A Novel Acetylated 3-Deoxyanthocyanidin Laminaribioside from the Fern *Blechnum novae-zelandiae*

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The major 3-deoxyanthocyanin isolated from the fern *Blechnum novae-zelandiae* was determined to be luteolinidin 5-*O*-β-D-[3-*O*-β-D-glucopyranosyl-2-*O*-acetylglucopyranoside] and was found to co-occur with quercetin 3-*O*-β-D-[6-*O*-caffeoylglucopyranoside], quercetin 3-*O*-β-D-[6-*O*-caffeoylgalactopyranoside] and kaempferol 3-*O*-β-D-glucuronopyranoside. These pigments were identified by using HPLC chromatography, NMR (1D, 2D) and electrospray mass spectroscopy.

Introduction

The 3-deoxyanthocyanins are a rare group of natural pigments known to occur in various Gesneriads (Harborne, 1966), mosses (Bendz *et al.*, 1962) and ferns (Crowden and Jarman, 1974). To date these are reported, often on the basis of limited evidence, to include the 5-*O*-glucosides of apigeninidin, luteolinidin, tricetinidin, and columnidin, as well as a series of 5-di-, 5,7-di-, 7-di- and 7-*O*-glycosides of apigeninidin and luteolinidin. A caffeic acid ester of apigeninidin 5-*O*-glucoside was reportedly induced in sorghum in response to fungal infection (Hipskind *et al.*, 1990). The identification of the above-mentioned brightly coloured pigments is in a number of cases only tentative and has been limited to thin layer and paper chromatographic evidence (Crowden and Jarman, 1974; Harborne, 1966; Stich and Forkmann, 1988) absorption spectroscopy (Harborne, 1966) and HPLC evidence (Hipskind *et al.*, 1990). More recently the NMR assignments and HPLC chromatographic data for two of the better known 3-deoxyanthocyanins luteolinidin 5-*O*-glucoside and apigeninidin 5-*O*-glucoside were reported for the first time (Swinny *et al.*, 2000). Two distinct downfield doublets in the ^{1}H NMR spectrum were characteristic of H-3 and H-4 of the flavonoid heterocycle, a feature which readily distinguishes 3-deoxyanthocyanins from normal C-3 oxygenated anthocyanins.

In this investigation, which is part of a broader strategy aimed at studying the hitherto relatively unexplored nature of 3-deoxyanthocyanins and their potential uses, the red-orange tipped fronds of the New Zealand endemic fern *Blechnum novae-zelandiae,* previously known as *Blechnum capense* (Chambers and Farrant, 1998), were examined. The new pigment luteolinidin 5-*O*-β-D-[3-*O*-β-D-glucopyranosyl-2-*O*-acetylglucopyranoside] (**1**) was isolated from the acidified (TFA) methanolic extract of the fronds together with the known flavonols quercetin 3-*O*-β-D-[6-O-caffeoylglucopyranoside] (**2**), quercetin 3-*O*-β-D-[6-*O*-caffeoylgalactopyranoside] (**3**) and kaempferol 3-*O*-β-D-glucuronopyranoside (**4**).

Results and Discussion

HPLC analysis of the methanolic extract of the *Blechnum novae-zelandiae* fronds showed four 3-deoxyanthocyanins and three main flavonols. The major 3-deoxyanthocyanin **1** and the flavonol glycosides **2, 3** and **4** were purified by CC on Diaion HP-20, cellulose, polyamide, Fractogel HW-40 and Lobar RP-18. Their structures were elucidated by using 1D (^{1}H, ^{13}C, ^{13}C DEPT) and 2D (DQF COSY, HSQC, HMBC, TOCSY) NMR techniques.

