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PROANTHOCYANIDINS FROM *LOTUS CORNICULATUS*

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Key Word Index—*Lotus corniculatus*; Leguminosae; birdsfoot trefoil; proanthocyanidin polymers; structure; phloroglucinol scission.

Abstract—The chemical structure of the purified proanthocyanidin polymers of *Lotus corniculatus* was analysed by ^{13}C NMR and by mild acid catalysed degradation in the presence of excess of phloroglucinol. The NMR data showed that the polymer was partially glycosidated with a number average M_r in the range 1800–2100 (six to seven flavanoid units). The products from phloroglucinol scission reaction indicated the extender flavan units to consist mostly of epicatechin (67%) and epigallocatechin (30%), with minor amounts of catechin and epiafzelechin units, which were linked together predominantly by C-4/C-8 interflavanoid bonds. The polymer chains were terminated mostly by catechin (83%) and, to a lesser extent, by epicatechin (16%).

INTRODUCTION

Proanthocyanidins or condensed tannins are widespread in nature, occurring in numerous plant species including many important plant-derived food materials and a number of economically significant forage legumes [1–5]. Their biological significance is still being debated and, in recent years, a great deal of attention has been focused on their role in ruminant nutrition. One of the most distinctive properties of proanthocyanidin polymers is their ability to complex with protein and, as a result, their presence had been associated with antinutritional properties in feeds [6–8]. This is certainly true when proanthocyanidins are present at high levels [9, 10], but, however, their presence at low levels in feeds is able to prevent bloat in cattle [11, 12] when given diets having a high soluble protein content. Bloat is a major problem worldwide, imposing a heavy cost to farmers amounting to hundred of millions of dollars in the U.S.A. alone [13]. Bloat most frequently occurs in dairy cattle in New Zealand, affecting up to 90% of the herds in a district, while deaths in an individual herd may exceed 15% [13].

It is now recognized that a low concentration of proanthocyanidins in ruminant diets can also be highly beneficial in many other ways, such as reducing the effects of parasites in the gastrointestinal tract [14], reducing problems relating to fly strike [15] and, paradoxically, by improving protein availability [16, 17]. Proanthocyanidins modify the microbial degradation of protein [18], as well as reducing the activity of microbial enzymes [19, 20]. A better understanding of the role of proanthocyanidins in this respect has been gained through extensive feeding trials [21], which provide evidence that proanthocyanidins reduce microbial degradation of plant protein in the rumen.

While microbes in the rumen are essential for degrading structural components, such as cellulose and hemicellulose, this service is provided at the expense of protein, and when fresh forages are given to ruminants *ca* 70% of the plant protein is lost to the animal in the form of ammonia. In the presence of proanthocyanidins, the concentration of ammonia in rumen digesta was reduced [22, 23] and the bacterial count in the rumen was also lower, as well as that of soluble protein in rumen liquor [11]. The increased availability of protein to ruminants appeared to be due to the protection of plant protein via complex formation with condensed tannins in the rumen and the subsequent dissociation of this complex under more acidic conditions of the abomasum to allow for enzyme digestion of the plant proteins in the proximal duodenum [21–27].

However, not all proanthocyanidins exhibit the same kind of beneficial affects to ruminant nutrition. They differ in chemical structure and, hence, their affinity for proteins; proteins, likewise, differ greatly in their affinity for various proanthocyanidins [27, 29]. Proanthocyanidins in *Lotus corniculatus*, for example, increased the absorption of essential amino acids from the small intestine [22], but absorption was not increased by the proanthocyanidins in this species [23]. There is, therefore, much interest in proanthocyanidin-containing herbages and, in particular, *L. corniculatus* because it tolerates low fertility conditions and is cultivated for pasture and hay in the northern U.S.A. and eastern Canada [30];

strains having a greater persistence under medium fertility conditions are currently undergoing selection and breeding in New Zealand. Observations have shown that proanthocyanidins from *L. corniculatus* and *L. pedunculatus* differ substantially in their effects upon digestion [22, 23] and in their ability to overcome effects of gastrointestinal parasites. These effects are not due to different amounts of proanthocyanidins in plant dry matter [31]. In view of the considerable benefits attributable to proanthocyanidins in *L. corniculatus*, and the apparent differences between proanthocyanidins from the two lotus species, it is important to quantify the differences between them. The present study was undertaken in order to determine the chemical structure of proanthocyanidins in *L. corniculatus* to provide a better understanding of their role in protein digestion.

