Semipreparative Isolation and Structure Determination of Pelargonidin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside and Other Anthocyanins from the Tree *Dacrycarpus dacrydioides*

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Pelargonidin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (Podocarpin B), a novel anthocyanin, was isolated from receptacles of *Dacrycarpus dacrydioides*. In addition, smaller amounts of cyanidin-, delphinidin- and pelargonidin 3-*O*-β-D-glucopyranosides were found. The seeds contained 56 %, 29 % and 15 %, respectively, of the latter three anthocyanins, while 74 %, 24 % and 2 % of the same pigments were found in the undeveloped ovules. The total anthocyanin content in receptacle, seed and undeveloped ovules was 16, 63 and 72 mg per 100 g of fresh material, respectively.

The anthocyanins were isolated by a combination of chromatography on Amberlite XAD-7 (an ion exchange resin), droplet counter-current chromatography and Sephadex LH-20 gel filtration. The scale of the isolation enabled recording of ^{13}C NMR spectra.

Despite a dramatic increase in the number of publications on or involving ^{13}C NMR spectroscopy of other flavonoid groups,[1] the use of ^{13}C NMR spectroscopy in analysis of anthocyanins is still very limited: Ikuta *et al.*[2] reported signal assignments in the spectra of synthetic cyanidin and cyanidin 3-*O*-glucoside isolated from *Morus alba*, Bridle *et al.*[3] used ^{13}C NMR spectroscopy for confirmation of the structure of cyanidin 3-*O*-β-(6-*O*-malonyl)glucoside isolated from *Cichorium intybus*, Goto *et al.*[4] have reported the signals in the ^{13}C NMR spectrum of 3-*O*-(β-sophorosyl)-5-*O*-(β-glucosyl)peonidin isolated from *Ipomoea*, and Strack *et al.*[5] have reported the corresponding signals of cyanidin 3-oxalylglucoside isolated from orchids. In this study, the tentative ^{13}C NMR spectra of three monoglucosides and one diglucoside are presented.

The Podocarpaceae, a large family of gymnosperms distributed in the southern hemisphere, are mostly evergreen trees. The endemic New Zealand white pine (*Dacrycarpus dacrydioides*), which has been examined in this study, has earlier been listed as *Podocarpus dacrydioides*. Markham and Whitehouse have recently[6] isolated a number of interesting flavones and flavonols from the foliage of *D. dacrydioides*. Lowry[7] has reported the presence of an unidentified anthocyanin, Podocarpin B, and pelargonidin-, cyanidin- and delphinidin-3-glucoside in the seed-bearing structures of the tree.

A combination of isolation methods is often required in the search for a few compounds in an extract containing several thousand components. For many years, the most commonly used methods for isolation of anthocyanins have been paper, thin-layer and occasionally column chromatography.[8] These methods enable isolation of the anthocyanins on a moderate scale, but may lead to losses due to irreversible adsorption, to incomplete removal of contaminants or to contamination with oligomeric extractives from the adsorbents. Recently, isolation of multi-milligram amounts of anthocyanins by high performance

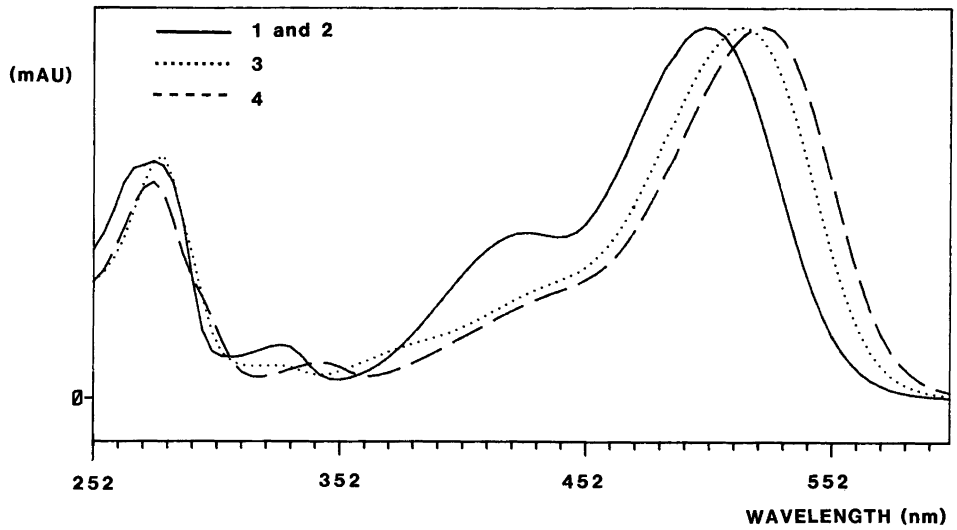


Fig. 1. Spectra of anthocyanins found in receptacles and seeds of *D. dacrydioides* recorded with a diode-array detector during HPLC analysis. Automatic scaling was used.

