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The phenols and prodelphinidins of white clover flowers

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Abstract

White clover flowers (*Trifolium repens* L.) contain an abundance of phenolics, namely *cis*- and *trans*-*p*-coumaric acid 4-*O*-β-D-glucopyranoside, the 3-*O*-β-D-galactopyranosides of myricetin, quercetin and kaempferol together with two new derivatives namely myricetin 3-O-(6″-acetyl)-β-D-galactopyranoside and kaempferol 3-*O*-(6″-acetyl)-β-D-galactopyranoside. Gallocatechin, epigallocatechin, gallocatechin-(4α-8)-epigallocatechin and their corresponding prodelphinidin polymers were also present. The ^{13}C-NMR spectra showed that the polymers consisted of only gallocatechin and epigallocatechin monomeric units with the latter being about twice as abundant in the extenders but only slightly more than that in the terminating units. The average degree of polymerization was estimated by ^{13}C-NMR and ES–MS, which gave a remarkably consistent result of about 5.8 flavanol units.

Keywords: *Trifolium repens*; Leguminosae; Flowers; Phenolics; Prodelphinidins; Molecular size

1. Introduction

The most common pasture for cattle and sheep grazing in New Zealand consists of a mixture of perennial ryegrass and white clover. White clover is desirable due to its high nutritive value and its nitrogen fixing properties which minimises the need for application of nitrogen fertilizer. Another advantage for inclusion of clover, particularly white clover (*Trifolium repens* L.), is the presence of condensed tannins or proanthocyanidins in the flower (Jones et al., 1976), although the leaves of both the white and red clover (*T. pratense* L.) have been shown to contain proanthocyanidins at only very low level and which can only be detected by using sensitive methods (Li et al., 1996). While the presence of flowers in white clover is seasonal there is potential for genetic manipulation for increased yield and frequency as proanthocyanidins play an important role in animal health and nutrition. The ability of proanthocyanidin containing forages to prevent bloat in cattle was known since 1711 and has been confirmed by more recent studies (Jones and Lyttelton, 1971; Clark and Reid, 1974) that have included dietary supplementation work (Waghorn and Jones, 1989). Although dietary proanthocyanidins are well known for their anti-nutritional effects, more recent studies suggest the contrary to be true if their levels in feeds are low. Feeding trials with both cattle and sheep have shown superior performance using *Lotus corniculatus* relative to lucerne (Marten et al., 1987; Douglas et al., 1995; Wang et al., 1996) and the benefits were shown to be due to the presence of proanthocyanidins (Jones and Mangan, 1977; Barry and Forss, 1983). The increased performance has been attributed to reduced protein losses from the rumen and a net increase of amino acid absorption in the intestine even though protein digestion was reduced. Additional benefits with proanthocyanidin containing forages include improved sheep health associated with fewer dags and an associated decrease in the incidence of flystrike (Leathwick and Atkinson, 1995; Robertson et al., 1995).

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However, not all proanthocyanidins provide the same nutritional effects to ruminants. Those from *Lotus corniculatus* which consisted of epicatechin (67%) and epigallocatechin (30%) extender units (Foo et al., 1996) did not appear to inhibit amino acid absorption (Waghorn et al., 1987, Wang et al., 1994) while those from *Lotus pedunculatus* with epigallocatechin (about 70%) as the dominant extender units (Foo et al., 1997) showed extensive inhibition of most amino acids (Waghorn et al., 1994). This significant difference in activity, even among apparently closely related plant species, highlights the need for more detailed information on the chemical structure of the proanthocyanidins in various forages. This report is concerned with the chemical nature of the proanthocyanidins and other low molecular phenolic compounds present in the flowers of white clover (*Trifolium repens* L.).

2. Results and discussion

The defatted 70% aqueous acetone extract of *Trifolium repens* was fractionated on a Sephadex LH20 column prepared in water and eluted first with water to wash out the sugars and then with aqueous methanol to yield four main chromatographic fractions (a)–(d). Examination of fraction (a) by HPLC showed that the fraction contained two main polar compounds **1** and **2** which were successfully separated by sequential chromatography on a Sephadex LH20 column using water as the eluant and followed by chromatography of the partially purified fractions on MCI CHP 20 with aqueous methanol. The ^{13}C-NMR spectra of both compounds were similar and showed two distinct systems to be present in each compound, namely a sugar moiety and an aromatic carboxylic acid portion. The presence of a *p*-coumaric acid moiety in the two compounds was evident from the observation of a pair of degenerate carbon resonances (δ 117.0 and 130.5 in **1** and 117.7 and 132.3 in **2**) and the corresponding proton resonances with their diagnostic *ortho* couplings (J = 8.6 and 8.8 Hz for **1** and **2**, respectively). The olefinic carbons were observed at δ 116.0 and 146.0 for **1** and δ 124.2 and 136.7 for **2**. Their respective protons had larger couplings (J = 16.1 Hz) in **1** than in **2** (J = 12.7 Hz) suggesting that the former was *trans* and the latter the *cis*-*p*-coumaric acid. Acid hydrolysis of both compounds yielded glucose establishing that **1** was *trans*-p-coumaric acid 4-*O*-β-D-glucopyranoside and **2** was *cis*-*p*-coumaric acid 4-*O*-β-D-glucopyranoside. The β-configuration was deduced from the proton–proton coupling constants of the anomeric protons observed at δ 5.00 (J = 7.1 Hz) and δ 4.87 (J = 7.3 Hz) for **1** and **2**, respectively. The ES–MS of both compounds gave $[M - H]^-$ peaks at m/z 325 which were consistent with the deduced chemical structures.

Fraction (b) was left standing overnight at ambient temperatures to allow the methanol to evaporate and yielded a yellow crystalline material. HPLC analysis using a reverse phase column showed the yellow solid to be a mixture of two major and four minor compounds (Fig. 1). Repeated chromatography of the yellow solid alternating between Sephadex LH20 and MCI CHP 20 resulted in the isolation of the six compounds, **3**–**8**.

