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DELPHINIDIN-3-NEOHESPERIDOSIDE AND CYANIDIN-3-NEOHESPERIDOSIDE FROM RECEPTACLES OF *PODOCARPUS* SPECIES

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Key Word Index—*Podocarpus*; Podocarpaceae; anthocyanins; delphinidin-3-neohesperidoside; cyanidin-3-neohesperidoside; podocarpin A; chemotaxonomy.

Abstract—Receptacles of *Podocarpus totara*, *P. nivalis* and *P.* cv *nivalis* × *acutifolius* were studied for their anthocyanins. Cyanidin-3-neohesperidoside (podocarpin A) was the major anthocyanin in all examined samples. The minor pigments were isolated and characterized as delphinidin-3-neohesperidoside (a new anthocyanin), cyanidin-3-rutinoside and cyanidin-3-glucoside. Anthocyanins with neohesperidose as the sugar unit have chemotaxonomic significance since they have never been reported to occur outside the Podocarpaceae.

INTRODUCTION

The gymnosperm family Podocarpaceae consists of eight genera, the largest being *Podocarpus* [1], distributed in the southern hemisphere. Although *Podocarpus* s.l. may have been a natural taxon [2], the revision by De Laubenfels [3] divided it into five genera. Anthocyanins have been reported from several species belonging to Podocarpaceae [4–6]. From *Podocarpus totara* and *P. nivalis* Lowry [4] isolated a labile pigment which was tentatively identified as cyanidin 3-acylglucoside, podocarpin A, as the only anthocyanin. This paper reports the anthocyanin content of three populations of *P. totara* and one population of *P. nivalis* and *P.* cv *nivalis* × *P. acutifolius*. A new anthocyanin, delphinidin-3-neohesperidoside, and cyanidin-3-neohesperidoside, cyanidin-3-rutinoside and cyanidin-3-glucoside were isolated and characterized. Podocarpin A was found to be cyanidin-3-neohesperidoside.

RESULTS AND DISCUSSION

The anthocyanin extract of *P. totara* receptacles (bulk sample) was partitioned against ethyl acetate 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 purity of the different pigments was tested by HPLC and TLC before analysis. The anthocyanin extracts of the other examined species and populations were purified by partition against EtOAc and then chromatographed on an Amberlite XAD-7 resin. The anthocyanins were separated by HPLC, and their identification were based on spectroscopic measurements and cochromatography with anthocyanins isolated from *P. totara* (bulk sample).

The UV/Vis spectrum of compound **1** in methanol (0.02% HCl) showed a visible maximum at 528 nm with A_{440}/A_{528} of 26%. The addition of a few drops of 5% aluminium chloride (in MeOH) resulted in a bathochromic shift of 25 nm. Thus the spectral data indicated the presence of a 3-glycoside with a cyanidin or peonidin nucleus [7]. Mild acid hydrolysis produced cyanidin-3-glucoside (**4**) with the liberation of rhamnose, while complete acid hydrolysis gave cyanidin, glucose and rhamnose. These data were confirmed by cochromatography (TLC of sugars and both TLC and HPLC of cyanidin and cyanidin-3-glucoside) with authentic markers.

The cyanidin group was confirmed as the chemical shifts for signals in the ^{1}H NMR spectrum of the aglycone moiety of **1** corresponded to those of **4** (Table 1). Since the chemical shifts of the signals for anomeric protons in ^{1}H NMR spectra are more downfield than signals for the other sugar protons [8], the two doublets at δ5.57 and 5.17 together with the integration data defined the cyanidin : 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-glucopyranose [6, 8]. The signal at δ5.17 indi-

Table 1. ^{1}H NMR data for **1** and **4** and pelargonidin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside[6] (**5**) (chemical shifts (δ) of aglycone, anomeric protons and methyl groups)