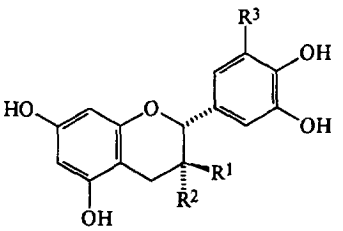
RESULTS AND DISCUSSION

The defatted 70% aqueous extract of *L. corniculatus* yielded only 1.5% of ethyl acetate extractables and 4.8% of purified proanthocyanidin polymers, the latter obtained by immobilization on a Sephadex LH20 column and washing off other accompanying plant constituents and low M_r phenolics with excess of methanol–H_2O before eluting off the polymers with aqueous acetone. Three flavanoids were identified from the ethylacetate extract and these were catechin, (**1**) epicatechin (**2**) and gallocatechin (**3**), in the approximate proportions of 8:3:1, respectively. Two procyanidin dimers, epicatechin-(4$\beta \rightarrow$8)-catechin (**5**) and epicatechin-(4$\beta \rightarrow$8)-epicatechin (**6**) in about equal proportion were also isolated. All these compounds were identified by ^{13}C NMR and confirmed by spectroscopic and chromatographic comparisons with authentic samples [32]. A procyanidin trimer (**7**), as shown by electrospray mass spectrometry (ES-MS), which gave a $[M - H]^-$ at *m/z* 865 on a negative-ion probe, was also isolated. The procyanidin constitution of **7** was apparent from the ^{13}C NMR spectrum, which showed B-ring signals at *ca* δ115 and 119, characteristic of unsubstituted catechol aromatic carbons. The presence of a degenerate upfield C-2 peak (δ76.7) confirmed the presence of two procyanidin units both with the 2,3-*cis*-configuration, while a signal at δ81.6, characteristic of the C-2 resonance of a catechin unit unsubstituted at C-4, suggested that this flavanoid was present as the terminal unit. The nature of the interflavanoid linkages in **7** was established by partial thiolysis using benzylmercaptan, which gave catechin, thus confirming its terminal position in the trimer. In addition, epicatechin-(4$\beta \rightarrow$*S*)-benzylthioether (**14**) was also detected, thus corroborating the epicatechin-extending role in the oligomer. In addition, (**5**) and epicatechin-(4$\beta \rightarrow$8)-epicatechin-(4$\beta \rightarrow$*S*)-benzylthioether (**15**) were also identified, thus establishing the interflavanoid linkages as the more frequently encountered C-4/C-8 bonds in proanthocyanidins. Compound **7** was therefore epicatechin-(4$\beta \rightarrow$8)-epicatechin-(4$\beta \rightarrow$8)-catechin.

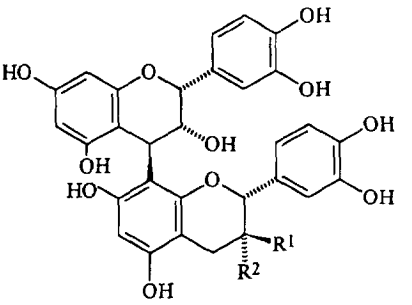
The ^{13}C NMR spectrum, of the purified proanthocyanidin polymer, $[\alpha]_D$ + 137° (*c* 0.18 methanol), showed an intense signal at δ76.4 and weak signals at lower-field in the heterocyclic region, indicating that flavanoid units with the 2,3-*cis* configuration were by far the most dominant extension units [33]. A signal at δ80.9 showed the polymers to be terminated mostly by flavanoid units with the 2,3-*trans* configuration. The observation of two upfield methine carbon signals (δ114–117), together with those of the hydroxylated carbon signals (δ144–145), were evidence for the presence of a catechol B-ring. The ratio of signal size, particularly those of the hydroxylated carbons (δ144–145), compared to the corresponding C-3′ and C-5′ hydroxylated pyrogallol carbon peaks (δ146.0), was in the approximate ratio of 2.5:1, respectively, indicating that the polymer was a mixture of procyanidin and prodelphinidin units, with the catechol units predominating. A relatively small signal in the upfield heterocyclic region at δ62.8 was shown by DEPT to be methylene carbons attributable to those of a sugar unit and corroborated by the presence of another equivalent sized signal at δ103.9, which were characteristic of those of the anomeric carbons, suggesting that a small fraction of the flavanoid units was glycosidated. The presence of glycosides was further confirmed by mild acid treatment of the polymer, which yielded glucose as a hydrolysis product. The low-field position of the anomeric carbon indicated that the sugar moieties were attached to the aliphatic 3-OH [34].

The C-3 signal (δ67.2) of the chain-terminating flavanoid units was well resolved from the corresponding C-3 signal (δ72.1) of the extender unit and with their comparable T_1, and NOE values allowed for an estimation of M_r by relative signal intensity, which gave a size of about six to seven extender units [35]. This value did not take into account the sugar signals, which could distort the signal sizes, but, because of their relative small contribution, the M_r was not expected to be affected significantly.

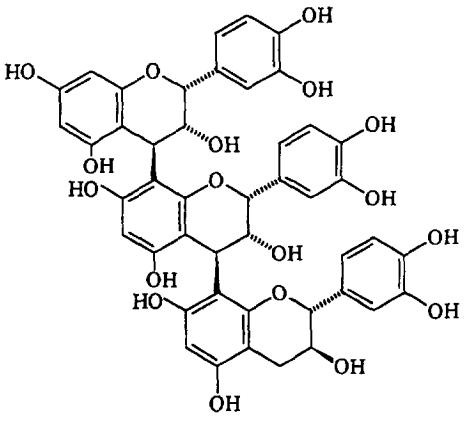
To elucidate the structure in more detail, the polymer was subjected to mild acid catalysed scission, and the generated extender flavan carbocations were captured with phloroglucinol [35]. This reaction resulted in the cleavage of the terminal flavanoid units, which were identified as catechin and epicatechin, together with a small amount of epigallocatechin, in the ratio of *ca* 50:10:1, respectively; this figure is consistent with the ^{13}C NMR data. A small amount of the procyanidin dimer, epicatechin-(4$\beta \rightarrow$8)-catechin [$[\alpha]_D$ − 30° (*c* 0.10, methanol)] was also isolated and identified from the reaction mixture, confirming the propensity of catechin as end unit. Among the monomeric phloroglucinol captured products, were epicatechin-(4$\beta \rightarrow$2)-phloroglucinol (**8**), epigallocatechin-(4$\beta \rightarrow$2)-phloroglucinol (**9**), catechin-(4$\alpha \rightarrow$2)-phloroglucinol (**10**) and epiefzelechin-(4$\beta \rightarrow$2) phloroglucinol (**11**). Compounds **8**–**10** were identified from their respective NMR chemical shift values and by chromatographic comparison on TLC with authentic materials [36]. The structure of **11** followed readily from these data because the ^{13}C NMR chemical shifts of **11** were very similar to those of **8** and **9**, except for the B-ring chemical shift values. For **11**, there



