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Delphinidin 3′-galloylgalactosides from blue flowers of *Nymphaéa caerulea*

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Abstract

Two novel acylated anthocyanins have been isolated from blue flowers of the African water lily *Nymphaéa caerulea* (= *Nymphaéa capensis*). By a combination of chromatography, electrospray MS and homo- and heteronuclear two-dimensional NMR techniques they were identified to be delphinidin 3′-*O*-(2″-*O*-galloyl-*β*-galactopyranoside) (**1**) and delphinidin 3′-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**2**). Contrary to other anthocyanidin 3′-glycosides reported, **1** and **2** have the monosaccharide galactose instead of glucose on the B-ring and no glycosyl moiety on the anthocyanidin 3-position. The chemotaxonomic significance of anthocyanidin galloylgalactosides in Nymphaéaceae is discussed.

Keywords: Water lily; *Nymphaéa caerulea*; Nymphaéaceae; Blue flowers; Anthocyanins; 3′-Galloylgalactosides; Delphinidin 3′-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside); Delphinidin 3′-*O*-(2″-*O*-galloyl-*β*-galactopyranoside)

1. Introduction

The family Nymphaéaceae comprises of some thirty-five species spread over the whole world. These aquatic perennials show flower colour ranging from white, yellow, red to blue and have achieved high horticultural value. As part of our work on colour variation due to acylated anthocyanins, we have recently reported the identification of five anthocyanins, including three anthocyanins acylated with gallic acid, from flowers and leaves of *Nymphaéa* × *marliacea* cultivars with white, pink and red flowers (Fossen, Larsen, & Andersen, 1998). In this paper we report on the isolation and structure identification of two anthocyanins from the blue flowers of the African water lily *Nymphaéa caerulea*. These anthocyanins are based on the same building blocks as found in delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) and delphinidin 3-*O*-(2″-*O*-galloyl-*β*-galactopyranoside) isolated from *Nymphaéa* × *marliacea*, however the glycosidic moieties are located on the anthocyanidin B-ring.

2. Results and discussion

The HPLC chromatogram of the methanolic extract from flowers of *Nymphaéa caerulea* detected in the visible spectral region, showed one major (**2**) and several minor anthocyanins. Pigments **1** and **2** were purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography. The pigments were purified by Sephadex LH-20 column chromatography and separated by preparative HPLC. The pure anthocyanins were checked for homogeneity by analytical HPLC (Table 1).

The UV–Vis spectrum of **2** taken on-line during HPLC showed a visible maximum at 532 nm with A_{440}/A_{532} and $A_{\text{UV-max}}/A_{532}$ of 31 and 173%, respectively, indicating an acylated anthocyanidin-glycoside with aromatic non-cinnamic acyl group(s) having unusual linkage points between the aglycone and sugar moiety (Strack, Wray, Metzger, & Grosse, 1992). The relative high mobility in both aqueous and alcoholic TLC systems and long retention time on ODS-HPLC column compared with non-acylated pigments (Table 1), supported aromatic acylation. A molecular ion $[M+H]^+$ at m/z 657 in the electrospray MS spectrum of **2** was in agreement with the aglycone delphinidin and one moiety of hexose, gallic acid and acetic acid. The singlet at δ 9.19 (H-4), the 2H *meta*-coupled AX system at δ 6.78 (H-6) and δ 6.96 (H-8) together with the 2H *meta*-coupled AX system at δ

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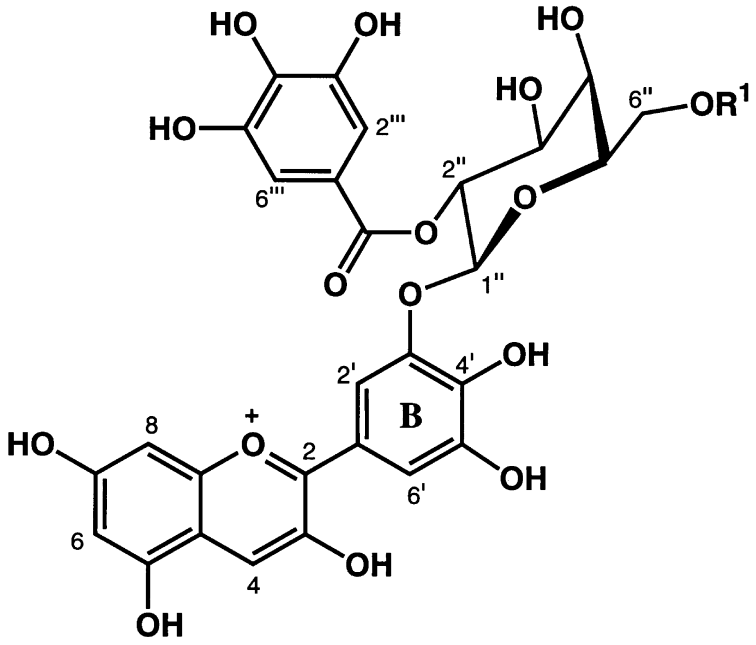