liquid chromatography (HPLC)[9,10,12] and droplet counter-current chromatography (DCCC)[11,12] has been described.

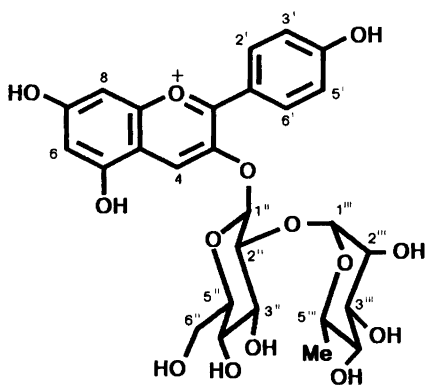
A very important step in any isolation sequence for plant phenolics from aqueous extracts is the elimination of the bulk of water-soluble contaminants. Various ion exchange resins have been reported to fulfil this task.[13,14]

This paper describes a convenient combination of methods for the isolation of anthocyanins on a semipreparative scale, and the identification of the anthocyanins in seeds, receptacles and undeveloped ovules from *D. dacrydioides*. Using chemical and spectroscopic procedures, Podocarpin B has been identified as pelargonidin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, a novel anthocyanin.

Results and discussion

The anthocyanin extracts of *D. dacrydioides* seeds and receptacles were purified by partition against EtOAc and then chromatographed on an Amberlite XAD-7 column. The anthocyanins were separated by DCCC, and finally purified by Sephadex LH-20 gel filtration. The purified pigments were checked for homogeneity by HPLC and TLC before analysis.

Compound **1** was only found in the receptacles. The UV/Vis spectrum of **1** in MeOH (0.02 % HCl) showed a visible maximum at 509 nm with A_{440}/A_{509} of 0.43. The addition of a few drops of 5 % $AlCl_3$ (in MeOH) did not result in any shift. Thus, the spectral data indicated the presence of a 3-glycoside with a pelargonidin nucleus.[8] Partial acid hydrolysis gave pelargonidin 3-*O*-glucoside (**2**) and pelargonidin, while complete acid hydrolysis gave pelargonidin, glucose and rhamnose. These data were confirmed by co-chromatography (TLC of sugars, and both TLC and HPLC of pelargonidin and pelargonidin 3-*O*-glucoside) with authentic markers.



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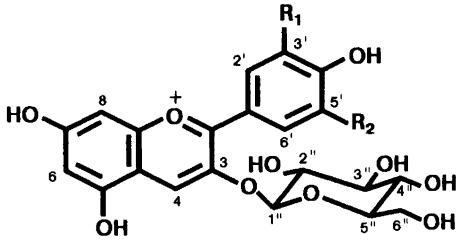
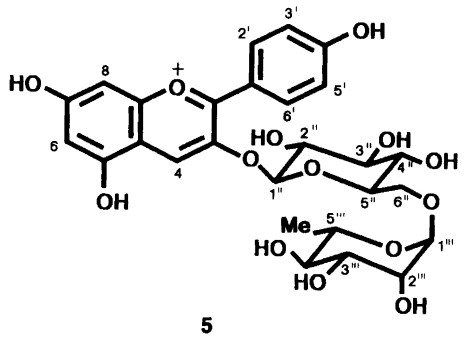
The attachment of the sugar to the 3-position of the aglycone was established by H_2O_2 oxidation of **1**, which provided a neohesperidose. This structure was confirmed by co-chromatography with authentic material prepared by similar oxidation of the flavanone naringin. Authentic pelargonidin 3-*O*-rutinoside (**5**), the only anthocyanin hitherto reported to contain pelargonidin, glucose and rhamnose in ratio 1:1:1, was then used for chromatographic (TLC and HPLC) comparison with **1**. R_f-values (0.93 for **1** and 0.73 for **5** in solvent C) and t_R-values (8.31 min for **1** and 10.41 min for **5** with system II), show the pigments to be different.

