *Acer* taxa [2, 3], and as the 3-(2″-galloylgalactosides) of delphinidin and cyanidin in two *Victoria* species [4]. In recent years different aliphatic acids have been found to be part of many anthocyanins. The linkage between the aliphatic acyl moiety and the sugar is however often sensitive to acids. Identification of the aliphatic acyl group is hampered by the lack of visible absorption and in some cases exchange of protons with deuterium in some NMR solvents [5]. As opposed to anthocyanins acylated with aliphatic dicarboxylic acids, the determination of acetylated anthocyanins is further complicated by no unusual electrophoretic mobility [6]. The following di- and polyacylated anthocyanins are reported to include acetyl moiety(ies): pelargonidin 3-(6-malonylglucoside)-5-(6-acetylglucoside) and the 3,5-di-(6-acetylglucoside) of pelargonidin, cyanidin and delphinidin from *Verbena* [7], pelargonidin 3-(6-*p*-coumarylglucoside)-5-(6-acetylglucoside) from *Hyacinthus orientalis* [8] and malvidin 3-coumarylglucoside-5-acetylxyloside from *Tibouchina urvilleana* [9].

Here we report on the unusual anthocyanin content in the leaves of a white-flowered variety of *Nymphaéa × marliacea*, including a delphinidin derivative acylated with both gallic and acetic acid on the galactosyl moiety.

RESULTS AND DISCUSSION

The HPLC chromatogram of the methanolic extract from leaves of *Nymphaéa × marliacea* detected in the visible spectral region, showed two major anthocyanins, **2** (24%) and **3** (69%), in addition to four minor anthocyanins (together 7%). Pigments **1**–**3** were purified by partition against both hexane and ethyl acetate followed by Amberlite XAD-7 column chromatography. The pigments were separated by Sephadex LH-20 gel filtration and preparative HPLC. The pure anthocyanins were checked for homogeneity by analytical HPLC and TLC (Table 1).

The UV-Vis spectrum of one of the minor anthocyanins, **1**, taken during on-line HPLC showed visible maximum at 527 nm with A_{440}/A_{527} of 26%, indicating the presence of an anthocyanin 3-glycoside with three oxygen functions on the B-ring [10]. The downfield part of the 1D ^{1}H NMR spectrum **1** showed a 1H

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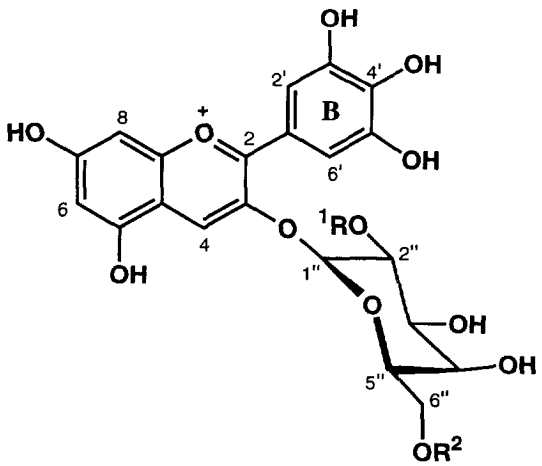

	R^1	R^2
1	H	H
2	H	acetyl
3	galloyl	acetyl

singlet at δ 9.07 (H-4), a 2H singlet at δ 7.88 (H-2′/6′) and a 2H AB system at δ 6.95 (*d*, J = 1.9 Hz, H-8), δ 6.74 (*d*, J = 1.9 Hz, H-6). The corresponding aglycone carbons were assigned by the heteronuclear shift correlation (HSC) experiment, and the signals of the quaternary carbon atoms (Table 3) were found by a heteronuclear coupling modulated spin echo (SEFT) experiment to be in accordance with a delphinidin aglycone [11]. The anomeric carbon signal appears considerably downfield from the other sugar resonances, and thus the crosspeak at δ 5.4/104.6 in the HSC spectrum of **1** together with 5 carbon resonances between δ 77.8 and 62.4 indicated a hexose with a pyranose form [12]. The identity of the sugar was found to be β-galactopyranosyl based on information regarding coupling constants, integration data (^{1}H NMR) and homo- and heteronuclear shift correlations (Table 2 and 3). Pigment **1** was co-chromatographed (HPLC and TLC) with authentic pigment from *Vaccinium* spp. [10], and a molecular ion $[M]^+$ of m/z 465 confirmed the structure to be delphinidin-3-*O*-β-galactopyranoside.

