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Variation in antimicrobial action of proanthocyanidins from *Dorycnium rectum* against rumen bacteria

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Abstract

The proanthocyanidin polymer fractions of the leaves of the forage legume *Dorycnium rectum* were analysed by acid catalysis with benzyl mercaptan, NMR and ES–MS. The results showed that *D. rectum* differs from other temperate proanthocyanidin-containing forage legumes in that the range of polymers extends up to very high degrees of polymerisation. Three fractions were characterised as low, medium, and high molecular weight proanthocyanidin fractions with mean degree of polymerisations of 10.3, 41 and 127, respectively. Epigallocatechin was the most abundant extension unit and the terminating flavan-3-ols comprised largely catechin and gallocatechin units in equal proportions. Formation of thiolyated dimer products showed the interflavan-linkages of the lower molecular weight proanthocyanidins to be predominantly C4 → C8 with a small amount of C4 → C6. ES–MS spectra distinguished lower from higher polymeric proanthocyanidins from $M2^{-}$ to $M8^{2-}$. The antibacterial activity of proanthocyanidin fractions against pure cultures of microbes selected from the ruminal population to represent fibre degrading, proteolytic and hyper ammonia producing bacteria in broth culture was evaluated. The activity of proanthocyanidin fractions against *Clostridium aminophilum*, *Butyrivibrio fibrisolvens* and *Clostridium proteoclasticum* was significantly dependent on their structure but not so against *Ruminococcus albus* and *Peptostreptococcus anaerobius*. The latter observation was unique in that they were sensitive to all proanthocyanidin fractions evaluated, even at the lowest concentration (100 μg/ml). The results suggest the effects of the extractable proanthocyanidins on rumen microbes should be considered when evaluating an alternative proanthocyanidin-containing forage source for ruminants, such as *D. rectum*.

Keywords: *Dorycnium rectum*; Leguminosae; Proanthocyanidins; Thiolysis; Mass spectrometry; Rumen bacteria; *Ruminococcus albus*; *Butyrivibrio fibrisolvens*; *Clostridium proteoclasticum*; *Clostridium aminophilum*; *Peptostreptococcus anaerobius*; Inhibitory activity

1. Introduction

Dorycnium rectum has been evaluated in New Zealand as an alternative legume species (Oppong et al., 2001) for use in semi-arid environments as forage (Douglas and Foote, 1994) and for dryland pasture rejuvenation and resource conservation (Douglas and Foote, 1994; Wills et al., 1989). Forage produced by *Dorycnium* spp. in Australia can provide livestock with a source of nutrition in areas of low rainfall during late summer and autumn where there are regular periods of feed shortage from conventional pastures (Davies and Lane, 2003) or where soils with high levels of aluminium

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are found (Dear et al., 2003). This shrub has high concentrations of proanthocyanidins in the leaves, typically 15–20% of the dry matter (Waghorn and Molan, 2001). The effects of proanthocyanidins on rumen microbes should be considered when evaluating the introduction of an alternative forage, such as *D. rectum*, for ruminants. It has been suggested that based on their concentration and activity proanthocyanidins suppress forage digestion by inhibiting both microbial enzymes in the rumen and mammalian enzymes in the small intestine (Bae et al., 1993). The effect of proanthocyanidins on reducing fibre digestion is regarded as a secondary anti-nutritional effect compared with nitrogen digestion (McSweeney et al., 2001).

The potential value of some proanthocyanidins as an anthelmintic for ruminants (Waghorn and Molan, 2001) has previously been the focus of investigation by our group. *D. rectum* has consistently exhibited high anti-parasitic activity in field trials with sheep and in vitro studies (Niezen et al., 2002). Proanthocyanidins extracted from *D. rectum* were more effective in vitro at inhibiting the motility and development of ovine gastro-intestinal parasites (Molan et al., 2000, 2002) than proanthocyanidins from other forage legumes. The structure and mean degree of polymerisation (mDP) of proanthocyanidin plants and fractions used in these studies have not been determined and information on these could provide a better understanding of the chemical structure and activity relationship.