Myricetin 3-*O*-β-D-galactopyranoside (**3**), quercetin 3-*O*-β-D-galactopyranoside (**4**) and kaempferol 3-*O*-β-D-galactopyranoside (**5**) were identified from their respective NMR spectra and confirmed by comparison with those of authentic samples or published data (Agrawal, 1989). The ^{13}C-NMR spectra of **6**, **7** and **8** were very similar to **3**, **4** and **5**, respectively and it appeared that they were of the same analogous series differing only in the degree of hydroxylation in the B-ring. In addition, two carbon signals arising from an aliphatic carbon (ca 20 ppm) and a carbonyl carbon (ca 170 ppm) were observed in the ^{13}C-NMR spectra of all the three compounds. The aliphatic carbon was identified as a methyl carbon by integration of the corresponding proton resonances thus indicating the presence of an acetyl moiety. Comparison of the carbon chemical shift values of **6**–**8** with those of the parent glycosides **3**–**5** showed the C6 carbon signals of the galactopyranoside moieties to have shifted downfield by about 3 ppm indicating that the acetyl groups were attached to this carbon atom. Compound **6** was therefore myricetin 3-*O*-(6″-acetyl)-β-D-galactopyranoside, **7** quercetin 3-*O*-(6″-acetyl)-β-D-galactopyranoside and **8** kaempferol 3-*O*-(6″-acetyl)-β-D-galactopyranoside. Further confirmation of the chemical structures was made by mild alkaline hydrolysis with 2N sodium hydroxide at ambient temperatures to yield the respective deacylated flavonoid galactosides **3**, **4** and **5** which were identified by HPLC comparison with authentic samples. Myricetin 3-*O*-(6″-acetyl)galactoside and kaempferol 3-*O*-(6″-acetyl)galactoside appear to be new compounds while quercetin 3-*O*-(6″-acetyl)galactoside along with its parent glycoside have been reported recently in white clover flower (Schittko et al., 1999).

Separation of fraction (c) on an MCI CHP 20 column yielded gallocatechin (**9**), epigallocatechin (**10**) and the associated prodelphinidin dimer gallocatechin-(4α-8)-epigallocatechin (**11**). These compounds were identified by their characteristic flavan-3-ol carbon chemical shifts and distinction between **9** and **10** was made from the relative position of the C2 and C3 resonances. The ^{13}C-NMR spectrum of the prodelphinidin dimer **11** showed similar chemical shift patterns for the phloroglucinol A-ring and the pyrogalloyl B-ring as those observed in **9** and **10**. The appearance of a

carbon resonance at 37.7 ppm indicated the presence of a flavanol unit with an aryl substituent at the benzylic C4 (Porter et al., 1982). This was corroborated by the observation of a corresponding downfield shift of the C3 (δ 72.9) and C2 (δ 83.3) resonances of the affected pyran ring. The large downfield shift of the C2 resonance indicated that the extender or upper flavanol unit had the 2,3-*trans* configuration (Porter et al., 1982). The magnitude of the coupling (J = 9.6 Hz) between H2 (δ 4.39) and H3 (δ 4.58), the protons associated with these carbons, confirmed this *trans* stereochemistry. The chemical shift values of the remaining C2 (δ 79.2) and C3 (δ 66.3) resonances were consistent with a terminating flavanol unit with a 2,3-*cis* configuration thus indicating that the prodelphinidin dimer consisted of a gallocatechin extender and an epigallocatechin terminating unit.

The nature of the inter-flavonoid linkage in **11** was established by HMBC and HMQC experiments which allowed for the assignment of the NMR chemical shifts, albeit with some ambiguity with respect to the upper A-ring as a result of the almost identical carbon chemical shift values observed for C6 and C8. The HMBC technique was used successfully to confirm the interflavonoid linkage in procyanidin B3 (Bruyne et al., 1996) in spite of complications arising from the multiplicity of the NMR signals due to the existence of rotational isomers. Although dimer **11** also possesses a similar 2,3-*trans* flavanol upper unit, thereby giving rise to conformational isomers as observed in procyanidin B3 (Foo and Porter, 1983), the NMR spectra of **11** in deuterated acetone containing a few drops of D_2O displayed no such multiplicity of signals. It was fortuitous that the oxygenated A-ring carbon chemical shifts, which were critical to establish the interflavonoid linkage, were well resolved, which facilitated assignment of structure **11**. However, structural assignment was made difficult by the absence of a signal arising from long range proton–carbon coupling with J optimised at 7.2 Hz between the lower unit H2 (δ 4.92) and the oxygenated C8a, the latter assignment was necessary before the location of the interflavonoid bond could be made. Using the unambiguous lower unit H4 methylene proton signals (δ 2.8–3.0) as the starting point, their long range H–C couplings to two oxygenated A-ring carbon atoms indicated that the carbon chemical shift of C8a was either 154.4 or 154.7 ppm (see Table 1). The isolated lower unit A-ring singlet (δ 6.08) was correlated to the carbons at 154.7 and 155.1 ppm, thereby establishing the lower field resonance at 155.1 ppm to be that of the oxygenated C7. This assignment was further confirmed by the observation of long range H–C coupling between carbon signal at 155.1 ppm and the upper unit H4 methine proton (δ 4.69) attached to the carbon (δ 37.7) involved in the interflavonoid linkage. In addition, this proton was also coupled to the oxygenated carbon at 154.4 ppm and successful assignment of this carbon will establish the location of the interflavonoid linkage. With the J value optimised at 7.2 Hz there was no