	R^1	R^2	R^3
1	OH	H	H
2	H	OH	H
3	OH	H	OH
4	H	OH	OH



	R^1	R^2
5	OH	H
6		OH



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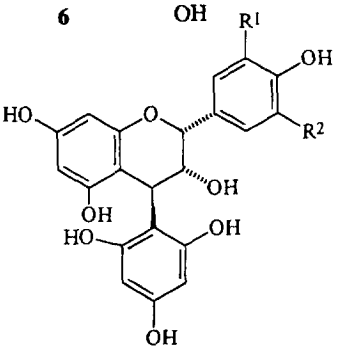
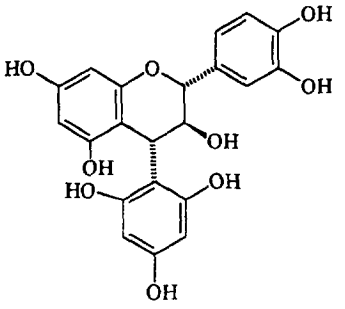


	R^1	R^2
8	H	H
9	OH	H
11	OH	OH



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were two sets of degenerated methine carbon signals (from DEPT studies) at δ 115.5 and 129.0, suggesting that the B-ring was symmetrical. This was confirmed by the observation of two sets of mutually coupled doublets (δ7.27, J = 6.8 Hz; δ6.82, J = 6.8 Hz) in the ^{1}H NMR spectrum, consistent with the presence of a 4-hydroxylated B-ring. The chemical shift values were also fully consistent with published NMR data for the B-ring of epiefzelechin [37]. The chemical structure of **11** was further corroborated by ES-MS, which gave a $[M - H]^-$ at m/z 397 using a negative-ion probe. The identification of this uncommon epiefzelechin monomeric unit, albeit at relatively low concentrations, and the presence of flavan glycoside monomers, indicated that the proanthocyanidin polymers of *L. corniculatus* were more heterogeneous than previously realized [2].

To establish the nature of the interflavanoid linkages in the polymers, it was necessary to isolate some larger scission products which contained these linkages. Three dimeric phloroglucinol adducts (**12**–**14**) were isolated by

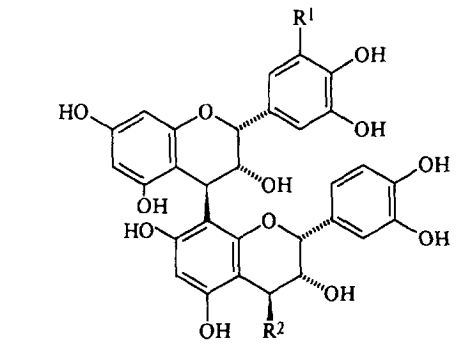
column chromatography alternating between Sephadex LH20 and high porosity polystyrene polymer (MC1 CHP 20P) using aqueous ethanol and aqueous methanol, respectively. Compound **12** gave $[M - H]^-$ peak at *m/z* 701 on negative-ion ES-MS, which was consistent with a procyanidin dimer phloroglucinol adduct. The ^{13}C NMR spectrum of **12** showed two sets of signals for C-2 (δ76.6 and 76.8) and C-3 (δ71.6 and 73.0), which were characteristic of two procyanidin units with the 2,3-*cis* configuration. Partial degradation of **12** with benzylmercaptan yielded phloroglucinol, epicatechin-(4$\beta \rightarrow$ 2)-phloroglucinol (**8**), epicatechin (4$\beta \rightarrow$ *S*)-benzylthioether (**14**) and epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4$\beta \rightarrow$ *S*)-benzylthioether (**15**). The identification of these products established that **12** was epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4β-2)-phloroglucinol.

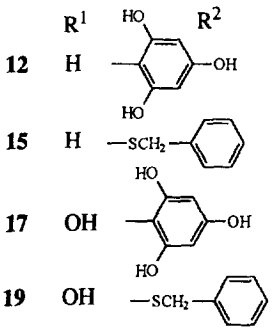
Compound **13**, also gave a $[M - H]^-$ at *m/z* 701 by negative-ion ES-MS, indicating that is also a procyanidin dimer–phloroglucinol adduct. Its ^{13}C NMR spectrum again showed twin peaks for C-2 (δ76.6 and 76.9), C-3 (72.3 and 72.4) and C-4 (δ36.9 and 37.1) indicating a similar chemical constitution to **12**, with both flavanoid units possessing the 2,3-*cis* configuration. This indicated that **13** must be the regioisomer with the less common C-4 to C-6 interflavanoid linkage. This linkage was confirmed by partial cleavage of **13** with benzylmercaptan, which yielded products identical to those obtained from **12**, with one exception; epicatechin-(4$\beta \rightarrow$ 6)-epicatechin-(4$\beta \rightarrow$ *S*)-benzylthioether (**16**) was produced instead of **15**.