The presence of the pelargonidin group was confirmed as the chemical shifts for signals in both the ^{1}H NMR and ^{13}C NMR spectra of the aglycone moiety of **1** corresponded to those of **2**. The assignments of the signals of the carbohydrate portion of the ^{13}C NMR spectra of **1** were deduced by reference to the spectrum of **2** and the literature. [15,16] Particularly distinctive for the rhamnose *C*-methyl was the doublet (J = 6.0 Hz) at 0.73 ppm in the ^{1}H NMR spectrum. Since the signals for anomeric protons in ^{1}H NMR spectra are further downfield than signals for the other sugar protons,[17] the two doublets at 5.57 ppm and 5.17 ppm together with the integration data defined the pelargonidin:glucose:rhamnose ratio as 1:1:1. The coupling constant of 7.4 Hz at 5.57 ppm agreed with a diaxial coupling between the protons on C-1 and C-2 in a β-linked D-glycopyranose.[17] The signal at 5.17 ppm corresponded to an L-rhamnopyranose C-1 proton.[17] A coupling constant of 1.8 Hz eliminated a diaxial coupling and indicated an α-glycosidic linkage.[18] The lack of signals between 4 ppm and 5 ppm in the ^{1}H NMR spectrum[17] and around 66.5 ppm in the ^{13}C



2: R_1 = H R_2 = H
3: R_1 = OH R_2 = H
4: R_1 = OH R_2 = OH



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NMR spectrum[1] of **1**, together with the signal for the anomeric proton of rhamnose at low field (5.17 ppm)[17] in the ^{1}H NMR spectrum, excluded the possibility of the usual 1,6-linkage between the rhamnose and glucose units. The downfield shift (3.65 ppm) of the C-2″ in the ^{13}C NMR spectrum of **1** relative to the C-2″ signal of **2** was due to the rhamnosylation, and indicated a 1.2-linkage between the rhamnose and glucose units. The upfield shift (2.55 ppm) of the C-1″ signal for glucose, compared to the same signal of **2**, also indicated a 1,2-linkage between the sugars. By using the established criteria for the distinction between methyl glycofuranosides and methyl glycopyranosides of the same sugar,[1] both the glucose and rhamnose unit in **1** were determined to be in the pyranose form.

Published ^{13}C NMR spectra of anthocyanins have hitherto been limited to three monoglucosides of cyanidin[2,3,5] and 3-*O*-(β-sophorosyl)-5-*O*-(β-glucosyl)peonidin.[4] Table 1 contains the ^{13}C NMR data for two pelargonidin derivatives (**1** and **2**) and one delphinidin derivative (**4**) in addition to that for cyanidin 3-*O*-β-glucoside (**3**). The assignments of the signals in the spectrum of **3** are based on published data.[2] The ^{1}H noise-decoupled spectra of glucoside **2** and **4** each consist of 19 discrete peaks. The nine peaks due to the carbons of the heterocyclic ring and the A-ring, and the six peaks due to the carbons of glucose can be assigned with reference to the spectrum of **3**. The remaining peaks, which represent the carbons of ring B, can be subdivided into one quaternary, one non-quaternary (sp^2) signal and two signals with intensity of approximately twice that of a single non-quaternary signal. The quaternary signal (119.2 and 118.3 ppm in the spectra of **2** and **4**, respectively) has to be

Table 1. ^{13}C NMR data for anthocyanins in *D. dacrydioides*.[a]

	1	2	3	4
Anthocyanidin				
C-2	161.5	161.3	161.4	161.1
C-3	143.8	143.7	144.1	144.1
C-4	134.3	134.3	134.7	134.4
C-5	157.6	157.6	157.7	157.6
C-6	102.5	102.7	102.5	102.5
C-7	168.8	169.0	168.6	168.4
C-8	94.2	94.0	94.1	94.0
C-9	156.0	156.0	155.7	155.7
C-10	111.9	111.7	111.8	111.7
C-1′	120.3	119.2	119.5	118.3
C-2′	134.0	134.4	117.4[c]	111.2
C-3′	116.8	117.0	146.2	146.5
C-4′	164.6	164.3	154.4	143.1
C-5′	116.8	117.0	116.8[c]	146.5
C-6′	134.0	134.4	126.7	111.2
Glucose				
C-1″	99.5	102.0	101.9	101.8
C-2″	76.6[b]	72.9	73.1	73.1
C-3″	76.5[b]	76.3[b]	76.5[b]	76.4[b]
C-4″	69.9[c]	69.5	69.6	69.6
C-5″	77.2[b]	77.2[b]	77.5[b]	77.5[b]
C-6″	60.3	60.6	60.7	60.6
Rhamnose				
C-1‴	100.9			
C-2‴	70.2[c]			
C-3‴	70.1[c]			
C-4‴	72.4			
C-5‴	68.2			
C-6‴	17.7			