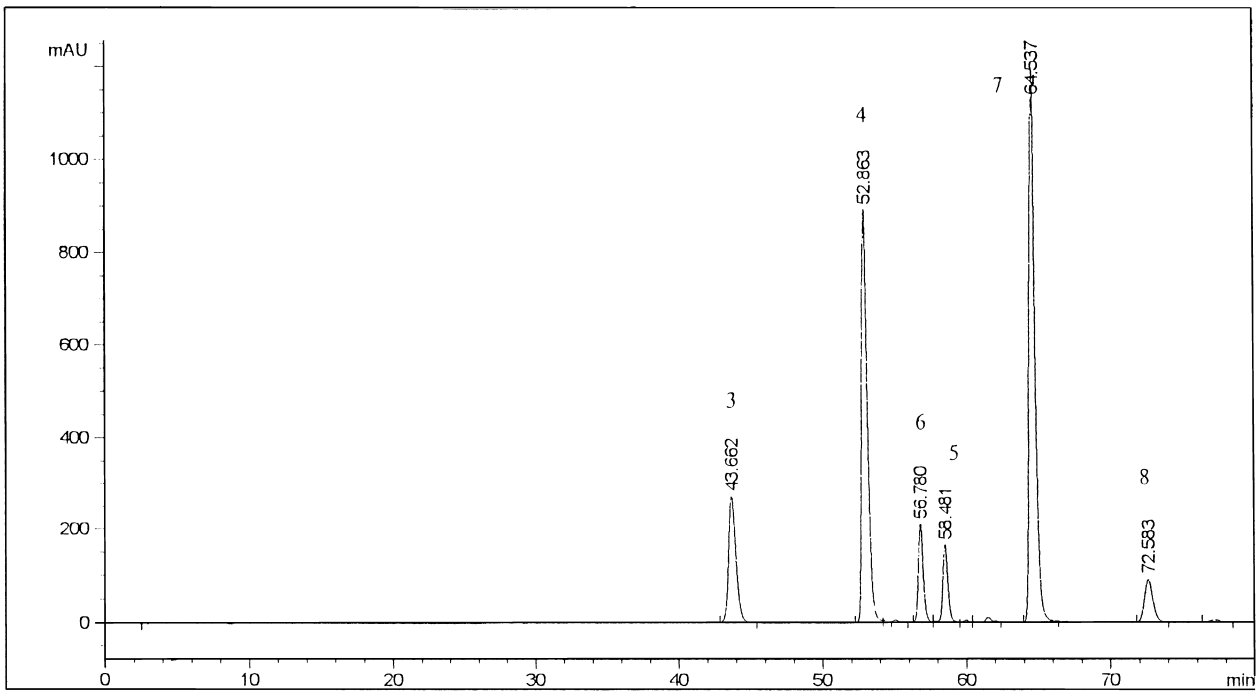


Fig. 1. HPLC of the flavonoid fraction from white clover flower. The peak numbers refer to the corresponding flavonoid chemical structures in the text.

clear long range coupling between H2 and the A-ring carbons in the lower flavonoid unit. The identical result was obtained with J = 6.6 Hz. However, at a higher value (J = 9.0 Hz) distinctive H–C coupling was observed between the H2 (δ 4.92) and the oxygenated carbon at 154.4 ppm. The result clearly established that the carbon chemical shift at 154.4 ppm was due to C8a and that the interflavonoid linkage was through C8. The prodelphinidin dimer **11** was therefore gallocatechin-(4α-8)-epigallocatechin.

The proanthocyanidin polymers were obtained by treatment of fraction (d) on a Sephadex LH20 column, washing of the column with excess 50% aqueous methanol until the washings were clear, and then eluting the polymers off with 70% aqueous acetone. The ^{13}C-NMR spectrum of this polymer was consistent with that of a prodelphinidin polymer, and showed pronounced chemical shifts at around 107 and 109 ppm which are consistent with C2″ and C6″ of a pyrogalloyl B-ring and confirm the absence of a catechol B-ring. This was confirmed by heating the polymer with 5% hydrochloric acid in *tert*-butanol which gave rise exclusively to delphinidin.

In spite of the broad nature of the peaks in the upfield region (δ 65–85) of the ^{13}C-NMR spectrum, the resolution was sufficient to allow for some structural interpretation to be made from the chemical shift values (see Fig. 2). The low intensity signals at 79.7 and 81.8 ppm were attributed to the C2 chemical shifts of the 2,3-*cis* and 2,3-*trans* flavonol unit respectively which had no substituent at the C4 position (Porter et al., 1982). Hence they were those of the terminating units. Their presence showed that the polymer had gallocatechin and epigallocatechin as end units with the latter being the more common as judged by the relative carbon signal size. The peaks at around 76.8 and 83.7 ppm were the C2 chemical shifts of the 2,3-*cis* and 2,3-*trans* extender units, respectively and their relative signal intensity suggested that there were at least twice as many epigallocatechin as there were gallocatechin extender units in the white clover prodelphinidin polymers. In view of the adequate resolution observed for all these carbon signals, it would therefore be possible to estimate the average chain length of the polymer using the ratio of the respective carbon signal intensity between the extending and terminating units as these are relatively low molecular weight polymers and their T1 and nOe are comparable for the respective carbons (Czochanska et al., 1980). In this instance it would be possible to employ either the C2 signals namely the ratio of the sum of the areas of 79.7 and 81.8 ppm peaks to the sum of the 76.8 and 83.7 ppm peaks. Alternatively the C3 signals could be used, involving the ratio of the peak at 66–68 to that at 72.8 ppm. Calculation based on the area of the C2 signals gave a ratio of 1 terminal unit to 4.90 extender units or an average chain length of about 5.90 flavanol units. With the C3 signals the ratio is 1:4.76 giving an average chain length of about 5.76, a value which is in reasonable agreement with that obtained using the C2 resonances.