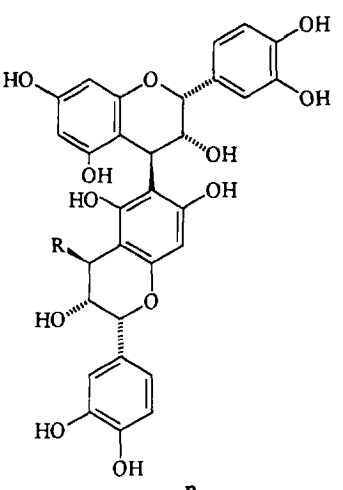
Compound **17** showed a $[M - H]^-$ peak at *m/z* 717 by negative-ion ES-MS, showing it to be another procyanidin dimer–phloroglucinol adduct where one of the B-ring flavanoid units carried additional hydroxylation. The ^{13}C NMR spectrum obtained in acetone showed an intense signal at δ76.7, which may be attributed to the coincidence of both C-3 flavanoid resonances, a medium-size signal at δ72.9, which accounted for one of the C-2 resonances, while the other C-2 appeared as a double peak (δ71.7 and 71.9) whose total peak size was equivalent to the signal size of the δ72.9 peak. The two substituted C-4 carbon resonances appeared at δ36.6 and 36.8; their intensity was consistent with a proanthocyanidin dimer–phloroglucinol adduct constitution. Evidence for the presence of a prodelphinidin unit was the presence of the signal at δ146.0 attributable to the oxygenated C-3′ and C-5′ of the pyrogallol B-rings. This was further confirmed by the presence of methine carbon (DEPT) signals at δ106.4 and 106.8 for the unsubstituted C-2′ and C-6′ carbons, although their magnetic nonequivalence, and the multiplicity of C-2 (δ71.7 and 71.9) for one of the C-2 carbons, was unexpected. These observations were viewed as a result of some restriction to the free rotation about the interflavanoid bond brought about by additional hydroxylation on one of the flavanoid units. Indeed, the Drieding model indicated some degree of crowding between the two B-rings, which was exacerbated by the presence of an additional hydroxyl group. Multiplicity of ^{13}C NMR signals were also evident among other B-ring carbons and further corroborated by ^1H NMR data, which exhibited multiplicity for the galloyl ring protons (δ6.54 and 6.62), as well as the catechol ring protons, in addition to other signal-line broadening. The H-2 protons appeared to be most affected, where the signals took on the appearance of broad multiplets at δ5.14–5.36. Normally, these signals appear as slightly broader singlets due to small vicinyl proton couplings. However, when the ^{13}C NMR of **17** was run in methanol, the multiplicity of the lower C-2 carbon chemical shifts disappeared to give rise to a single peak at δ72.4, confirming that the observed multiplicity was due to restricted rotation about the interflavanoid bond. How this was brought about remained unclear, but could be related to differences in hydrogen-bonding effects of the solvents, resulting in changes to the preferred conformation of **17**. In addition, the ^{13}C chemical shift values of the A- and C-ring carbons in methanol were almost identical to those of epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4$\beta \rightarrow$ 2)-phloroglucinol in methanol [36], thus establishing the mode of interflavanoid linkage as C-4 to C-8.

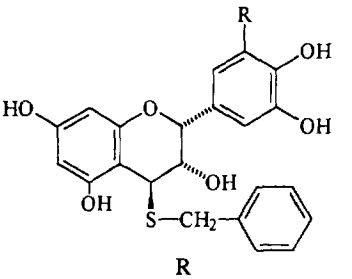
Although it was possible to make most assignment of carbon chemical shifts through the use of homo- and hetero-nuclear NMR correlation spectroscopic techniques, such as HMQC and HMBC, the lack of resolution of the critical C-2 and H-2 NMR resonances between the two flavanoid units prohibited any distinction to be made from their long-range correlation data with the B-ring signals. While the positional sequence of the flavanoid units in **17** could not be established by these techniques, the data were completely consistent with the chemical constitution of the phloroglucinol product. The sequence by which the epicatechin and epigallocatechin units manifested themselves in **17** was obtained by partial cleavage with benzylmercaptan. This reaction resulted in the identification of epicatechin-(4$\beta \rightarrow$ *S*)-benzylthioether (**14**), epigallocatechin-(4$\beta \rightarrow$ *S*)-benzylthioether (**18**), epicatechin-(4$\beta \rightarrow$ 2)-phloroglucinol (**9**) and epigallocatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4$\beta \rightarrow$ *S*)-benzylthioether (**19**), thus establishing the structure of **17** as epigallocatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4$\beta \rightarrow$ 2)-phloroglucinol.

The isolation of the dimeric phloroglucinol adducts, **12**, **13** and **17**, in the relative proportions of *ca* 3:1:6, respectively, in addition to a small amount of the procyanidin dimer (**5**) (Table 1) could be assumed, in the light of the mild conditions employed in their production, to reflect the relative frequency of the type of interflavanoid linkages in the original polymer. In the same manner, both the monomeric (**8**–**11**) and the dimeric phloroglucinol adducts were representative of the polymer extender units; these were characterized by flavanoid units consisting almost exclusively of the 2,3-*cis* configuration, as deduced earlier from the ^{13}C NMR spectrum of the polymer. The only 2,3-*trans* product was catechin-(4$\beta \rightarrow$ 2)-phloroglucinol, which was isolated as a minor component. Epicatechin units were the most prevalent species, comprising some 65–70% of the extender units, followed by epigallocatechin (30%) and a small amount of epiefzelechin (1–2%). It was apparent from ^{13}C NMR data that a small proportion of the flavanoid units were glycosidated, but, because of these being acid labile, it

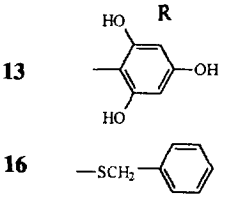








R
14 H
18 OH



was expected that no phloroglucinol condensation products containing a sugar unit would be detected [34]. Their presence in the purified *L. corniculatus* polymers was confirmed by mild acid treatment with 0.7% HCl in ethanol at ambient temperatures, which resulted in the liberation of glucose. The nature of the proanthocyanidin distribution in many plant species has been observed to be correlated with the co-occurring monomeric flavans, the latter providing an insight into the terminal units in the biosynthetically related oligomers. Thus, when catechin is present, there is a preponderance of oligomers with catechin as the end unit [38]. The presence of catechin, and to a lesser extent epicatechin, in *L. corniculatus* was consistent with this observation, where they were found to be distributed in a generally similar pattern as terminal units in the polymers. An exception to this was the presence of gallocatechin as the free flavan and of epigallocatechin as a product liberated from the phloroglucinol scission reaction. However, these were minor components, and the related evidence for the