[a]Spectra were determined in DMSO-d_6/trace HCl at 20 MHz. δ-values in ppm ref. to TMS. [b,c]Assignment bearing the same superscript in any one spectrum may be reversed.

assigned to C-1′. Because of the symmetry in the B-ring, it is expected that the signals of C-2′ and C-6′ are superimposed, and that C-3′ and C-5′ also are represented by one signal. By comparing with published chemical shifts of analogous B-rings of flavonols,[1] the signal at 134.4 ppm in the spectrum of **2** and at 111.2 ppm in the spectrum of **4** are assigned to C-2′ and C-6′. Similar arguments lead to the assignment of the signal at 117.0 ppm in the spectrum of **2** and at 146.5 ppm in the spectrum of **4** to C-3′ and C-5′. The remaining signal at 164.3 and 143.1 ppm in the spectra of **2** and **4**, respectively, is then assigned to C-4′. All the sugars in the anthocyanins found in *D. dacrydioides,* were determined to be in the pyranose form.[1]

In the majority of anthocyanins which have been studied, the sugars and the points of attachment to the aglycone are normally established. The ring size of each sugar, the anomeric form of the sugar and the glycosidic linkages between sugars are, however, generally unknown or poorly defined. The elucidation of the structure of pelargonidin 3-*O*-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranoside (**1**) shows what contribution ^{13}C NMR spectroscopy, often in combination with ^1H NMR spectroscopy, can make in these problem areas.

The evolutionary tendency in higher plants has been to select for the pigments which give flowers and fruits of the colour range most appreciated by the predominant pollinating and seed-dispersing animals. The occurrence of anthocyanins in the gymnosperms is rather rare.[19] A typical ripe female ovule of *D. dacrydioides,* which consists of an orange-red receptacle, atop a bluish seed and two blue undeveloped ovules, must be one of the most striking anthocyanin structures. It gives the appearance of an angiosperm fruit, and the anthocyanins obviously render the structure more readily detectable and aid in animal dispersion of the seed. While compound **1** was the major pigment in receptacles, compounds **2**, **3** and **4** constituted the anthocyanins in the seeds and undeveloped ovules (Table 2). Since the undeveloped ovules are non-mature seeds, it is not

Table 2. The total amount and relative proportions of anthocyanins in receptacle, seed and undeveloped ovule in *D. dacrydioides.*

Anthocyanin	Area/%		
	Receptacle	Seed	Ovule[a]
1	94	–	–
2	1	15	2
3	4	56	74
4	1	29	24
Total amount (mg per 100 g fresh material)	16	63	72

[a]Undeveloped.

unexpected that the anthocyanin content in seed and ovules are rather similar; however, the relative proportions of **2** and **3** were clearly different.