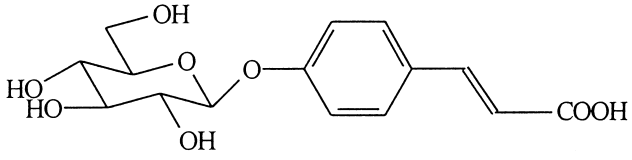
Table 1
The long range H–C couplings (HMBC) observed in prodelphinidin 11 optimised at J = 9.0 Hz

Proton (δ)	Correlated carbon (δ)
2.80–3.00	66.3, 79.2, 99.0, 154.4, 154.7
4.29	79.2, 99.0
4.39	37.7, 72.9, 108.1, 131.3, 157.9
4.58	37.7, 83.3, 107.9, 131.3
4.69	72.9, 83.3, 97.1, 106.2, 107.9, 155.1, 157.9
4.92	66.3, 106.4, 131.5, 154.4
5.86	95.8, 106.2, 156.4
5.89	97.1, 106.2, 156.6
6.08	99.0, 107.9, 154.7, 155.1
6.62	83.3, 108.1, 131.3, 133.4, 145.8
6.73	79.2, 106.4, 131.5, 132.6, 146.1

The validity of estimating the average molecular weight of proanthocyanidin polymers using ^{13}C-NMR signal intensity as described above could be verified by examining the electrospray mass spectral (ES–MS) data of the sample. The ES–MS spectrum (Fig. 3) showed a distribution of ion peaks from m/z 607.3 to 1368.9 with each peak being separated by a mass of about 152 mass units. For prodelphinidin polymers the constituent flavanol units are made up of either gallocatechin or epigallocatechin, hence the ion peak separation is expected to be 304 mass units, which is twice that observed. This halving in mass separation suggested that the observed ion peaks in the mass spectrum are doubly charged, a situation commonly observed in oligomeric proanthocyanidins consisting of more than four monomeric units (Roux et al., 1998; Fulcrand et al., 1999; Foo and Lu, 1999). Confirmation of the presence of doubly charged ions was gleaned from the observation of peaks at m/z 760.3, 1064.5 and 1368.9 which could most likely arise from the pentameric, heptameric and nonameric prodelphinidins, respectively. The more or less normal distribution pattern of the mass ions observed indicated that the degree of polymerization (DP) ranged from 4 to 9 with a DP of six being the most common.

As there was minimal fragmentation observed in the ES–MS, the relative ion peak intensity is, therefore, a reflection of the relative abundance of each molecular species in the sample. The presence of singly charged species could distort the relative ion peak intensity observed and complicate molecular weight estimation. If these did occur, peaks at around m/z 1217 ($DP4^-$), 912 ($DP3^-$) and 607 ($DP2^-$) will be the ones that would be affected. However, most of these prodelphi-

nidin dimers, trimers and tetramers would have been removed during the chromatographic purification of the polymers so their contribution to the ion peak intensity would be minimal. Calculations based on the relative peak heights of the mass ions gave an average DP for the prodelphinidin polymer of 5.79, a value which was remarkably consistent with that obtained by ^{13}C-NMR.



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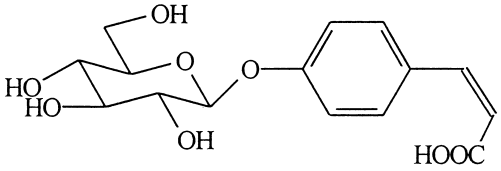
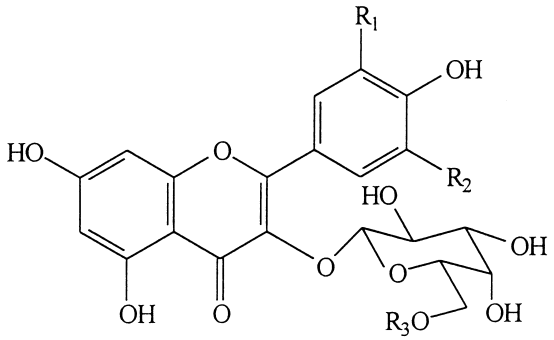
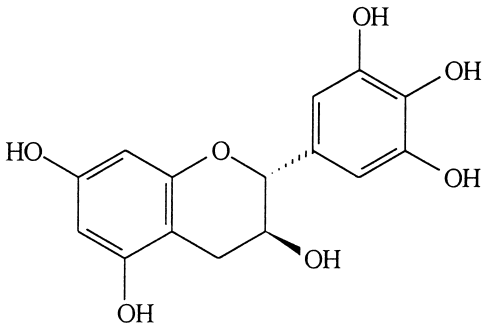


(2)



(3) R_1=R_2=OH, R_3=H
(4) R_1=OH, R_2=R_3=H
(5) R_1=R_2=R_3=H
(6) R_1=R_2=OH, R_3=$COCH_3$
(7) R_1=OH, R_2=H, R_3=$COCH_3$
(8) R_1=R_2=H, R_3=$COCH_3$



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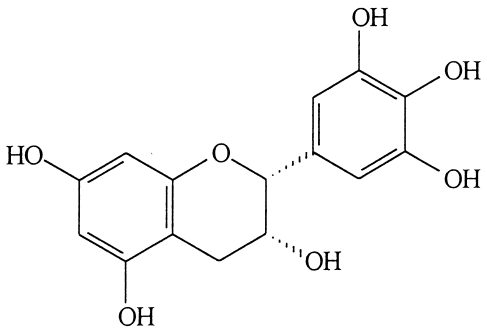

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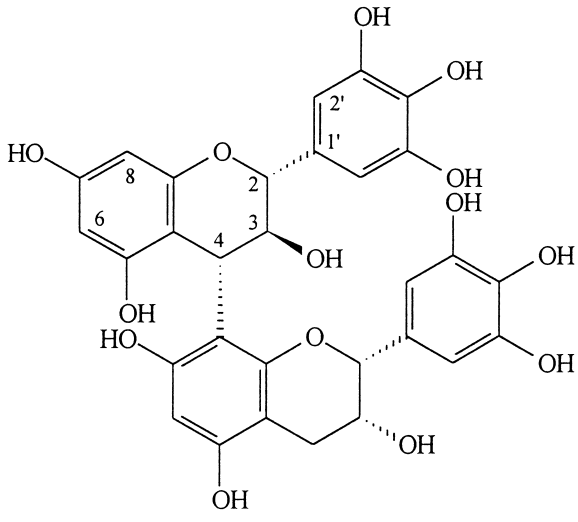