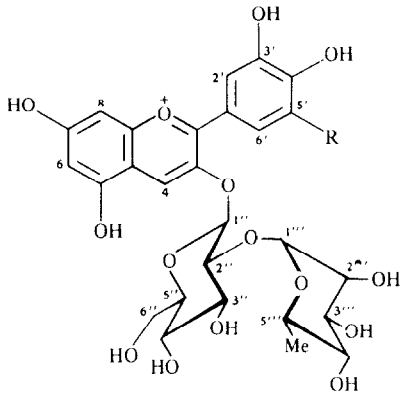
H	**2**	**4**	**5**
4	8.84 *s*	8.81 *s*	8.89 *s*
6	6.69 *d* (2.0)	6.66 *d* (1.8)	6.71 *d* (1.8)
8	6.79 *d* (2.0)	6.74 *d* (1.4)	6.83 *d* (1.8)
2′	8.04 *m*	8.02 *m*	8.43 *d* (8.9)
3′	—	—	6.99 *d* (8.8)
5′	6.98 *d* (9.2)	6.94 *d* (9.2)	6.99 *d* (8.8)
6′	8.04 *m*	8.02 *m*	8.43 *d* (8.9)
Glc-H-1	5.57 *d* (7.4)	5.21 *d* (7.4)	5.56 *d* (7.4)
Rha-H-1	5.17 *d* (1.6)	—	5.16 *d* (1.8)
-Me	0.73 *d* (6.1)	—	0.73 *d* (6.0)

The spectra were recorded at 200 MHz in DMSO-d_6 solutions with 35% TFA-*d* using TMS ($\delta = 0$) as internal standard. Coupling constants in Hz are given in parantheses. The remaining sugar protons were found in region δ3.2–3.9.

cated an L-rhamnopyranose C-1 proton [6, 8]. A coupling constant of 1.6 Hz eliminated a diaxial coupling and indicated an α-glycosidic linkage [9]. The lack of signals between δ4 and 5 together with the signal for the anomeric proton of rhamnose at low field (5.17 ppm) excluded the possibility of the usual 1,6-linkage between the rhamnose and glucose units [8]. Particularly distinctive for the rhamnose C-methyl was the doublet (J = 6.1 Hz) at δ0.73.

The UV/Vis spectrum of compound **2** in methanol (0.02% HCl) showed a visible maximum at 537 nm with A_{440}/A_{537} of 0.19. The addition of a few drops of 5% aluminium chloride (in MeOH) resulted in a bathochromic shift of 27 nm. Thus the spectral data indicated the presence of a 3-glycoside with a delphinidin or petunidin nucleus [7]. Mild acid hydrolysis produced delphinidin-3-glucoside with the liberation of rhamnose, while complete acid hydrolysis gave delphinidin, glucose and rhamnose. These data were confirmed by cochromatography (TLC of sugars and both TLC and HPLC of delphinidin and delphinidin-3-glucoside) with authentic markers.

Authentic delphinidin-3-rutinoside (isolated from *Ribes nigrum*) [10], the only anthocyanin hitherto reported to contain delphinidin, glucose and rhamnose in the ratio 1 : 1 : 1, was then used for chromatographic (TLC and HPLC) comparison with **2**. R_f values (0.81 for **2** and 0.58 for delphinidin-3-rutinoside in solvent C) and R_t values (5.78 min for **2** and 7.51 min for delphinidin-3-rutinoside with system II), show the pigments to be different.



1 R = OH
2 R = H

The attachment of the sugars of both **1** and **2** to the 3-position of the aglycones was established by hydrogen peroxide oxidation, which provided neohesperidose in both cases. This structure was confirmed by cochromatography (TLC) with material prepared by similar oxidation of pelargonidin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside [6]. Thereby **1** and **2** were defined to be delphinidin-3-neohesperidoside and cyanidin-3-neohesperidoside, respectively.

The other pigments which were isolated from receptacles of *P. totara*, cyanidin-3-rutinoside (**3**), and cyanidin-3-glucoside (**4**) were identified by standard procedures. The other examined populations and species revealed the same anthocyanins. Only minor quantitative variation between samples was evident (Table 2). Thus cyanidin-3-neohesperidoside was dominant in all four examined species.