The 3-deoxyanthocyanin nature of **1** was indicated by the HPLC on-line UV-Vis spectrum which showed a visible maximum (λ_{max} 491 nm) in the same region as that reported (λ_{max} 486 nm) for luteolinidin 5-*O*-glucoside (Swinny *et al.*, 2000). Acid hydrolysis of **1** produced luteolinidin and glucose. The luteolinidin was identified by

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HPLC co-chromatography and on-line spectral comparison while the sugar was determined to be glucose by paper chromatography against an authentic standard (Markham, 1982). The presence of an acetyl group was indicated by the three-proton singlet at δ 2.03 in the ^{1}H NMR spectrum together with a methyl carbon and carbonyl carbon signal at δ 20.8 and δ 170.2 respectively in the ^{13}C NMR spectrum. A long-range HMBC experiment showed correlation between the proton singlet and the carbonyl carbon. Two distinct doublets in the downfield part of the ^{1}H NMR spectrum at δ 8.29 (J = 8.9 Hz) and δ 8.64 (J = 8.9 Hz) were assigned to H-3 and H-4 respectively. These proton signals were correlated to the corresponding carbon signals at δ 111.9 (C-3) and δ 146.5 (C-4) in the HSQC spectrum. This structural arrangement in the heterocycle of **1** was confirmed by the significant long-range correlations (summarized in Fig. 1) which were observed between C-2 (δ 176.6) and the protons H-3 and H-4 in the HMBC spectrum. Two *meta*-coupled doublets in the ^{1}H NMR spectrum at δ 6.97 (J < 1.0 Hz) and δ 7.11 (J < 1.0 Hz) were assigned to H-6 and H-8 respectively of the A-ring. The remaining B-ring of the luteolinidin portion of the molecule was defined by an ABX system at δ 7.05 (1H, d, J = 8.6 Hz, H-5′), δ 7.81 (1H, d, J = 2.0 Hz, H-2′) and δ 7.94 (1H, d, J = 2.0, 8.6 Hz, H-6′).

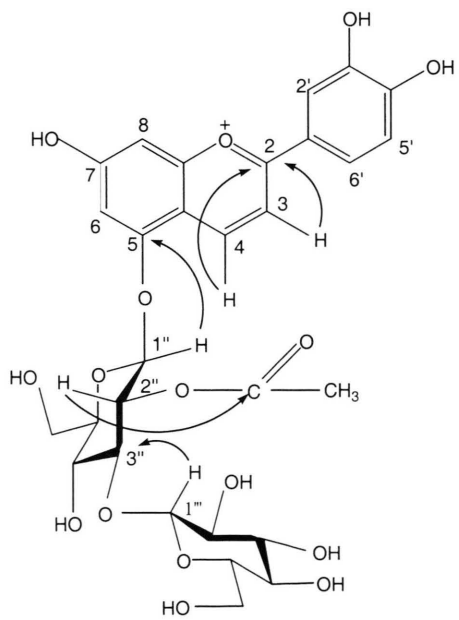

Fig. 1. The structure of **1** showing the main HMBC correlations (↷).

Two anomeric protons were detected in the ^{1}H NMR spectrum and the proton and carbon signals (Table I) which belonged to the respective sugar units were sequentially assigned from the DEPT, DQF COSY, HSQC, HMBC and TOCSY experiments. The NMR data in conjunction with the evidence from acid hydrolysis indicated that **1** is a luteolinidin diglucoside. The magnitude of the anomeric proton coupling constants (J = 7.9 and 7.8

Table I. ^{1}H NMR and ^{13}C NMR spectral data for luteolinidin 5-*O*-β-D-[3-*O*-β-D-glucopyranosyl-2-*O*-acetylglucopyranoside].

	1	
Carbon	δ_C (ppm)	δ_H (ppm), *m*, *J* (Hz)
Aglycone		
2	176.6	
3	111.9	8.29, *d*, 8.9
4	146.5	8.64, *d*, 8.9
5	155.5	
6	103.6	6.97, *d*, <1.0
7	169.6	
8	97.2	7.11, *d*, <1.0
9	NC	
10	111.7	
1′	120.1	
2′	115.7	7.81, *d*, 2.0
3′	147.1	
4′	155.9	
5′	117.2	7.05, *d*, 8.6
6′	125.1	7.94, *dd*, 2.0, 8.6
5-O-β-D-glucopyranoide		
1	98.3	5.54, *d*, 7.9
2	71.8	5.09, *t*, 8.62, 8.86
3	83.8	3.91–3.81, *m*
4	68.3	3.46–3.50, *m*
5	77.3	3.67–3.76, *m*
6	60.3	[a]3.67–3.80, *m* [b]3.59, *dd*, 5.1, 11.7
3″-O-β-D-Glucopyranosyl		
1	104.1	4.32, *d*, 7.8
2	73.3	3.00–3.05, *m*
3	76.8	3.10–3.26, *m*
4	70.2	3.06–3.13, *m*
5	77.1	3.10–3.26, *m*
6	61.2	[a]3.67–3.81, *m* [b]3.45, *dd*, 6.3, 11.6
2″-O-Acetyl		
CH3	20.8	2.03, s
C=O	170.2	