(11)

3. Experimental

^{13}C-NMR spectra were obtained on a Bruker Avance 300 NMR spectrometer operating at 300.13 MHz for ^{1}H and 75.45 MHz for ^{13}C using the XWINNMR software package. DEPT (distortionless enhancement by polarization transfer) experiments were performed using a transfer pulse of 135° to obtain positive for CH and CH_3 and negative ones for CH_2. Polarization transfer delays were adjusted to an average CH coupling of 135 Hz. Gradient version of ^{1}H–^{1}H DQF–COSY, ^{1}H–^{13}C HSQC and HMBC experiments were carried out using the standard pulse sequences as supplied by Bruker. Deuterated NMR solvents were used, DMSO for flavonoid glycosides, methanol for the coumaric acid glycosides and acetone for the gallocatechins, the prodelphinidin dimer and the polymer. HPLC analyses were performed on a Hewlett Packard HP-1100 instrument using a LiChrospher® 100 RP-18 (5 μm) column (125 × 4 mm) heated at 30°C with solvent A consisting of AcOH/H_2O (2:98 v/v) and B consisting of AcOH/CH_3CN/H_2O (2/20/78 v/v) with a flow rate of 0.5 ml/min. Solvent programming was set at 80% solvent A and 20% B at 0 time increasing to 40% A and 60% B at 40 min, and 100% B at 60 min and then isocratic for another 20 min. Peak detection was made using a UV detector at 280 nm. Mass spectra were obtained from a VG Plateform Electrospray mass spectrometer operating in the negative ion detection mode.

3.1. Material

White clover flowers were collected during summer 1997/98, from Palmerston North and Lincoln. Flowers were collected from more than 600 plants of an experimental population derived from both locations. This experimental population resulted from 3 cycles of selection for elevated floral proanthocyanin content within Grasslands Huia (Woodfield et al., 1998). Flowers were bulked and stored on ice prior to transfer to a −20°C freezer.

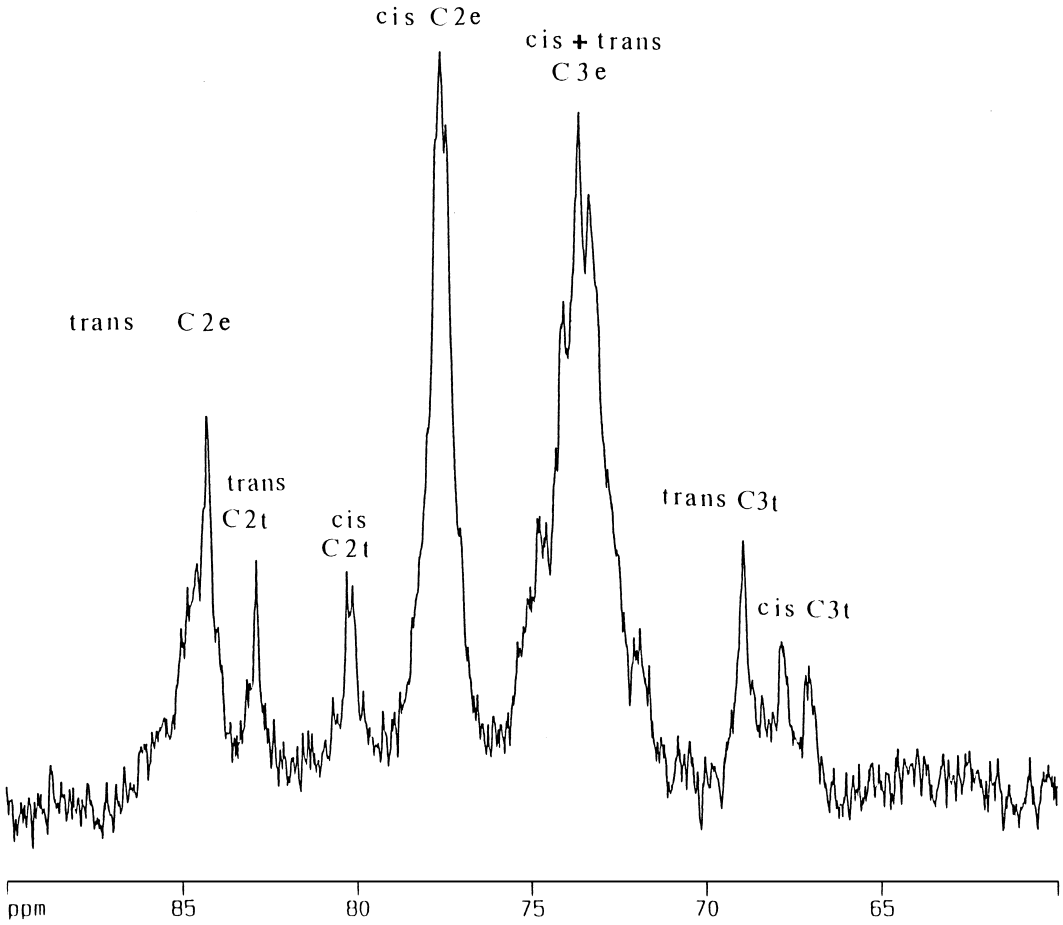

Fig. 2. ^{13}C-NMR spectrum of the heterocyclic region of the polymeric prodelphinidin from white clover flower.