De Laubenfels [3] and Quinn [11] have transformed the generic classification of the 17 species included in the family Podocarpaceae by Allan [12]. Relevant to this study is the segregation of *Dacrycarpus* from *Podocarpus*. While genera within the Podocarpaceae have largely been recognized on character-states drawn from morphology of the female cone, Markham *et al.* [13] in a study of the flavonoids in *Podocarpus* s.l., give support to De Laubenfels' recognition of the genera *Podocarpus* s.s., *Prumnopitys* and *Dacrycarpus*. Recently, pelargonidin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside was isolated as the major anthocyanin in receptacles of *Dacrycarpus dacrydioides* [6]. The sugar unit of this pigment, neohesperidose (L-rhamnopyranosyl-(1→2)-β-D-glucopyranose), is the same as found in cyanidin-3-neohesperidoside isolated from *Podocarpus lawrencii* [5] and cyanidin-3-neohesperidoside and delphinidin-3-neohesperidoside isolated from all the examined samples in this study. Since this sugar unit has never been found connected to an anthocyanin nucleus outside Podocarpaceae, it is valuable as a chemotaxonomic marker. It links the genera *Dacrycarpus* and *Podocarpus* to Podocarpaceae. On the other hand, the finding of pelargonidin-3-neohesperidoside in receptacles of *Dacrycarpus dacrydioides* [6] and cyanidin-3-neohesperidoside in receptacles of *P. totara*, *P. nivalis* and *P.* cv *nivalis* × *acutifolius* will support De Laubenfels' segregation of *Dacrycarpus* and *Podocarpus*. Presumably, podocarpin A and podocarpin

Table 2. Qualitative and quantitative distribution of anthocyanins in *Podocarpus totara*, *P. nivalis* and *P.* cv *nivalis* × *acutifolius*

Species*		Compound†				Total amount (mg/100 g fr. wt)
		Cy-neo	Dp-neo	Cy-rut	Cy-glc	
P. totara	(1)	92	3	2	3	12
P. totara	(2)	97	<1	<1	2	19
P. totara	(3)	92	2	2	4	15
P. nivalis		98	<1	1	1	14
P. nivalis × *acutifolius*		96	<1	2	2	11

* Plant localization are listed under the Experimental.

† Cy = cyanidin, Dp = delphinidin, neo = 3-neohesperidoside, rut = 3-rutinoside, glc = 3-glucoside. Numbers represent percentages of total anthocyanins.

B, which Lowry [4] reckoned as cyanidin 3-acylglucoside and pelargonidin 3-acylglucoside, are cyanidin-3-neohesperidoside and pelargonidin-3-neohesperidoside, respectively. The anthocyanin patterns of the species examined by Lowry [4] will then further support De Laubenfels' transformation of the Podocarpaceae.

EXPERIMENTAL

Plant sources. Podocarpus totara bulk supply: Maidstone Park and Totara Park, Upper Hutt, New Zealand, April 1987 (popul. 1). Other *P. totara* samples: Butterfly Creek, Eastbourne, New Zealand, April 1987 (popul. 2), Rimataku Forest Park, New Zealand, May 1987 (popul. 3). *P. nivalis* and *P.* cv *nivalis* × *acutifolius* were cultivated in the Botanical Gardens of the University of Bergen, Norway and harvested Sept. 1987. They orginated from seeds collected on Mt. Robert, Nelson Lakes Nat. Park, New Zealand 15/2–75. Voucher specimens of the two latter species are deposited in BG, Norway.

Extraction. Female cones were divided into seeds and receptacles. The receptacles (151 g of the bulk sample of *P. totara*) were extracted at 4° with MeOH containing 3% TFA. The extractions were repeated until anthocyanin colour in the tissue was very weak. The MeOH was removed on a rotary evaporator, some H_2O was added and the aq. extract washed twice with petrol (bp 40–60°) and EtOAc. The petrol and EtOAc-solubles were discarded.

Chromatography. The extract was passed through an Amberlite XAD-7 column with H_2O, 50% aq. MeOH and dry MeOH as solvents respectively. The two latter solvents contained in addition 0.5% TFA. The fractions which showed anthocyanin colour were collected together and then concd to *ca* 20 ml in a rotary evaporater. 20 ml of the upper layer of BAW (*n*-BuOH–HOAc–H_2O; 4:1:5), was added and the mixture was subjected to droplet counter-current chromatography (DCCC). DCCC was carried out using a Model DCC-300 (Tokyo Rikakikai Eyela) chromatograph fitted with 250 glass capillaries (400 × 3.4 mm id). The lower phase of BAW, was used as mobile phase. A flow rate of *ca* 50 ml/hr was maintained throughout the experiment. *Ca* 310 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 cellulose TLC (C, D) and HPLC (Syst. II). Fractions were grouped according to their constituents. Each combined group was separately re-chromatographed on Sephadex LH-20 using 0.1% HCl as eluent. The eluate was monitored by eye and by TLC.