Table 1. Composition of isolated scission products of proanthocyanidin polymers from *Lotus corniculatus* with phloroglucinol

Product	Yield (mg)
Catechin (**1**)	253
Epicatechin (**2**)	50
Epigallocatechin (**4**)	5
Epicatechin-(4β → 2)-phloroglucinol (**8**)	637
Epigallocatechin-(4β → 2)-phloroglucinol (**9**)	290
Catechin-(4α → 2)-phloroglucinol (**10**)	14
Epiefzelechin-(4β →)-phloroglucinol (**11**)	16
Epicatechin-(4β → 8)-epicatechin-(4β → 2)-phloroglucinol (**12**)	86
Epicatechin-(4β → 6)-epicatechin-(4β → 2)-phloroglucinol (**13**)	32
Epigallocatechin-(4β → 8)-epicatechin (4β → 2)-phloroglucinol (**17**)	176
Epicatechin-(4β → 8)-catechin (**5**)	8

suggested association to the polymer constitution was likely to be obscured during such experimental work-up.

In contrast, the proanthocyanidin polymers of *L. pedunculatus* are much more heterogenous than those of *L. corniculatus*, both in terms of the variety of terminal and extender flavanoid units. The chemical structure of this polymer is currently being investigated in some detail.

EXPERIMENTAL

^{13}C NMR spectra were generally obtained in Me_2CO-d_6 with a small amount of D_2O. Optical rotation measurements were made in MeOH. Mass spectra were obtained by ES-MS. TLC were performed on Schleicher and Schuell cellulose plates and developed with *t*-BuOH–HOAc–H_2O (3:1:1) (solvent A) and HOAc–H_2O (3:47) (solvent B).

Extraction and isolation. Ground leaves of *L. corniculatus* (cv. Grasslands. Goldie) from *ca* 80 plants were exhaustively extracted with 70% aq. Me_2CO and the extract concd on a rotatory evaporator under red. pres. The residual extract was diluted with H_2O and washed exhaustively with CH_2Cl_2 before freeze-drying. A sample of the freeze-dried extract (92 g) was suspended in H_2O (1 l), extracted with EtOAc (5 × 300 ml) and the combined extracts dried (Na_2SO_4) and concd to yield the dried extract (1.38 g). The solid was dissolved in 50% aq. EtOH and fractionated on a Sephadex LH 20 column. Frs were monitored by TLC using solvent B and visualized with $FeCl_3$–$K_3Fe(CN)_6$. Frs containing mixts were further treated chromatographically alternating between MCI CHP 20 (aq. MeOH) and Sephadex LH 20 (aq. EtOH) until chromatographically pure samples were obtained.

Residual EtOAc was removed from the aq. fr. from the EtOAC extraction, the residue diluted with an equal vol. of MeOH and the resulting mixt. applied directly on to a Sephadex LH 20 column. The column was washed exhaustively with MeOH–H_2O (1:1) until the eluate was almost colourless and the proanthocyanidin polymers eluted with acetone–H_2O (3:2). The eluates were concd to small vol. and freeze-dried to give a light brown fluffy powder (4.43 g).

Degradation with phloroglucinol. Purified proanthocyanidin polymers (4 g) were reacted with phloroglucinol (2.8 g) in 1% HCl in EtOH (35 ml) with continuous shaking until complete soln was affected; the time taken for complete soln was *ca* 15 min. The resulting soln was concd under a stream of N_2 to *ca* 25 ml and fractionated on a Sephadex LH 20 column using EtOH as eluent. Frs were monitored by TLC, developed with solvent B, and visualized with $FeCl_3$–$K_3Fe(CN)_6$ spray. These frs were further chromatographically treated, alternating between MC1 CHP 20 (MeOH–H_2O, 3:7, and Sephadex LH 20 (EtOH–H_2O, 19:1 to 1:1) until homogenous products were obtained.

Reaction with benzylmercaptan. Benzylmercaptan (3 mg) and HOAc (1 drop) were added to a sample of procyanidin or flavanoid–phloroglucinol adduct (*ca* 5 mg) in EtOH (2 ml) in a vial. The vial was flushed with N_2 for 1 min, sealed and heated at 95° for 45 min. Reaction products were analysed by 2D-TLC, developed with solvents A and B, and visualized by spraying with vanillin–HCl and heating.

(+)-*Catechin* (**1**). Freeze-dried powder (102 mg from EtOAc extract, 253 mg from phloroglucinol degradation reaction). $[\alpha]_D$ + 3° (*c* 0.10, MeOH). R_f 0.67 (A), 0.48 (B). ^{13}C NMR: δ28.8 (C-4), 68.2 (C-3), 82.5 (C-2), 95.2 (C-8), 96.2 (C-6), 100.5 (C-4a), 115.4 (C-2′), 115.8 (C-5′), 119.9 (C-6′), 131.9 (C-1′), 145.6, 145.7 (C-3′, C-4′), 156.7, 157.2, 157.6 (C-5, C-7, C-8a). ^{1}H NMR: δ2.43–2.95 (*m*, H-4), 4.0 (*m*, H-3), 4.55 (*d*, *J* = 6.1 Hz, H-2), 5.88 (*bs*, H-6), 6.04 (*bs*, H-8), 6.80 (*m*, H-2′, H.5′), 6.91 (*s*, H.6′).

(−)-*Epicatechin* (**2**). Freeze-dried powder (40 mg from EtOAc extract, 50 mg from phloroglucinol degradation). $[\alpha]_D$ − 34° (*c* 0.08, MeOH). R_f 0.50 (A), 0.30 (B). ^{13}C NMR: δ29.1 (C-4), 66.9 (C-3), 79.2 (C-2), 95.7 (C-8), 96.3 (C-6), 115.1 (C-2′), 115.6 (C-5′), 119.3 (C-6′), 132.0 (C-1′), 145.1, 146.5 (C-3′, C-4′), 156.9–158.4 (C-5, C-7, C-8a). ^{1}H NMR: δ2.7–3.1 (*m*, H-4), 4.23 (*bs*, H-3), 4.90 (H-2), 6.98 (*bs*, H-6), 6.10 (*bs*, H-8), 6.80–6.90 (*m*, H-2′, H-5′), 7.08 (*s*, H-6′).