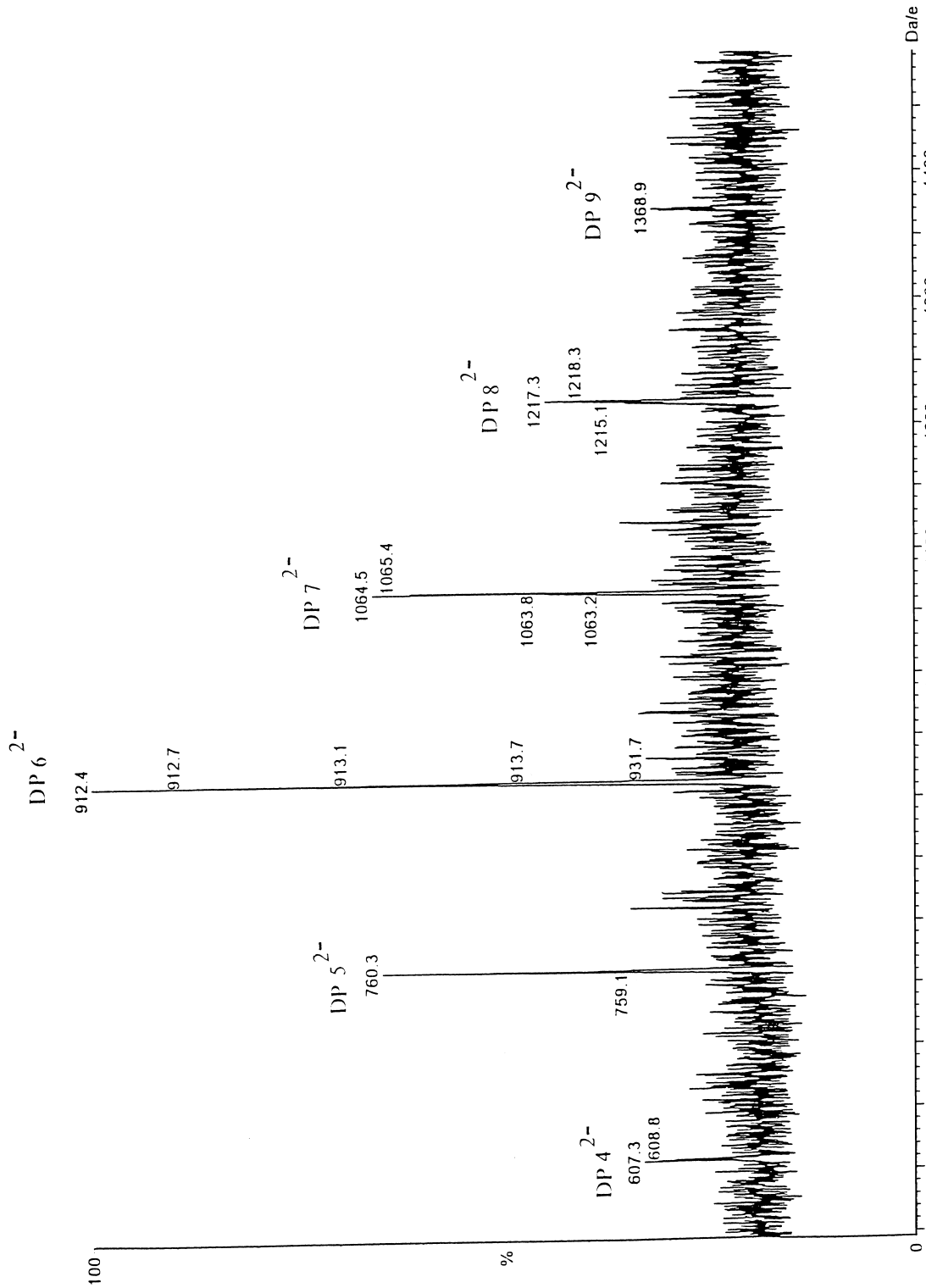

Fig. 3. ES–MS of the polymeric prodelphinidin from white clover flower.

3.2. Extraction and isolation

Ground dried flowers of white clover (500 g) were extracted exhaustively with 70% aq. Me_2CO and the extract concd on a rotatory evaporator under reduced pressure. The residual extract was diluted with water and defatted with CH_2Cl_2 and freeze-dried to give a light brown powder (21 g). The dried extract was dissolved in 5% MeOH and the solution applied onto a Sephadex LH20 column and the column eluted initially with H_2O and then aq. methanol containing increasing volume of MeOH. The prodelphinidin polymers were obtained by eluting the column with aqueous acetone (65%). Fractions were further treated chromatographically on Sephadex LH20 and MCI HP 20 until sufficiently pure compounds were separated. Fractions were monitored by HPLC.

3.3. trans-p-Coumaric acid 4-O-β-D-glucopyranoside (*1*)

Freeze-dried powder (400 mg). HPLC R_t 12.7 min. ^{13}C-NMR (75 MHz, CD_3OD) δ 61.0 (C6″), 69.8 (C4″) 73.3 (C2″), 75.9 (C5″), 76.5 (C3″), 100.2 (C1″), 116.0 (C8), 117.0 (C3, C5), 129.1 (C1), 130.5 (C2, C6), 146.0 (C7), 158.8 (C4), 171.2 (C9). ^{1}H-NMR (300 MHz, CD_3OD) δ 3.89–3.40 (*m*, sugar Hs), 5.00 (*d*, J = 7.1 Hz, H1″), 6.16 (*d*, J = 16.1 Hz, H8), 6.97 (*d*, J = 8.6 Hz, H3, H5), 7.36 (*d*, J = 8.6 Hz, H2, H6) 7.41 (*d*, J = 16.1 Hz, H7). Hydrolysis by heating with 2N HCl yielded *p*-coumaric acid and glucose.