	R^1
1	H
2	CH_3CO

Table 1
Chromatographic and spectral data of delphinidin 3′-*O*-(2″-*O*-galloyl-*β*-galactopyranoside) (**1**), delphinidin 3′-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**2**), delphinidin 3-*O*-(2″-*O*-galloyl-*β*-galactopyranoside) (**3**), delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**4**) and delphinidin 3-*O*-*β*-galactopyranoside (**5**)

	TLC (R_f)		UV–Vis			
Compound	FHW	BAW	Vis-max (nm)	$A_{440}/A_{\text{Vis-max}}$ (%)	HPLC R_t (min)	ES-MS m/z
1	0.63	0.45	528	30	7.30	617
2	0.68	0.60	532	31	9.39	659
3	0.58	0.36	541	18	8.58	617
4	0.60	0.54	545	18	11.46	659
5	0.26	0.19	534	19	7.80	465

7.66 (H-2′) and δ 7.40 (H-6′) in the ^{1}H NMR spectrum of **2**, were in accordance with a delphinidin aglycone with the B-ring protons as non-equivalent doublets indicating an asymmetrically substituted B-ring. The protons in the sugar region showed typical coupling constants for a *β*-galactopyranose unit (Table 2). This identification was confirmed by the carbon shift values in the ^{13}C SEFT and the HSQC spectra of **2**. The singlet at δ 7.0 (H-2‴, 6‴) in the ^{1}H NMR spectrum, which was correlated to a carbon at δ 107.7 (C-2‴, 6‴) in the HSQC-spectrum, was in agreement with gallic acid (3,4,5-trihydroxybenzoic acid) (Fossen et al., 1998). The acetyl group was recognised by the 3H singlet at δ 2.2 (H-2⁗) which was correlated to carbon shifts at δ 20.5 (C-2⁗) and δ 172.4 (C-1⁗) in the HMBC-spectrum of **2**.

The crosspeak at δ 7.7/5.6 (H-2′/H-1″) in the ROESY spectrum of **2** revealed the linkage between the sugar and the aglycone at the 3′-hydroxyl. The pronounced downfield shift of H-2″ compared to the analogous H-2″-signal of unsubstituted galactose (1.6 ppm) indicated that the galloyl moiety was connected to C-2″ on the galactose ring (Fossen et al., 1998). The crosspeaks at δ 7.0/5.7 (H-2‴, 6‴/H-2″) and δ 7.0/5.6 (H-2‴, 6‴/H-1″) in the ROESY-spectrum of **2** confirmed this linkage. The downfield shifts of H-6A″ and H6B″ (0.5 ppm) and C-6″ (2.8 ppm) compared to the analogous resonances of unsub-

Table 2
^{1}H NMR spectral data for delphinidin 3′-*O*-(2″-*O*-galloyl-*β*-galactopyranoside) (**1**), delphinidin 3′-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**2**), delphinidin 3-*O*-(2″-*O*-galloyl-*β*-galactopyranoside) (**3**), delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**4**) in CD_3OD: CF_3COOD (19:1) at 25°C

	1 (δ (ppm), J (Hz))	**2** (δ (ppm), J (Hz))	**3** (Fossen et al., 1998) (δ (ppm), J (Hz))	**4** (Fossen et al., 1998) (δ (ppm), J (Hz))
Aglycone				
4	9.30 s	9.19 s	9.05 s (broad)	8.99 d 0.8
6	6.75 d, 1.9	6.78 d, 1.9	6.71 d 2.0	6.73 d 1.9
8	6.95 d, 1.9	6.96 d, 1.9	6.89 dd 2.09, 0.8	6.90 dd 0.8, 1.9
2′	7.71 d, 2.2	7.66 d, 2.2	7.68 s	7.64 s
6′	7.42 d, 2.2	7.40 d, 2.2	7.68 s	7.64 s
O-Galactoside				
1″	5.55 d, 8.0	5.61 d, 7.8	5.65 d 7.9	5.63 d 7.9
2″	5.69 dd, 8.0, 10.0	5.69 dd, 7.8, 10.0	5.77 dd 7.9, 9.9	5.78 dd 7.9, 9.9
3″	4.11 dd, 10.0, 3.5	4.12 dd, 10.0, 3.5	4.07 dd 9.9, 3.4	4.07 dd 9.9, 3.7
4″	4.15 d, 3.5	4.15 d, 3.5	4.13 d 3.4	4.13 d 3.7
5″	4.04 m	4.30 dd, 4.4, 7.3	4.02 ddd 0.9, 4.8, 7.2	4.27 dd 3.5, 8.8
6A″	4.00 dd, 7.4, 11.4	4.48 m	3.97 dd 7.2, 11.4	4.52 dd 8.5, 11.9
6B″	3.93 dd, 4.6, 11.4	4.47 m	3.92 dd 4.8, 11.4	4.44 dd 3.4, 11.9
2″-Galloyl				
2‴, 6‴	7.01 s	6.99 s	7.07 s	7.06 s
6″-Acetyl				
2⁗		2.23 s		2.18 s