Experimental

General techniques. Samples were passed through a 18×2.6 cm Amberlite XAD-7 column (an ion exchange resin from BDH Chemicals Ltd.), which had been washed in advance with H_2O. The column was washed with 2 l of H_2O. To elute the anthocyanins, 300 ml each of 50 % aqueous MeOH and anhydrous MeOH (both containing 0.5 % CF_3COOH) were used successively. DCCC (droplet counter-current chromatography) was carried out using a Tokyo Rikakikai Eyela Model DCC-300 chromatograph linked to a Cecil CE212A monitor and a fraction collector. The instrument was fitted with 225 glass capillaries (BAW, 40 cm × 3.4 mm i.d.), and the lower phase of 1-butanol-acetic acid-water (BAW; 4:1:5, v/v), was used as mobile phase. A flow rate of ca 50 ml h^{-1} was maintained throughout the experiment. Ca. 280 ml of stationary phase was displaced prior to elution of the first drop of mobile phase. Fractions of 9–12 ml were collected and monitored at 280 nm, and by HPLC and TLC. Gel filtration was performed on a 25×1.5 cm Sephadex LH-20 column using 0.1 % HCl as eluent. The eluate was monitored visually and by TLC. TLC analyses on cellulose were performed on Schleicher and Schüll F1440 sheets in the following solvent systems: (A) AcOH–H_2O (15:85, v/v), (B) conc. HCl–H_2O (3:97, v/v), (C) HCOOH-conc. HCl–H_2O (5:1:5, v/v) and (D) upper phase of BAW. HPLC was carried out with two systems. I: on an Alltech-Applied Science Econosphere C-18 column (250×4.6 mm), 5 μm, fitted with an RP-18 (Brownlee) precolumn with the solvents: HCOOH–H_2O (1:8, v/v) (E) and MeOH H_2O (19:1, v/v) (F). A programmed mix was used beginning with 10 % F and changing linearly to 50 % over a period of 20 min. The detector was set at 280 nm, and the flow rate was 1.5 ml min^{-1}. The equipment constituting system II is mostly as published.[12] Two solvents were used for elution: (G) HCOOH–H_2O (1:9, v/v) and (H) HCOH–H_2O MeOH (1:4:5, v/v). The elution profile was 10 % H to 80 % H in G over a period of 20 min. UV/Vis absorption spectra were measured in MeOH containing 0.02 % conc. HCl with a Hewlett Packard HP-8451A spectrophotometer, and in eluates during HPLC analysis with a Hewlett Packard HP-1040 diode-array detector.

^{13}C NMR spectra were recorded on a Varian FT-80A spectrometer operating at 20 MHz. The spectra were recorded on samples (10–32 mg) in DMSO-d_6/trace conc. HCl at 30 °C in 5 mm tubes using acquisition times of ca. 0.8 s. The central peak of the solvent septet was used as an internal reference, and all data were converted to chemical shifts relative to TMS using the equation:

$$\delta = \delta(\text{DMSO-}d_6) + 39.5 \text{ ppm}.$$

^1H NMR spectra were obtained on a Varian XL-200 instrument at ambient probe temperature, using 35 % CF_3COOD – 65 % DMSO-d_6 (v/v) as solvent and TMS as an internal reference.

Acid and partial hydrolysis were carried out with one single modification of published procedure:[12] The aqueous solutions which contained the sugars, were not de-acidified with *N*-methyl-*N,N*-dioctylamine prior to being evaporated to dryness. H_2O_2 oxidation was according to standard procedure.[8]

Extraction. Female cones (517 g) of *D. dacrydioides* were collected 16/4-87 in Upper Hutt, New Zealand. Fresh cones were divided into seeds (206 g) and receptacles (311 g). The black ovules were parted from the receptacles. The seeds, receptacles and ovules were extracted separately with MeOH containing 0.1 % HCl (3×1 l, 3×1 l and 3×0.1 l, respectively).

Quantitative analysis. The total amount of anthocyanins in the extracts was calculated as cyanidin 3-*O*-β-D-glucoside from a standard curve. This curve was prepared by plotting different concentrations of cyanidin 3-*O*-β-D-glucoside in acidified MeOH (ε = 29500) versus A_{537} measured on a Hewlwtt Packard HP-8451A spectrophotometer.

Isolation. The extracts, except the extract of undeveloped ovules, were concentrated under reduced pressure and then partitioned twice against EtOAc. In order to eliminate polar non-phenolic compounds, the concentrates of the aqueous layers from the EtOAc partitions were passed through an Amberlite XAD-7 column. The el-

uates were taken to dryness under reduced pressure, and the residues were dissolved in 20 ml of equal parts of upper and lower layers of BAW and subjected to DCCC. Compound **1** (16 mg) was obtained in the fractions 25 to 36 of the DCCC eluate of the receptacles. Compounds **2**, **3** and **4** were collected from the DCCC eluate of the seeds. **4** (19 mg) was obtained in fractions 31 to 47, followed by a mixture of **4** and **3** (5 mg) in fractions 48 to 53. Pure **3** (41 mg) was eluted in fractions 54 to 65, followed by a mixture of **3** and **2** (3 mg) in fractions 66 to 78, and finally pure **2** (14 mg) from fractions 79 to 102. All the compounds were gel filtered on Sephadex LH-20 before analysis.