The UV-Vis spectra of pigments **2** and **3** taken during on-line HPLC showed visible maxima at 532 nm and 537 nm with $A_{440}/A_{\text{Vis.max}}$ of 22% and 20%, respectively, indicating the presence of two anthocyanidin 3-glycosides with extended chromophores. The relative high mobilities in both aqueous and alcoholic TLC systems and the relatively long retention times (HPLC) of **2** and **3** (Table 1) indicated that these pigments were acylated. The ^{1}H and ^{13}C resonances in the one- and two-dimensional NMR spectra of **2** and **3** were assigned in a similar way as those of **1** (Table 2 and 3). The signal resolution in the 1D ^{1}H NMR-spectra was remarkably good showing even the long-range couplings between H-4 and H-8 (0.8 Hz). Pigment **2** and **3** were thus found to contain the same aglycone (delphinidin) and sugar (β-galactopyranosyl) as **1**, however, the SEFT NMR spectra of **2** and **3** showed two and seven more resonances than the corresponding spectrum of **1**, respectively.

The acyl group of **2** was identified as acetic acid by the signals of δ 20.69 and 172.76 in the SEFT spectrum, the 3H singlet at δ 2.13 in the ^{1}H NMR spectrum [13] and the presence of the crosspeak at δ 2.1/20.7 in the HSC spectrum. In the same HSC spectrum the carbon signal at δ 65.20 was correlated to the proton resonances at both δ 4.43 (H-6A″) and δ 4.37 (H-6B″). The acetyl group was thus determined to be situated in the 6″-position on the galactosyl since the chemicals shifts of H-6A″, H-6B″ and C-6″ were shifted 0.5, 0.5, and 2.8 ppm downfield, respectively, and C-5″ was shifted 2.6 ppm upfield, compared to the corresponding signals of **1** (Table 2 and 3). A molecular ion $[M]^+$ of m/z 507 confirmed the structure of **2** to be delphinidin-3-*O*-(6″-*O*-acetyl-β-galactopyranoside), a novel anthocyanin. Gao and Mazza [14] have previously indicated the presence of an acetylated delphinidin-3-galactoside in an anthocyanin mixture isolated from lowbush blueberry, *Vaccinium angustifolium* Ait.

The UV-Vis spectrum of **3** showed higher absorbances around 278 nm ($A_{278}/A_{\text{Vis.max}}$ = 90%) than those of delphinidin 3-galactoside ($A_{278}/A_{\text{Vis.max}}$ = 69%), indicating the presence of an aromatic acyl group. The SEFT spectrum of **3** showed in addition to the corresponding signals of **2**, four positive and one negative carbon signals (Table 3). Two of the signals (δ 146.3 and 110.5) represented each two carbon atoms. The latter signal was correlated (HSC) with a 2H singlet at δ 7.06, in accordance with a galloyl (3,4,5-trihydroxybenzoyl) moiety. Starting from the anomeric proton and the two 6″-sugar protons we could, through the crosspeaks in the DQF-COSY spectrum of **3** assign all the sugar protons (Table 2). The pro-

Table 1. Chromatographic and spectral data of anthocyanins from leaves of *Nymphaéa* × *marliacea*; delphinidin 3-*O*-β-galactopyranoside (**1**), delphinidin 3-*O*-(6″-*O*-acetyl-β-galactopyranoside) (**2**), delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside) (**3**)

Compound	TLC (R_f) FHW	BAW	On-line HPLC Vis. max. (nm)	$A_{440}/A_{\max}$(%)	R_t(min)	ES-MS $[M]^+$
1	0.23	0.17	527	26	12.87	465
2	0.30	0.40	532	22	16.67	507
3	0.58	0.52	537	20	17.60	659