(+)-*Gallocatechin* (**3**). Freeze-dried powder (13 mg from EtOAc extract). $[\alpha]_D$ + 17° (*c* 0.09, MeOH). R_f 0.48 (A), 0.40 (B). ^{13}C NMR: δ28.5 (C-4), 68.0 (C-3), 82.5 (C-2),

95.3 (C-8), 96.2 (C-6), 100.5 (C-4a), 107.4 (C-2′, C-6′), 131.1 (C-1′), 133.3 (C-4′), 146.2 (C-3′, C-5′), 156.5, 157.0, 157.4 (C-5, C-7, C-8a).

Epicatechin-(4β → 8)-*epicatechin* (**6**). Freeze dried powder (15 mg from EtOAc extract. $[\alpha]_D$ + 15° (*c* 0.16, MeOH). R_f 0.43 (A), 0.63 (B). ES-MS: $[M - H]^-$ *m/z* 577. ^{13}C NMR: δ29.0, 66.2, 72.8, 76.4, 79.1, 95.7, 97.1, 100.5, 101.3, 115.3, 115.5, 115.6, 115.9, 119.1, 132.2, 132.4, 145.2–145.4, 156–158. Reaction with benzylmercaptan gave epicatechin and epicatechin-(4*β* → *S*)-benzylthioether.

Epicatechin-(4β → 8)-*catechin* (**5**). Freeze-dried powder (8 mg from EtOAc extract and from phloroglucinol-scission reaction with the polymer). $[\alpha]_D$ − 30° (*c* 0.10, MeOH). R_f 0.42 (A), 0.60 (B). ES-MS: $[M - H]^-$ *m/z* 577. ^{13}C NMR: δ29.0, 68.0, 72.0, 76.8, 82.3, 95.7, 96.1, 96.5, 101.2, 106.9, 115.2–115.9, 119.2, 120.1, 131.6, 131.8, 145.2–146.1, 154.5–158.9. Treatment with benzylmercaptan yielded catechin and epicatechin-(4*β* → *S*)-benzylthioether.

Epicatechin-(4β → 2)-*phloroglucinol* (**8**). Freeze-dried solid (637 mg). $[\alpha]_D$ + 98° (*c* 0.35, MeOH). R_f 0.50 (A), 0.60 (B). ES-MS: $[M - H]^-$ *m/z* 413. ^{13}C NMR: δ36.8, 72.4, 76.8, 95.5, 96.1, 96.2, 100.5, 106.8, 115.2, 115.6, 119.1, 132.2, 145.2, 145.4, 157.5, 157.6, 157.8, 158.3, 158.5. ^{1}H NMR: δ3.99 (*s*, H-3), 4.54 (*s*, H-4), 5.07 (*s*, H-2), 5.94 (*s*, phloroglucinol-H), 6.02, 6.05 (each *d*, *J* = 2.3 Hz, H-6 and H-8), 6.70 (*dd*, *J* = 1.8, 8.18 Hz, H-6′), 6.79 (*d*, *J* = 8.18 Hz, H-5′), 7.0 (*d*, *J* = 1.8 Hz, H-2′).

Epigallocatechin-(4β → 2)-*phloroglucinol* (**9**). Freeze-dried powder (290 mg). $[\alpha]_D$ + 91° (*c* 0.18, MeOH). R_f 0.32 (A), 0.54 (B). ES-MS: $[M - H]^-$ *m/z* 429. ^{13}C NMR: δ36.7 (C-4), 72.6 (C-3), 76.9 (C-2), 95.9, 96.4, 96.5 (unsubstituted A-ring and phloroglucinol carbons), 100.6 (C-4a), 106.7 (C-2′, C-6′), 106.9 (substituted phloroglucinol carbon), 131.7 (C-1′), 132.8 (C-4′), 146.2 (C-3′, C-5′), 157.6, 157.8, 158.0, 158.5, 158.6 (oxygenated A-ring and phloroglucinol carbons). ^{1}H NMR: δ4.03 (*bs*, H-3), 4.61 (*s*, H-4), 5.01 (*s*, H-2), 5.94, 6.04 (*bs* each, A-ring and phloroglucinol H), 6.50 (*s*, H-2′, H6′).

Catechin-(4α → 2)-*phloroglucinol* (**10**). Freeze-dried powder (14 mg). $[\alpha]_D$ − 150° (*c* 0.10, MeOH). R_f 0.53 (A), 0.62 (B). ^{13}C NMR: δ38.1, 73.3, 83.4, 95.8, 97.3, 100.6, 107.1, 115.1, 115.9, 120.7, 132.1, 145.5, 145.7, 157.4–158.4. ^{1}H NMR: δ4.4–4.6 (*m*, H-2, H_3, H_4), 5.8–6.0 (*m*, A-ring and phloroglucinol H), 6.7–7.0 (*m*, B-ring H).