Pelargonidin 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**1**); $[\alpha]_D^{20}$ −23° (*c* 0.12; H_2O); λ_{max} nm (ε) (0.02% HCl–MeOH): 272 (17400), 509 (28100), A_{440}/A_{509} 0.43; λ_{max} nm (during HPLC Syst. II) 276, 500, A_{440}/A_{500} 0.43; TLC R_f: 0.83 (A), 0.64 (B), 0.93 (C) and 0.64 (D); HPLC t_R min: 15.8 (I), 8.31 (II); ^{1}H NMR: δ 8.89 (1H, s, H-4), 8.43 (2H, d, *J* = 8.9 Hz, H-2′ and H-6′), 6.99 (2H, d, *J* = 8.8 Hz, H-3′ and H-5′), 6.83 (1H, d, *J* = 1.8 Hz, H-8), 6.71 (1H, d, *J* = 1.8 Hz, H-6), 5.57 (1H, d, *J* = 7.4 Hz, H-1 glc), 5.17 (1H, d, *J* = 1.8 Hz, H-1 rha), 3.2–3.9 (10H, m, sugar protons), 0.73 (3H, d, *J* = 6.0 Hz, Me rhamnose); ^{13}C NMR: see Table 1.

Pelargonidin 3-O-β-D-glucopyranoside (**2**); $[\alpha]_D^{20}$ −11° (*c* 0.11; H_2O): λ_{max} nm (ε) (0.02% HCl–MeOH): 272 (16400), 509 (26900), A_{440}/A_{509} 0.41; λ_{max} nm (during HPLC Syst. II) 276, 500, A_{440}/A_{500} 0.43; TLC R_f: 0.33 (A), 0.09 (B), 0.65 (C) and 0.58 (D); HPLC t_R min: 17.2 (I), 9.85 (II); ^{1}H NMR: δ 8.87 (1H, s, H-4), 8.44 (2H, d, *J* = 8.8 Hz, H-2′ and H-6′), 6.98 (2H, d, *J* = 8.8 Hz, H-3′ and H-5′), 6.81 (1H, d, *J* = 1.8 Hz, H-8), 6.70 (1H, d, *J* = 1.8 Hz, H-6), 5.21 (1H, d, *J* = 7.4 Hz, H-1 glc), 3.2–3.9 (6H, m, sugar protons); ^{13}C NMR: see Table 1.

Cyanidin 3-O-β-D-glucopyranoside (**3**); $[\alpha]_D^{20}$ −8° (*c* 0.08; H_2O); λ_{max} nm (ε) (0.02% HCl–MeOH): 277 (17500), 526 (27700), A_{440}/A_{526} 0.25; λ_{max} nm (during HPLC Syst. II) 280, 516, A_{440}/A_{516} 0.31; TLC R_f: 0.30 (A), 0.07 (B), 0.52 (C) and 0.43 (D); HPLC t_R min: 15.6 (I), 7.90 (II); ^{1}H NMR: δ 8.81 (1H, s, H-4), 8.02 (2H, m, H-2′ and H-6′), 6.94 (1H, d, *J* = 9.2 Hz, H-5′), 6.74 (1H, d, *J* = 1.4 Hz, H-8), 6.66 (1H, d, *J* = 1.8 Hz, H-6), 5.21 (1H, d, *J* = 7.4 Hz, H-1 glc), 3.2–3.9 (6H, m, sugar protons); ^{13}C NMR: See Table 1.

Delphinidin 3-O-β-D-glucopyranoside (**4**); $[\alpha]_D^{20}$ −18° (*c* 0.09; H_2O); λ_{max} nm (ε) (0.02% HCl–MeOH): 279 (17000), 537 (27300), A_{440}/A_{537} 0.19; λ_{max} nm (during HPLC Syst. II) 277, 524, A_{440}/A_{524} 0.28; TLC R_f: 0.16 (A), 0.02 (B), 0.37 (C) and 0.22 (D); HPLC t_R min: 14.2 (I), 6.51 (II); ^{1}H NMR: δ 8.76 (1H, s, H-4), 7.74 (2H, s, H-2′ and H-6′), 6.73 (1H, d, *J* = 1.8 Hz, H-8), 6.67 (1H, d, *J* = 1.6 Hz, H-6), 5.25 (1H, d, *J* = 7.4 Hz, H-1 glc), 3.2–3.9 (6H, m, sugar protons); ^{13}C NMR: see Table 1.

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