3.4. cis-p-Coumaric acid 4-O-β-D-glucopyranoside (*2*)

Freeze dried powder (210 mg). HPLC R_t 17.0 min. ^{13}C-NMR (75 MHz, CD_3OD) δ 62.7 (C6″), 71.6 (C4″), 75.1 (C2″), 78.0 (C5″), 78.3 (C3″), 102.3 (C1″), 117.7 (C3, C5), 124.2 (C8), 132.1 (C1), 132.3 (C2, C6), 136.7 (C7), 159.1 (C4), 174.8 (C9). ^{1}H-NMR (300 MHz, CD_3OD) δ 3.18–3.81 (m, sugar Hs), 4.87 (*d*, J = 7.3 Hz, H1″), 5.82 (*d*, J = 12.7 Hz, H8), 6.49 (*d*, J = 12.7 Hz, H7), 6.94 (*d*, J = 8.8 Hz, H3, H5), 7.42 (*d*, J = 8.8 Hz, H2, H6). ESMS gave $(M - H)^-$ at m/z 325.

3.5. Myricetin 3-O-β-D-galactopyranoside (*3*)

Freeze dried powder (60 mg). HPLC R_t 43.7 min. ^{13}C-NMR (75 MHz, DMSO-d_6) δ 60.4 (C6″), 68.3 (C4″), 71.6 (C2″), 73.7 (C3″), 76.3 (C5″), 93.7 (C8), 99.1 (C6), 102.4 (C1″), 104.2 (C10), 108.9 (C2″, C6″), 120.3 (C1″), 134.1 (C3), 137.0 (C4″), 145.7 (C3″, C5″), 156.6 (C2, C9), 161.6 (C5), 164.7 (C7), 177.7 (C4). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.17–3.65 (m, sugar Hs), 5.34 (*d*, J = 7.7 Hz, H1″), 6.20 (*d*, J = 1.6 Hz, H6), 6.39 (*d*, J = 1.6 Hz, H8), 7.22 (*s*, H2″, H6″).

3.6. Quercetin 3-O-β-D-galactopyranoside (*4*)

Freeze-dried powder (130 mg). HPLC R_t 52.6 min. ^{13}C-NMR (75 MHz, DMSO-d_6) δ 60.2 (C6″), 68.0 (C4″), 71.3 (C2″), 73.3 (C3″), 75.9 (C5″), 93.6 (C8), 98.8 (C6), 101.9 (C1″), 103.8 (C10), 115.2 (C2″), 116.0 (C5″), 121.1 (C1″), 122.0 (C6″), 133.5 (C3), 144.9 (C3″), 148.6 (C4″), 156.2 (C2), 156.4 (C9), 161.3 (C5), 164.6 (C7), 177.5 (C4). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.28–3.68 (*m*, sugar Hs), 5.38 (*d*, J = 7.7 Hz, H-1″), 6.21 (*d*, J = 1.9 Hz, H6), 6.41 (*d*, J = 1.9 Hz, H8), 6.83 (*d*, J = 8.3 Hz, H5″), 7.55 (*d*, J = 2.1 Hz, H2″), 7.67 (*dd*, J = 8.5, 2.1 Hz, H6″).

3.7. Kaempferol 3-O-β-D-galactopyranoside (*5*)

Freeze-dried powder (32 mg). HPLC R_t 58.5 min. ^{13}C-NMR(75 MHz, DMSO-d_6) δ 60.5 (C6″), 68.2 (C4″), 71.6 (C2″), 73.5 (C3″), 76.1 (C5″), 94.0 (C8), 99.0 (C6), 102.1 (C1″), 104.1 (C10), 115.4 (C3″, C5″), 121.1 (C1″), 131.3 (C2″, C6″), 133.9 (C3), 156.6 (C2, C9), 160.3 (C4″), 161.6 (C5), 164.5 (C7), 177.7 (C4). ^{1}H-NMR(300 MHz, DMSO-d_6) d 3.37-4.07 (*m*, sugar Hs), 5.4 (*d*, J = 7.6 Hz, H1″), 6.22 (*d*, J = 2.1 Hz, H6), 6.44 (*d*, J = 2.1 Hz, H8), 6.86 (*d*, J = 8.7 Hz, H3″, H5″), 8.08 (*d*, J = 8.7 Hz, H2″, H6″).

3.8. Myricetin 3-O-(6″-acetyl)-β-D-galactopyranoside (*6*)

Freeze dried powder (48 mg). HPLC R_t 56.7 min. ^{13}C-NMR (75 MHz, DMSO-d_6) δ 20.4 (CH_3), 63.5 (C6″), 68.6 (C4″), 71.3 (C2″), 73.3 (C3″, C5″), 93.7 (C8), 99.0 (C6), 102.1 (C1″), 104.1 (C10), 108.8 (C2″, C6″), 120.3 (C1″), 134.0 (C3), 137.1 (C4″), 145.7 (C3″, C5″), 156.6 (C2, C9), 161.6 (C5), 164.5 (C7), 170.2 ($COCH_3$), 177.7 (C4). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.75 (*s*, CH_3), 3.37–4.07 (*m*, sugar Hs), 5.31 (*d*, J = 7.8 Hz, H1″), 6.20 (*d*, J = 2.0 Hz, H6), 6.39 (*d*, J = 2.0 Hz, H8), 7.19 (*s*, H2″, H6″).

3.9. Quercetin 3-O-(6″-acetyl)-β-D-galactopyranoside (*7*)

Freeze-dried powder (235 mg). HPLC R_t 64.3 min. ^{13}C-NMR (75 MHz, DMSO-d_6) δ 20.5 (COCH3), 63.5 (C6″), 68.5 (C4″), 71.3 (C2″), 73.2 (C3″, C5″), 93.8 (C8), 99.0 (C6), 102.1 (C1″), 104.2 (C10), 115.5 (C2″), 116.2 (C5″), 121.4 (C1″) 122.3 (C6″), 133.8 (C3), 145.2 (C3″), 148.8 (C4″), 156.6 (C2, C9), 161.6 (C5), 164.5 (C7), 170.2 ($COCH_3$), 177.8 (C4). ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.74 (*s*, $COCH_3$), 3.30–4.10 (*m*, sugar Hs), 4.67, 4.90, 5.7 (3 × OH), 5.31 (*d*, J = 7.7 Hz, H1″), 6.20 (*d*, J = 1.9 Hz, H6), 6.41 (*d*, J = 1.9 Hz, H8), 6.82 (*d*, J = 8.3 Hz, H5″), 7.51 (*d*, J = 2.1 Hz, H2″), 7.64 (*dd*, J = 8.5, 2.1 Hz, H6″). The sample

was subjected to alkaline hydrolysis with 2N NaOH at room temperature for 2 h. The reaction mixture was neutralised with 2N HCl and the resulting mixture analysed by HPLC which showed quercetin-3-*O*-galactoside (R_t 52.4 min) as the principal product.