Earlier studies of proanthocyanidin polymer fractions of forage legumes (Foo et al., 1982), did not examine *D. rectum*. The proanthocyanidins of *Lotus* species (Foo et al., 1996, 1997), and sainfoin (*Onobrychis viciifolia*) (Koupai-Abyazani et al., 1993; Marais et al., 2000) were found to be varying mixtures of procyanidin (PC) and prodelphinidin (PD) units. The flavonoid composition of *D. rectum* has been reported (Moreno et al., 2002).

^{13}C NMR analysis of proanthocyanidin fractions provides information on the mDP, the procyanidin to prodelphinidin unit ratio of the mixture, and on the nature of terminal units in the proanthocyanidin fractions (Czochanska et al., 1980). The identification of the individual units that make up the proanthocyanidin fraction can be characterised by acid-catalysed cleavage of the polymer chains in the presence of benzyl mercaptan (Foo et al., 1982). The reaction results in the release of terminating units as free flavan-3-ols, whereas extender units are distinguished as thiolated adducts. From the reaction product ratios the overall composition of the polymer and the mDP can be estimated. Partial thiolysis in the presence of benzyl mercaptan with acetic acid lead to the formation of a series of thiol dimers. These were isolated by preparative-HPLC and characterised by negative ES–MS, NMR and subjected to further degradation to monomeric thiol adducts. These thiolated dimer products provide information on the interflavan-linkages of the polymer fraction. HPLC with negative ES–MS provides evidence of the procyanidin to prodelphinidin ratios and molecular dispersion for oligomeric proanthocyanidin components. In this report we describe the application of these methodologies to provide the first chemical characterisation of the structure of the proanthocyanidin oligomers and polymers of the leaves of the forage legume *D. rectum*.

Five strains of rumen bacteria (*Ruminococcus albus*, a fibre-degrader; *Butyrivibrio fibriosolvens* CF3 and *Clostridium proteoclasticum*, xylan and protein degraders; and *C. aminophilum*, *C. proteoclasticum*, and *Peptostreptococcus anaerobius*, ammonia hyper-producing bacteria) were grown in the presence of extractable proanthocyanidin fractions. These selected strains were used to investigate the effect of proanthocyanidins on microbial growth and to evaluate the relationship between the chemical structure (mDP) and possible selectivity in their antibacterial activity in pure cultures.

2. Results and discussion

While there has been considerable interest in *D. rectum* as an agronomic temperate forage legume, no study to date has investigated the chemical composition of the proanthocyanidin oligomers and polymers of *D. rectum*.

2.1. Extraction and chromatography

The established method (Foo and Porter, 1980) for the preparation of proanthocyanidin extracts from forage legumes involves extraction of frozen plant material with $Me_2CO–H_2O$ (7:3), partitioning with CH_2Cl_2, and chromatography of the remaining aq. phase on Sephadex LH-20. The proanthocyanidin-enriched fraction is usually obtained by elution of the Sephadex LH-20 column with $Me_2CO–H_2O$ (7:3) after first eluting with 50% aq. MeOH. This method was used in the present study. All fractions eluting from the LH-20 column were analysed by RP-HPLC with PDA detection, and an unexpected pattern of elution was found. Broad unresolved peaks with a UV maxima ca. 280 nm were observed in the chromatograms of both the 50% aq. MeOH and the $Me_2CO–H_2O$ (7:3) fractions suggesting the presence of proanthocyanidin material in both fractions. The $Me_2CO–H_2O$ (7:3) fraction was subsequently characterised by ^{13}C NMR and thiolytic cleavage as low molecular weight proanthocyanidin fraction (LMWPA). The 50% aq. MeOH fraction was subjected to re-chromatography on LH-20 with 50% aq. MeOH giving an initial fraction subsequently characterised as a high molecular weight proanthocyanidin fraction (HMWPA). This result suggests that the Sephadex LH-20 column was performing in a size exclusion manner for the HMWPA fraction which was not retained, and in the expected polarity based manner for the LMWPA fraction. A further proanthocyandin fraction was eluted with Me_2CO–H_2O (7:3) and characterised as a medium molecular weight proanthocyanidin fraction (MMWPA).