Cellulose TLC analyses were performed on Schleicher and Schüll F1440 sheets in the solvent systems: HOAc–H_2O (3:17) (A), conc HCl–H_2O (3:97) (B), HCO_2H–conc HCl–H_2O (5:1:5) (C) and upper phase of BAW (D).

HPLC was carried out with the two systems. (I): An Alltech-Applied Science Econosphere C-18 column, (250 × 4.6 mm), 5 μm, fitted with an RP-18 precolumn. Two solvents were used for elution: HCO_2H–H_2O (1:8) (E) and MeOH–H_2O (19:1) (F). Elution profile: A programmed mix beginning with 10% F and changing linearly to 50% F over a period of 20 min. The detector was set at 280 nm, and the flow rate was 1.5 ml/min. System II utilized a slurry packed ODS-Hypersil column (10 × 0.5 cm, 3 μm) with a Pelliguard LC-18 precolumn (2.5 × 0.5 cm, 40 μm). Two solvents were used for elution: HCO_2H–H_2O (1:9) (G) and HCO_2H–H_2O–MeOH (1:4:5) (H). The elution profile was 10% H to 80% H in G in 20 min, flowrate was 1.5 ml/min, and the photodiode array detector was set at 516 ± 25 nm. Further chromatographic details were as given in ref [6].

General. Techniques such as absorption spectroscopy, acid and partial hydrolysis, H_2O_2 oxidation and sugar analysis were carried out according to published procedures [6]. ^{1}H NMR spectra were obtained on a Varian XL-200 instrument at ambient probe temp., using 35% TFA-*d*–65% DMSO-d_6 and TMS as an int. ref. The total amounts of anthocyanins in the purified extracts were calculated as cyanidin-3-glucoside from a standard curve. This curve was prepared by plotting different concns of cyanidin-3-glucoside (isolated from *Ribes nigrum*) in acidified MeOH (log ε 4.44) versus A_{537} measured on a Hewlett Packard HP-8450A spectrophotometer. Aliquots of the raw extracts were analysed by HPLC, and the area percentage of each peak was calculated.

Cyanidin-3-neohesperidoside (**1**). $\lambda_{max}^{0.02\%HCl-MeOH}$ nm (log ε) 279 (4.24), 528 (4.43), A_{440}/A_{528} 0.26; λ_{max}nm (during HPLC Syst. II) 280, 516, A_{440}/A_{516} 0.31; TLC R_f: 0.75 (A), 0.56 (B), 0.89 (C) and 0.53 (D); HPLC R_t min: 13.6 (I), 7.14 (II); ^{1}H NMR: see Table 1.

Delphinidin-3-neohesperidoside (**2**). $\lambda_{max}^{0.02\%HCl-MeOH}$ nm (log ε) 279 (4.23), 537 (4.43), A_{440}/A_{537} 0.19; λ_{max}nm (during HPLC Syst. II) 277, 524, A_{440}/A_{524} 0.28; TLC R_f: 0.59 (A), 0.41 (B), 0.81 (C) and 0.40 (D); HPLC R_t min: 12.0 (I), 5.78 (II).

Cyanidin-3-rutinoside (**3**). $\lambda_{max}^{0.02\%HCl-MeOH}$ nm (log ε) 279 (4.14), 528 (4.33), A_{440}/A_{528} 0.25; λ_{max}nm (during HPLC Syst. II) 280, 516, A_{440}/A_{516} 0.31; TLC R_f: 0.35 (A), 0.15 (B), 0.69 (C) and 0.37 (D); HPLC R_t min: 17.5 (I), 9.23 (II).

Cyanidin-3-glucoside (**4**). $\lambda_{max}^{0.02\%HCl-MeOH}$ nm (log ε) 279 (4.19), 528 (4.39), A_{440}/A_{528} 0.25; λ_{max}nm (during HPLC Syst. II) 280, 516, A_{440}/A_{516} 0.31; TLC R_f: 0.22 (A), 0.05 (B), 0.47 (C) and 0.38 (D); HPLC R_t min: 15.7 (I), 8.25 (II); ^{1}H NMR: see Table 1.

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