[a] H-6A; [b] H-6b
NC = not clear

Hz) in the ^{1}H NMR spectrum showed that both sugars had β-glycosidic linkage. A cross-peak between the anomeric proton at δ 5.54 and the carbon at δ 155.5 (C-5) in the HMBC spectrum indicated that one of the glucopyranose units was attached to the 5-position of luteolinidin. A cross-peak between the second anomeric proton at δ 4.32 and the carbon at δ 83.8 (C-3″) revealed that the second β-D-glucose unit was attached to the first glucose unit at position-3 to form a laminaribiose. A downfield triplet at δ 5.09 (J = 8.62, 8.86 Hz) which correlated to H-1″ in the DQF COSY spectrum was assigned to H-2″ and a cross-peak between this proton and the acetyl carbonyl carbon signal (δ 170.2) in the HMBC spectrum established that the acetyl group was connected to C-2″ via an ester bond. The HR ESMS of **1** afforded a molecular ion at *m/z* 637.1747 consistent with that expected for a mono-acetate of a luteolinidin diglucoside ($C_{29}H_{33}O_{16}$; calcd.: 637.1763).

Thus the 3-deoxyanthocyanin **1** is defined as luteolinidin 5-*O*-β-D-[3-*O*-β-D-glucopyranosyl-2-*O*-acetylglucopyranoside].

HPLC analysis and paper chromatography showed that acid hydrolysis of **2** produced quercetin, glucose and caffeic acid. NMR data of **2** showed that a caffeoyl group was attached to the 6-position of a glucopyranoside unit which in turn was connected to the 3-position of quercetin. Compound **2** was therefore established to be the known quercetin 3-*O*-β-D-[6-O-caffeoylglucopyranoside] (Kunzemann and Herrmann, 1977). The structure of compound **3** was similarly examined and found to be quercetin 3-*O*-β-D-[6-*O*-caffeoylgalactoside] which has previously been isolated from the common Japanese weed *Hydrocotyle Sibthorpoides* (Shigematsu *et al.*, 1982). Compound **4** was identified as kaempferol-3-*O*-β-D-glucuronopyranoside by comparison against published NMR data (Nanwar *et al.*, 1984). This glucuronide was also found in *Adiantum* ferns together with other unidentified caffeoyl flavonol glycosides (Cooper-Driver and Swain, 1977). The present report is the first description of the glycosides **2, 3** and **4** in the fern *Blechnum novae-zelandiae.*

The HPLC on-line measured absorption maxima (λ_{max} 480–490 nm) of the other three minor 3-deoxyanthocyanins detected in the methanolic extract indicated that they also possessed luteolinidin nucleii.

In conclusion, the isolation and identification of luteolinidin 5-*O*-β-D-[3-*O*-β-D-glucopyranosyl-2-*O*-acetylglucopyranoside] in *Blechnum novae-zelandiae* described here provides the first substantive proof that glycosides of 3-deoxyanthocyanidins are the major cyanic colourants in *Blechnum* fronds. Additionally, the structure determination is the first for any acylated 3-deoxyanthocyanidin diglycoside.

Experimental

Plant material

Fronds of *Blechnum novae-zelandiae* were collected along the R3 road cuttings near Rotorua, New Zealand and authenticated by Dr. P. Brownsey, Curator of Botany at Te Papa Museum, Wellington, New Zealand. A voucher specimen (WELT P20084) is deposited at the Te Papa Herbarium.

Extraction and isolation

Clippings (500 g fresh wt.) of the red-orange tipped *Blechnum novae-zelandiae* fronds were extracted with 3.5 *l* of 80% MeOH (0.5% TFA) for 24 h. The MeOH was removed under reduced pressure at 40 °C and the aq. phase absorbed onto Diaion HP-20. The pigment extract was eluted with 90% MeOH (0.1% TFA), concentrated under reduced pressure, freeze-dried and subsequently chromatographed on a cellulose column (5% HOAc)). The 3-deoxyanthocyanin fraction was then applied to a polyamide column and eluted with MeOH-H_2O-TFA (10:90:0.1 increasing to 90:10:0.1 v/v). Final purification of **1** was obtained on a Lobar RP-18 column eluting with 15% MeCN (0.1% TFA).