Epiefzelechin-(4β → 2)-*phloroglucinol* (**11**). Light brown freeze-dried powder (16 mg). $[\alpha]_D$ + 113° (*c* 0.10, MeOH). R_F 0.52 (A), 0.63 (B). ES-MS: $[M - H]^-$ *m/z* 397. ^{13}C NMR: δ36.8 (C-4), 72.3 (C-3), 76.8 (C-2), 95.5 (C-8), 96.1 (phloroglucinol carbons), 96.2 (C-6), 100.6 (C-4a), 106.8 (substituted phloroglucinol carbon), 115.5 (C-3′, C-5′), 129.0 (C-2′, C-6′), 131.4 (C-1′), 157.4, 157.5, 157.8, 158.2, 158.5 (oxygenated A-ring and phloroglucinol carbons). ^{1}H NMR: δ3.95 (partly obscured by H_2O peak), 4.55 (*s*, H-4), 5.12 (*s*, H2), 5.94 (phloroglucinol H), 6.03, 6.08 (*s*, H-6, H-8), 6.82 (*d*, *J* = 6.9 Hz, H-3′, H-5′), 7.27 (*d*, *J* = 6.9 Hz, H-2′, H-6′).

Epicatechin-(4β → 8)-*epicatechin*-(4β → 2)-*phloroglucinol* (**12**). Freeze-dried powder (86 mg). $[\alpha]_D$ + 135° (*c* 0.11, MeOH). R_F 0.38 (A), 0.55 (B). ES-MS: $[M - H]^-$ *m/z* 701. ^{13}C NMR: δ36.9, 71.6, 76.6, 76.8, 96.1, 96.5, 96.7, 97.3, 100.6, 101.6, 106.4, 107.1, 114.8, 115.1, 115.6, 119.0, 119.1, 132.1, 132.3, 144.9, 145.0, 145.3, 146.1, 156.7–157.9. ^{1}H NMR: δ4.12 (*bs*), 4.16 (*bs*), 4.68 (*s*), 4.82 (*s*), 5.12 (*bs*), 5.22 (*bs*), 5.96 (*bs*), 6.06 (*bs*), 6.75 (*m*), 7.00 (*s*). Reaction with benzylmercaptan gave phloroglucinol, epicatechin-(4*β* → *S*)-benzythioether, epicatechin-(4*β* → 2)-phloroglucinol and epicatechin-(4*β* → 8)-epicatechin-(4*β* → *S*)-benzylthioether.

Epicatechin-(4β → 6)-*epicatechin*-(4β → 2)-*phloroglucinol* (**13**). Freeze-dried powder (32 mg). $[\alpha]_D$ + 141° (*c* 0.09, MeOH). R_f 0.36 (A), 0.43 (B). ES-MS: $[M - H]^-$ *m/z* 701. ^{13}C NMR: δ36.9, 37.1, 72.3, 72.4, 76.6, 76.9, 95.9–96.6, 99.0, 102.0, 106.6, 107.2, 115.2–115.9, 119.2, 119.7, 131.8, 132.0, 144.9, 145.0, 145.1, 155.0–158.7. ^{1}H NMR: δ3.93 (*s*), 3.98 (*s*), 4.43 (*s*), 4.61 (*s*), 4.86 (*s*), 5.02 (*s*), 5.9–6.24 (*m*), 6.71–6.83 (*m*), 7.02 (*m*). Treatment with benzylmercaptan gave phloroglucinol, epicatechin-(4*β* → *S*)-benzylthioether, epicatechin-(4*β* → 2)-phloroglucinol and epicatechin-(4*β* → 6)-epicatechin benzylthioether.

Epigallocatechin-(4β → 8)-*epicatechin*-(4β → 2)-*phloroglucinol* (**17**). Freeze-dried powder ((176 mg). $[\alpha]_D$ + 120°C (*c* 0.12, MeOH). ES-MS: $[M - H]^-$ *m/z* 717. ^{13}C NMR (Me_2CO-d_6): δ36.6, 36.8, 71.8 (*d*), 72.9, 76.7, 96.1, 96.5, 97.3, 100.8, 101.6, 106.4, 106.8, 107.1, 114.7, 115.1, 115.7, 119.1, 131.5, 132.2, 132.5, 132.7, 144.8, 144.9, 145.2, 146.0, 154.6–158.1. ^{1}H NMR (Me_2CO-d_6): δ3.88 (*br*), 4.14 (*bs*), 4.71 (*bs*), 4.85 (*bs*), 5.30 (*br m*), 5.99 (*bs*), 6.08 (*bs*), 6.54, 6.02, 6.75 (*bs*), 7.00 (*m*). ^{13}C NMR (MeOH-d_4): δ37.3 (×2), 72.4, 73.4, 76.9, 96.3, 96.7, 97.4, 101.5, 102.3, 106.7, 106.9, 107.8, 115.1, 116.2, 118.9, 119.3, 131.9, 132.5, 133.2, 133.3, 145.4, 145.7, 146.5 (×2), 154.7, 157.1, 157.6, 158.2. Reaction with benzylmercaptan gave phloroglucinol, epicatechin-(4*β* → 2)-phloroglucinol, epicatechin-(4*β* → *S*)-benzylthioether, epigallocatechin-(4*β* → *S*)-benzylthioether and epigallocatechin-(4*β* → 8)-epicatechin-(4*β* → *S*)-benzylthioether.

Epicatechin-(4β → 8)-*epicatechin*-(4β → 8)-*catechin* (**7**). Freeze-dried powder (10 mg from EtOAc extract). $[\alpha]_D$ + 47° (*c* 0.12, MeOH). R_F 0.27 (A), 0.54 (B). ES-MS: $[M - H]^-$ *m/z* 865. ^{13}C NMR: δ27.9, 36.8, 67.6, 71.9, 72.9, 76.7, 81.6, 95.8, 96.4, 97.0, 100.7, 101.2, 107.4, 114.6–115.8, 118.9, 119.1, 132.1, 145.0–145.5, 153.5–158.1. ^{1}H NMR: δ2.5–2.9 (*m*), 4.1–4.3 (*m*), 4.76, 4.92 (*d*, *J* = 6.0 Hz), 5.09, 5.24, 6.00, 6.07, 6.75–7.20. Reaction with benzylmercaptan gave catechin, epicatechin-(4*β* → 8)-catechin, epicatechin-(4*β* → *S*)-benzylthioether and epicatechin-(4*β* → 8)-epicatechin-(4*β* → *S*)-benzylthioether.