2.2. NMR analysis

Examination of the ^{13}C NMR spectra of the three proanthocyanidin fractions revealed qualitative and quantitative structural differences. The ^{13}C NMR of the LMWPA is consistent with a mixed prodelphinidin to procyanidin polymer (Fig. 1) with an approximate prodelphinidin to procyanidin ratio of 6:4 as estimated from the relative signal intensity of the B-ring carbons at 145 ppm (procyanidin) and at 146 ppm (prodelphinidin). The proanthocyanidins consist of predominately 2,3-*cis* extension units and is terminated with approximately equal amount of *cis* and *trans* units. Based on the relative signal intensity of the respective C3 chemical shifts of the extender (72.7 ppm) and terminal flavan-3-ol units (68.0 ppm) the LMWPA polymer has an average molecular weight of approximately 2500 Da. The ^{13}C NMR of the MMWPA is characteristic of a predominately prodelphidinin polymer with some procyanidin units, similar to that of the LMWPA, with an approximate prodelphinidin to procyanidin ratio of 7:3. The extender units consist of the 2,3-*cis* units (epicatechin and epigallocatechin) with small amounts of 2,3-*trans* units (catechin and gallocatechin). Very weak terminal carbon signals relative to the corresponding extender carbons indicated that this was a fairly large polymer with an average molecular weight of 3000 Da or higher. The HMWPA formed a gel-like consistency with the NMR solvent (d_6-acetone–D_2O). Consequently, the carbon signals were broad in spite of many hours of data acquisition. The polymer extender units are predominantly prodelphinidin with a small proportion of procyanidin units in an approximate prodelphinidin to procyanidin ratio of 8:2. Additional broad carbon signals in the heterocyclic shift region were also discernable, albeit much weaker than those of proanthocyanidin. This included a signal at 62.7 ppm which could be attributable to the C-6 of sugars, suggesting the sample was a proanthocyanidin glycoside. These extraneous peaks are analogous to those observed for *L. penduncul-atus* proanthocyanidin fractions where a small signal at 62.6 ppm consistent with the sugar methylene carbons (Foo et al., 1997). This observation was corroborated by the presence of other anomeric signals (101–102 ppm). The presence of a small degree of glycosylation observed in the proanthocyanidin polymer spectrum could not be confirmed by degradation with benzyl mercaptan. This may be due to a low level present in the fraction making their detection difficult. It may also be