stituted galactose, revealed the linkage of the acetyl group at the 6″-hydroxyl (Fossen et al., 1998). The crosspeak at δ 4.5/2.2 (H-6A″,6B″/H-2⁗) in the ROESY-spectrum confirmed this linkage. Thus, the structure of **2** was determined to be delphinidin 3′-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside).

The ^{1}H NMR resonances of **1** (Table 2) showed that this pigment contained the same aglycone (delphinidin), sugar (*β*-galactopyranoside) and aromatic acyl group (galloyl) as **2**. The 2H *meta*-coupled AX system at δ 7.71 (H-2′) and δ 7.42 (H-6′) were in accordance with an asymmetric B-ring having the galactoside in the 3′-position. The pronounced downfield shift of H-2″ (1.6 ppm) compared to the analogous H-2″-signal of unsubstituted galactose (Fossen et al., 1998), showed that the galloyl moiety was connected to C-2″ of the galactose moiety. A molecular ion $[M+H]^+$ at m/z 617 confirmed the structure of **1** to be the novel pigment delphinidin 3′-*O*-(2″-*O*-galloyl-*β*-galactopyranoside).

A transfer of the same glycosyl moiety from the anthocyanidin 3-position to the 3′-position seems to give the anthocyanins notable different spectroscopic and chromatographic properties. The two delphinidin 3′-glycosides, **1** and **2**, show a hypsochromic shift of 13 nm in the UV–Vis spectrum compared to the analogous delphinidin 3-glycosides (Table 1). The same glycosyl transfer implies that the $A_{440}/A_{\text{Vis-max}}$ increases with more than 66%. With respect to pigment colours, it is interesting to note that an acetyl unit positioned in the galactosyl 6-position also causes a bathochromic shift of 4 nm (Table 1), indicating influence of this acetyl moiety on intra- or intermolecular association. The delphinidin 3′-glycosides (**1** and **2**) have significantly higher R_f (TLC) and shorter R_t (ODS-HPLC) values than the corresponding delphinidin 3-glycosides (Table 1).

In this paper we report on two anthocyanins containing a galloylgalactosyl moiety. This rare moiety, however linked to different positions and aglycones, has been identified in one or more anthocyanins isolated from all examined species belonging to the family Nymphaéaceae (Strack et al., 1992; Fossen et al., 1998). Around 50 anthocyanins with a glycosyl moiety on the aglycone 3′-position have been identified. These pigments have been restricted to the families Bromeliaceae (Saito & Harborne, 1983), Commelinaceae (Yoshitama, 1978), Compositae (Yoshitama, Hayashi, Abe, & Kakisawa, 1975), Dioscoreaceae (Yoshida et al., 1991), Gentianaceae (Goto et al., 1982), Leguminosae (Saito, Abe, Honda, Timberlake, & Bridle, 1985), Lobeliaceae (Yoshitama, 1977), Orchidaceae (Griesbach, 1990) and Rhamnaceae (Bloor, 1997). Contrary to the anthocyanins identified in *N. caerulea*, **1** and **2**, all these pigments have another glycosyl moiety in the aglycone 3-position. The only anthocyanin previously reported to be glycosylated on the B-ring without a glycosyl moiety in the 3-position, is cyanidin 4′-glucoside. The glucosyl moiety of this pigment, which has been indicated to occur in flower petals of *Hibiscus esculentus*, was provisionally assigned to the

aglycone 4′-position mainly based on the lack of bathochromic shift after addition of aluminum chloride (Hedin, Lamar, Thompson, & Minyard, 1968). All other papers concerning the anthocyanin content of species in the genus *Hibiscus* or family Malvaceae, are reporting anthocyanidin 3-glycosides without a glycosyl on the B-ring. Anthocyanins like **1** and **2**, containing other monosaccharides than glucose connected to the anthocyanidin B-ring, have previously not been reported.