The flavonol fraction was separated on a Fractogel HW-40 column eluting with 40% MeOH (0.1% TFA). The flavonols **2–4** were finally purified on a Lobar RP-8 column eluting with 5–15% MeCN (0.1% TFA).

NMR and mass spectroscopy

The pigments were dissolved in 1% CF_3COOD in DMSO-d_6 and NMR spectra were recorded on a Bruker Avance spectrometer at 300.13 MHz (^{1}H) and 75.47 MHz (^{13}C) equipped with a 5 mm quad nuclei probe at 30 °C. In the ^{1}H and ^{13}C

spectra the residual solvent peaks for DMSO-d_6 were referenced to 2.50 ppm and 39.26 ppm, respectively.

Mass spectra were recorded on a PE Biosystems Mariner System 5158 CI TOF mass spectrometer.

Analytical HPLC

HPLC was conducted on a Waters 600E solvent delivery system coupled to a Waters 996 PDA detector. A Merck Supersphere Lichrocart 125-4 RP-18 endcapped (4 μm, 4 mm×119 mm) column was used with a solvent system comprising solvent A [1.5% H_3PO_4] and solvent B [HOAc-MeCN-H_3PO_4-H_2O (20:24:1.5:54.5 v/v)] using a linear gradient starting with 80% A, decreasing to 33% A at 30 min, 10% A at 33 min and 0% at 39.3 min. The 3-deoxyanthocyanins and flavonols were detected at 480 and 352 nm respectively. Retention times of **1, 2, 3** and **4** were 19.3, 28.6, 28.9 and 24.1 min, respectively.

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Bendz G., Martensson O. and Terenius L. (1962), Moss pigments, the anthocyanins of *Bryum cryophilum* O. Mårt. Acta, Chem. Scand. **16**, 1183–1190.

Chambers T. C. and Farrant P. A. (1998), The *Blechnum procerum ("capense")* (Blechnaceae) complex in New Zealand. New Zealand Journal of Botany **36**, 1–19.

Cooper-Driver G. and Swain T. (1977), Phenolic chemotaxonomy and phytogeography of *Adiantum.* Bot. J. Linn. Soc. **74**, 1–21.

Crowden R. K. and Jarman S. J. (1974), 3-Deoxyanthocyanins from *Blechnum procerum.* Phytochemistry **13**, 1947–1948.

Harborne J. B. (1966), Comparative biochemistry of flavonoids – II; 3-desoxyanthocyanins and their systematic distribution in ferns and gesnerads. Phytochemistry **5**, 589–600.

Hipskind J. D., Hanau R., Leite B. and Nicholson R. L. (1990), Phytoalexin accumulation in sorghum: identification of an apigeninidin acyl ester. Physiol. Molec. Plant Pathol. **36**, 381–396.

Kunzemann J. and Herrmann K. (1977), Isolierung und Identifizierung der Flavon(ol)-O-glykoside in Kümmel (*Carum carvi* L.), Fenchel (*Foeniculum vulgare* Mill.), Anis (*Pimpinella anisum* L.) und Koriander (*Coriandrum sativum* L.) und von Flavon-C-glykosiden im Anis. Z. Lebensm. Unters. Forsch. **164**, 194–200.

Markham K. R. (1982). Techniques of Flavonoid Identification. Academic Press, London.

Nanwar M. A. M., Souleman A. M. A., Buddus J. and Linsheid M. (1984), Flavonoids of the flowers *Tamarix nilotica,* Phytochemistry **23**, 2347–2349.

Stich K. and Forkmann G. (1988), Biosynthesis of 3-deoxyanthocyanins with flower extracts from *Sinningia cardinalis*, Phytochemistry 27, 785–789.

Swinny E. E., Bloor S. J. and Wong H. (2000), ^{1}H and ^{13}C NMR assignments for the 3-deoxyanthocyanins, luteolinidin-5-glucoside and apigeninidin-5-glucoside. Magn. Res. Chem. **38**, 1031–1033.