Table 2. ^{1}H NMR spectral data for delphinidin 3-*O*-*β*-galactopyranoside (**1**), delphinidin 3-*O*-(6″-*O*-acetyl-*β*-galactopyranoside) (**2**) and delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**3**) in CD_3OD–CF_3COOD (95:5) at 25°

	1 *δ* (ppm) *J* (Hz)	**2** *δ* (ppm) *J* (Hz)	**3** *δ* (ppm) *J* (Hz)
Delphinidin			
4	9.07 *s*	9.00 *d* 0.8	8.99 *d* 0.8
6	6.74 *d* 1.9	6.75 *d* 1.9	6.73 *d* 1.9
8	6.95 *s* (*broad*)	6.96 *dd* 1.9, 0.8	6.90 *dd* 0.8, 1.9
2′,6′	7.88 *s*	7.87 *s*	7.64 *s*
3-*O*-*β*-Galactopyranoside			
1″	5.36 *d* 7.8	5.34 *d* 7.7	5.63 *d* 7.9
2″	4.11 *dd* 7.8, 9.7	4.11 *dd* 7.7, 9.6	5.78 *dd* 7.9, 9.9
3″	3.78 *dd* 9.7, 3.7	3.78 *dd* 9.6, 3.4	4.07 *dd* 9.9, 3.7
4″	4.05 *d* 3.7	4.04 *dd* 3.4, 1.0	4.13 *d* (*broad*) 3.7
5″	3.85 *m*	4.15 *ddd* 1.0, 3.7, 8.3	4.27 *dd* (*broad*) 3.5, 8.8
6″A	3.86 *m*	4.43 *dd* 8.3, 11.8	4.52 *dd* 8.5, 11.9
6″B	3.86 *m*	4.37 *dd* 3.7, 11.8	4.44 *dd* 3.4, 11.9
2″-Galloyl			
2‴,6‴			7.06 *s*
6″-Acetyl			
2⁗		2.13 *s*	2.18 *s*

Table 3. ^{13}C NMR spectral data for delphinidin 3-*O*-*β*-galactopyranoside (**1**), delphinidin 3-*O*-(6″-*O*-acetyl-*β*-galactopyranoside) (**2**) and delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**3**) in CD_3OD–CF_3COOD (95:5) at 25°

	1 *δ* (ppm)	**2** *δ* (ppm)	**3** *δ* (ppm)	SEFT*
Delphinidin				
2	164.49	164.38	164.51	↑
3	145.96†	145.98†	145.62†	↑
4	136.61	135.68	135.34	↓
5	159.03‡	159.38‡	159.12‡	↑
6	103.29	103.30	103.30	↓
7	170.38	170.24	168.14	↑
8	95.03	95.13	95.18	↓
9	157.72‡	157.64‡	157.66‡	↑
10	113.29	113.87	112.97	↑
1′	120.07	120.01	119.53	↑
2′,6′	112.62	112.71	112.68	↓
3′,5′	147.56	147.57	147.38	↑
4′	144.71†	144.91†	144.79†	↑
3-*O*-*β*-Galactopyranoside				
1″	104.63	104.06	102.41	↓
2″	72.16	71.89	73.20	↓
3″	74.87	74.60	72.74	↓
4″	70.14	70.31	70.48	↓
5″	77.80	75.15	75.46	↓
6″	62.35	65.20	65.17	↑
2″-Galloyl				
1‴			120.82	↑
2‴,6‴			110.50	↓
3‴,5‴			146.30	↑
4‴			140.15	↑
7‴			170.33	↑
6″-Acetyl				
1⁗		172.76	172.83	↑
2⁗		20.69	20.74	↓

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

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

nounced downfield shift of H-2″ (1.7 ppm) compared to the analogous H-2″ signal of **1** and **2** (Table 2), showed that the galloyl moiety was connected to C-2″ on the galactose ring. This linkage was also confirmed by the observation that H-1″ and H-3″ of **3** were *ca* 0.3 ppm more deshielded than the corresponding signals of **1** and **2** (Table 2). Similarly, C-2″ was deshielded (more than 1 ppm) and C-1″ and C-3″ were shielded (more than 1.6 ppm) compared to the corresponding signals of **1** and **2** (Table 3). A molecular ion $[M]^+$ of *m/z* 659 confirmed the structure of **3** to be delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside), a novel anthocyanin. Anthocyanins containing a galloyl moiety are very rare in plants (see Introduction). Diacylation including gallic acid as one acyl moiety has previously not been reported for any flavonoid. This is the first report of a 2″,6″-diacylated anthocyanin involving an aliphatic acid.