3. Experimental

3.1. Plant material

Flowers of *Nymphaéa caerulea* (=*Nymphaéa capensis*) were collected near Kampala, Uganda in September 1997. Voucher specimens have been deposited in Kampala.

3.2. Isolation of pigments

The flowers were cut into pieces with a pair of scissors and extracted with 5% HOAc in MeOH. The filtered extract was concd under red. pres., purified by partition (several times) against EtOAc and applied to an Amberlite XAD-7 column (Andersen, 1988). The anthocyanins were further purified by Sephadex LH-20 column chromatography and separated by prep. HPLC according to the published procedure (Fossen et al., 1998).

3.3. Analytical chromatography

TLC was carried out on microcrystalline cellulose (F1440, Schleicher and Schüll) with the solvents BAW (1-BuOH–HOAc–H_2O; 4:1:5 v/v, upper phase) and FHW (HCO_2H–conc. HCl–H_2O; 1:1:2 v/v). Analyt. HPLC was performed with a ODS-Hypersil column (20×0.5 cm, 5 μm) using the solvents HCOOH–H_2O (1:9) (A) and HCOOH–H_2O–MeOH (1:4:5) (B). The elution profile consisted of a linear gradient from 10 to 100% B for 17 min, isocratic elution (100% B) for 4 min followed by linear gradient from 100 to 10% B for 1 min. The flow rate was 1.3 ml min^{-1} and aliquots of 15 μl were injected.

3.4. Spectroscopy

UV–Vis absorption spectra were recorded in 0.1% conc. HCl in MeOH. Spectral measurements were made over the wavelength range 240–600 nm in steps of 2 nm. The NMR experiments (DQF-COSY, ROESY, HMBC, HSQC, SEFT) were obtained at 600.13 and 150.92 MHz for ^1H and ^{13}C, respectively, on a Bruker DRX-600 instrument at 25°C. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CF_3CO_2D–CD_3OD; 1:19, v/v) were used as secondary references (δ 49.0 and δ 3.4 from TMS, respectively) (see Fossen et al. (1998) for more experimental details). ^{13}C NMR on the sugar moiety of **2**: δ 101.69 (1″), 72.67 (2″), 71.96 (3″), 70.24 (4″), 75.34 (5″), 65.13 (6″).

The mass spectra were obtained on a Quattro II (Micromass, UK) by flow injection into the electrospray source. The instrument was operated in the positive ion mode and the mobile phase carrier was a MeOH–H_2O (1:1) mixture containing 0.1% HCOOH. Data acquisition was obtained by scanning with the first quadrupole only from 50–1000 Da in 3 s scans. The carrier was pumped into the source at a flow rate of 100 μl min^{-1}. The samples were dissolved in 3% HCOOH (in MeOH) prior to analysis.

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References

Andersen, Ø. M. (1988). *Acta Chem. Scand.*, *42*, 462.
Bloor, S. J. (1997). *Phytochemistry*, *45*, 1399.
Fossen, T., Larsen, Å., & Andersen, Ø. M. (1998). *Phytochemistry*, *48*, 823.
Goto, T., Kondo, T., Tamura, H., Imagawa, H., Iino, A., & Takeda, K. (1982). *Tetrahedron Lett.*, *23*, 3695.
Griesbach, R. J. (1990). *Lindleyana*, *5*, 231.
Hedin, P. A., Lamar, P. L., Thompson, A. C., & Minyard, J. P. (1968). *Am. J. Bot.*, *55*, 431.
Saito, N., Abe, K., Honda, T., Timberlake, C. F., & Bridle, P. (1985). *Phytochemistry*, *24*, 1583.
Saito, N., & Harborne, J. B. (1983). *Phytochemistry*, *22*, 1735.
Strack, D., Wray, V., Metzger, J. W., & Grosse, W. (1992). *Phytochemistry*, *31*, 989.
Yoshida, K., Kondo, T., Kameda, K., Kawakishi, S., Lubag, A. J. M., Mendoza, E. M. T., & Goto, T. (1991). *Tetrahedron Lett.*, *32*, 5575.
Yoshitama, K. (1977). *Phytochemistry*, *16*, 1857.
Yoshitama, K. (1978). *Bot. Mag. Tokyo*, *91*, 207.
Yoshitama, K., Hayashi, K., Abe, K., & Kakisawa, H. (1975). *Bot. Mag. Tokyo*, *88*, 213.