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REFERENCES

1. Jones, W. T., Broadhurst, R. B. and Lyttleton, J. W. (1976) *Phytochemistry* **15**, 1407.
2. Foo, L. Y., Jones, W. T., Porter, L. J. and Williams, V. M. (1982) *Phytochemistry* **21**, 933.
3. Foo, L. Y. and Porter, L. J. (1980) *Phytochemistry* **19**, 1747.
4. Sarkar, S. K., Howarth, R. E. and Goplen, B. P. (1976) *Crop Sci.* **16**, 543.
5. Terrill, T. H., Rowan, A. M., Douglas, G. B. and Barry, T. N. (1992) *J. Sci. Food Agric.* **58**, 321.
6. Mitaru, B. N., Reichert, R. D. and Blair, R. (1984) *J. Nutr.* **114**, 1787.
7. Singleton, V. L. and Kratzer, F. H. (1973) *Toxicants Occurring Naturally in Foods*, p. 327. 2nd edn, National Academy Science, New York.
8. Deshpande, S. S., Sathe, S. K. and Salunkhe, D. K. (1984) in *Nutritional and Toxicological Aspects of Food Safety* (Freidman, M., ed.), p. 457. Plenum Press, New York.
9. Wiseman, J. and Cole, D. J. A. (1988) in *Recent Advances in Animal Nutrition* (Haresign, W. and Cole, D. J. A., eds) p. 13. Butterworths, London.
10. Barry, T. N. and Duncan, S. J. (1984) *Br. J. Nutr.* **51**, 485.
11. Waghorn, G. C. and Jones, W. T. (1989) *N. Z. J. Agri. Res.* **32**, 227.
12. Lees, G. L. (1992) in *Plant Polyphenols* (Hemingway, R. W. and Laks, P. E., eds), p. 915. Plenum Press, New York.
13. Reids, C. S. W. (1976) *Bovine Practitioner* **1**, 24.
14. Niezer, J. H., Waghorn, T. S., Charleston, W. A. G. L. and Waghorn, G. C. (1995) *J. Agric. Sci. Cambridge* (in press).
15. Leathwick, D. M. and Atkinson, D. S. (1995) *N.Z. Soc. Anim. Prod.* **55** (in press).
16. Ulyatt, M. J. (1981) *N.Z. Agri. Sci.* **15**, 200.
17. Reid, C. S. W., Ulyatt, M. J. and Wilson, J. M. (1974) *N.Z. Soc. Anim. Prod.* **34**, 82.
18. Sadanandan, K. P. and Arora, S. P. (1979) *J. Nucl. Agric. Biol.* **8**, 1.
19. Makkar, H. P. S., Singh, B. and Bawra, R. K. (1988) *Br. J. Nutr.* **60**, 287.
20. Makkar, H. P. S., Dawra, R. K. and Singh, B. (1990) *Indian J. Anim. Nutr.* **7**, 207.
21. Waghorn, G. C., Jones, W. T., Shelton, I. D. and McNabb, W. C. (1990) *Proc. N.Z. Grassl. Assoc.* **51**, 171.
22. Waghorn, G. C., Ulyatt, M. J., John, A. and Fisher, M. T. (1987) *Br. J. Nutr.* **59**, 115.
23. Waghorn, G. C., Shelton, I. D. and McNabb, W. C. (1994) *J. Agric. Sci. Cambridge* **123**, 109.
24. Waghorn, G. C., John, A., Jones, W. T. and Shelton, I. D. (1987) *Proc. N.Z. Soc. Anim. Prod.* **47**, 25.
25. Jones, W. T. and Mangan, I. L. (1977) *J. Sci. Food Agric.* **28**, 126.
26. John, A., Lancashire, J. A. (1981) *Proc. N.Z. Grassl. Assoc.* **42**, 152.
27. Mangan, J. L. (1988) *Nutr. Res. Rev.* **1**, 209.
28. Hagerman, A. E. (1989) in *Chemistry and Significance of Condensed Tannins* (Hemingway, R. W., Karchesy, J. J. and Branham, S. J., eds), p. 323. Plenum Press, New York.
29. Asguith, T. N. and Butler, L. G. (1986) *Phytochemistry* **25**, 1591.
30. Seany, R. R. and Henson, P. R. (1970) *Adv. Agron.* **22**, 119.
31. Waghorn, G. C. and Shelton, I. D. (1995) *J. Agric. Sci. Cambridge* (in press).
32. Foo, L. Y. and Karchesy, J. J. (1989) *Phytochemistry* **28**, 1743.
33. Porter, L. J., Newman, R. H., Foo, L. Y., Wong, H. and Hemingway, R. W. (1982) *J. Chem. Soc., Perkin 1* 1217.
34. Porter, L. J., Foo, L. Y. and Furneaux, R. H. (1985) *Phytochemistry* **24**, 567.
35. Czochanska, Z., Foo, L. Y., Newman, R. H. and Porter, L. J. (1980) *J. Chem. Soc., Perkin Trans. 1* 2278.
36. Foo, L. Y. and Karchesy, J. J. (1989) *Phytochemistry* **28**, 3185.
37. Baek, N. I., Kennelly, E. J., Kardono, L. B. S., Tsauri, S., Padmawinata, K., Soejarto, D. D. and Kinghorn, A. D. (1994) *Phytochemistry* **36**, 513.
38. Thompson, R. S., Jacques, D., Haslam, E. and Tanner, R. J. N. (1972) *J. Chem. Soc., Perkin Trans. 1* 1387.