3.10. Kaempferol 3-O-(6″-acetyl)-β-D-galactopyranoside (**8**)

Freeze-dried powder (18 mg). HRLC R_t 72.0 min. ^{13}C NMR (75 MHz, DMSO-d_6) δ 20.5 (CH_3), 63.5 (C6″), 68.5 (C4″), 71.3 (C2″), 73.1 (C3″, C5″), 94.0 (C8), 99.0 (C6), 102.1 (C1″), 103.4 (C10), 115.4 (C3″, C5″), 120.9 (C1″), 131.3 (C2″, C6″), 133.5 (C3), 156.7 (C2, C9), 160.3 (C4″), 161.5 (C5), 164.5 (C7), 170.1 ($COCH_3$), 177.9 (C4). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.79 (*s*, CH_3), 3.38–4.10 (m, sugar Hs), 5.38 (*d*, J = 7.7 Hz, H1″), 6.27 (*d*, J = 2.0 Hz, H6), 6.50 (*d*, J = 2.0 Hz, H8), 6.92 (*d*, J = 8.8 Hz, H3″, H5″), 8.10 (*d*, J = 8.8 Hz, H2″, H6″).

3.11. Gallocatechin (**9**)

Freeze-dried solid (30 mg). R_f 0.40 on cellulose TLC developed with HOAc–H_2O (6:94 v/v). ^{13}C-NMR(75 MHz, acetone-d_6) δ 28.5 (C4), 69.2 (C3), 83.3 (C2), 95.9 (C8), 96.7 (C6), 101.2 (C4a), 107.6 (C2″, C6″), 132.0 (C1″), 134.4 (C4″), 147.3 (C3″, C5″), 157.2, 158.0, 158.2 (C5, C7 and C8a). ^{1}H-NMR (300 MHz, acetone-d_6) δ 2.4–2.9 (*m*, H4), 3.97 (m, H3), 4.55 (*d*, J = 7.2, Hz, H2), 5.88 (*d*, J = 2.2 Hz, H8), 5.94 (*d*, J = 2.2 Hz, H6), 6.4 (*s*, H2″, H6″).

3.12. Epigallocatechin (**10**)

Freeze-dried powder (13 mg). R_f 0.23 on cellulose TLC developed with HOAc–H_2O (6:94 v/v). ^{13}C-NMR (75 MHz, acetone-d_6) δ 29.2 (C4), 67.6 (C3), 79.9 (C2), 95.9 (C8), 96.5 (C6), 101.0 (C4a), 107.1 (C2″, C6″), 131.6 (C1″), 133.7 (C4″), 146.8 (C3″, C5″), 157.4, 157.7, 158.0 (C5, C7, C8a). ^{1}H-NMR (300 MHz, acetone-d_6) δ 2.70–2.89 (*m*, H4), 4.17 (*bs*, H3), 4.50 (obscured by H_2O, H2), 5.93 (*m*, H6, H8), 6.52 (*s*, H2″, H6″).

3.13. Gallocatechin-(4α-8)-epigallocatechin (**11**)

Freeze-dried powder (24 mg). [α] = +54° (*c* 0.08, MeOH). R_f 0.50 on cellulose TLC with HOAc–H_2O (6:94 v/v). ^{13}C-NMR(75 MHz, acetone-d_6) δ 30.0 (C4″, obscured by acetone peaks), 37.7 (C4), 66.3 (C3″), 72.9 (C3), 79.2 (C2″), 83.3 (C2), 95.8 (C6), 97.1 (C8), 97.2 (C6″), 99.0 (C4a″), 106.2 (C4a), 106.4 (C2‴, C6‴), 107.9 (C8″), 108.1 (C2, C6), 131.3 (C1″), 131.5 (C1‴), 132.6 (C4‴), 133.4 (C4″), 145.8, (C3″, C5″), 146.1 (C3‴, C5‴), 154.4 (C8a″), 154.7 (C5″), 155.1 (C7″), 156.4 (C7), 156.6 (C5), 157.9 (C8a). ^{1}H-NMR (300 MHz, acetone-d_6) δ 2.80–3.00 (*m*, H4″), 4.29 (*bs*, H3″), 4.39 (*d*, J = 9.6 Hz, H2), 4.58 (*dd*, J = 9.6, 7.7 Hz, H3), 4.69 (*d*, J = 7.7 Hz H4), 4.92 (*s*, H2″), 5.86–5.89 (*m*, H6, H8), 6.08 (*s*, H6″), 6.62 (*s*, H2″, H6″), 6.73 (*s*, H2‴, H6‴).

3.14. Prodelphinidin polymer

Freeze-dried powder (1200 mg) with [α] = +4.0° (*c* 0.05, MeOH) which yielded delphinidin on heating with 5% HCl in *tert*-BuOH. ^{13}C-NMR (75 MHz, acetone-d_6) δ all broad peaks at 29.4, 37.4, 38.9, 67–69, 72.8, 76.8, 79.7, 81.8, 83.7, 97.7, 100.9, 102.6, 107.1, 108.9, 131.5–134.3, 146.6 and 154.8–156.7. ES–MS gave doubly charged ion peaks at m/z 1368.9 (5.3%), 1217.3 (11.2%), 1064.5 (20.1%), 912.4 (35.8%), 760.3 (20.7%) and 607.3 (6.3%).

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