It is interesting to note that the galloyl group in the galactosyl 2″-position (pigment **3**) causes a significant upfield shift effect (0.2 ppm) on H-2′,6′ (aglycone) (Table 2) and a bathochromic shift of 5 nm on the visible absorption maxima (Table 1), compared to the corresponding signal of **2**. This indicates that in acidified methanolic solutions the galloyl group influences the chemical environment of the aglycone B-ring, most probably by molecular association.

EXPERIMENTAL

Extraction and sepn. Leaves of the water lily, *Nymphaéa* × *marliacea.* (white flower cultivar) were collected in June 1996 from the Botanical Garden of the University of Bergen, and a voucher specimen has been deposited in BG. The leaves were cut into pieces and extracted with 0.5 M citric acid in MeOH at 5°. The filtered extract was concd under red. pres., purified by partition against hexane and EtOAc and applied to an Amberlite XAD-7 column [15]. The anthocyanins were further purified on a Sephadex LH-20 column (100 × 1.0 cm, Pharmacia) using MeOH–H_2O–TFA (49.5:50:0.5) as eluent. The anthocyanins were sepd by prep. HPLC on an Econosil C18 column (25 × 2.2 cm, 10 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 consisted of isocratic elution (90% A, 10% B) in 4 min, linear gradient from 10% B to 100% B during the next 17 min, isocratic elution (100% B) in 12 min followed by linear gradient from 100% B to 10% B over 1 min. The flow rate was 4.0 ml min^{-1}.

Analytical chromatography. TLC was carried out on microcrystalline cellulose (F1440, Schleicher & Schüll) with the solvents BAW (1-butanol–HOAc–H_2O; 4:1:5, upper phase) and FHW (HCO_2H–conc HCl–H_2O; 1:1:2). Analytical HPLC was performed with a ODS-Hypersil column (20 × 0.5 cm, 5 μm) using isocratic elution (90% A, 10% B) in 4 min, linear gradient from 10% B to 100% B during the next 17 min, isocratic elution (100% B) in 4 min followed by linear gradient from 100% B to 10% B over 1 min. The flow rate was 1.0 ml min^{-1}, and aliquots of 15 μl were injected.

Spectroscopy. UV-Vis absorption spectra were recorded on-line during HPLC analysis using a photodiode array detector (HP 1050, Hewlett-Packard). Spectral measurements were made over the wavelength range 240–600 nm in steps of 2 nm. The relative 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. The NMR experiments were obtained at 600.13 MHz and 150.92 MHz for ^1H and ^{13}C, respectively, on a Bruker DRX-600 instrument at 25°. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CF_3CO_2D–CD_3OD; 5:95,) were used as secondary references (*δ* 49.0 and *δ* 3.4 from TMS, respectively). The 1D ^1H and the 2D homonuclear correlation experiment (DQF-COSY) were performed on a 5 mm multinuclear TXI-probe. The heteronuclear spin-echo experiments (SEFT) and the 2D heteronuclear shift correlation (HSC) experiments were obtained on a 5 mm BBO probe. The mass spectra were obtained on a Quattro II MS/MS (Micromass, U.K.) by flow injection into the electrospray source. The instrument was operated in the positive ion mode and calibrated by NaI. The mobile phase carrier was a MeOH–H_2O (1:1) mixt. containing 0.1% formic acid. The carrier was pumped into the source at a flow rate of 100 μl min. Data acquisition was obtained by scanning from to 50–1000 Da in 3 second scans. The samples were dissolved in 3% formic acid (in MeOH) prior to analysis.

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