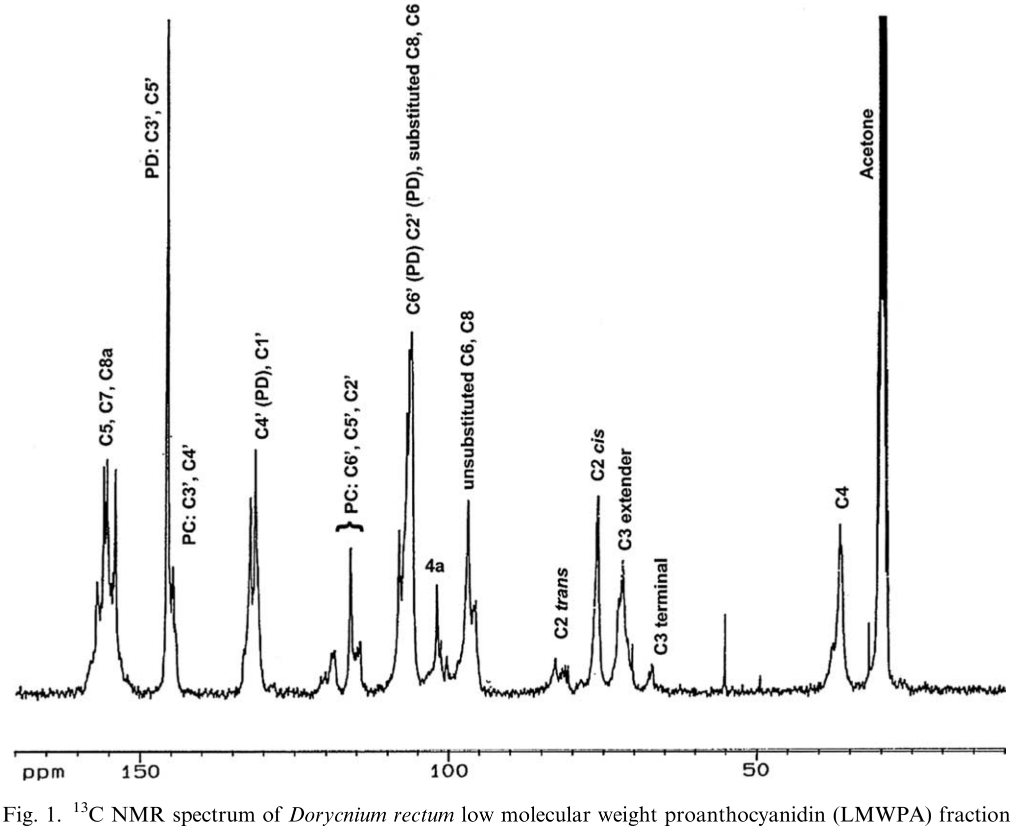

Fig. 1. ^{13}C NMR spectrum of *Dorycnium rectum* low molecular weight proanthocyanidin (LMWPA) fraction.

possible that these weak signals are due to the presence of a small amount of carbohydrate impurity. The signals observed in the ^{13}C NMR for the terminal flavan-3-ol carbons of the HMWPA were small and broad, which made more detailed analysis difficult. The presence of sugar carbon signals compounded this problem as they occur in the region of the heterocyclic carbons of the proanthocyanidin thereby precluding a meaningful estimation of mDP.

2.3. Thiolysis

The ^{13}C NMR data were in good agreement with the prodelphinidin to procyanidin ratios determined by RP-HPLC quantification from degradation of proanthocyanidin polymers in the presence of the nucleophilic reagent benzyl mercaptan (Czochanska et al., 1980). Thiolysis was performed independently for all three *D. rectum* proanthocyanidin fractions (Table 1) and quantification achieved through the use of authentic standards according to the method of Gu et al. (2002). The terminal units released as free flavan-3-ols were found to have catechol (procyanidin) and pyrogallol (prodelphinidin) B-rings in almost equal proportions. The polymer extender units released as flavan-3-ol thiol adducts showed a similar pattern in the LMWPA fraction (Fig. 2) with a higher proportion of prodelphinidin units (Table 1) consistent with the ^{13}C NMR data. By comparison with an authentic epigallocatechin benzyl ether standard, the thiolysis data indicate that the dominant extender units derive from epigallocatechin. The value (Table 1) obtained for the LMWPA (mDP 10.3) is consistent with the ^{13}C NMR prediction and similar to that observed for proanthocyanidins from other forage legumes (Foo et al., 1982). The very high mDP (127) found by thiolysis of the *D. rectum* HMWPA has not been reported for forage legumes.

The nature of the interflavanoid linkages in the LMWPA fraction were investigated using partial degradation and isolation of the dimer thiol products by RP-HPLC chromatography. Treatment of the LMWPA fraction with benzyl mercaptan in the presence of HOAc gave five dimer thiol fractions that contained three early-eluting homo-prodelphinidin thiol adducts (**1**, **2**, **3**; Fig. 3) *m/z* 731, one late-eluting hetero-prodelphinidin-procyanidin thiol adduct (**4**; Fig. 3) *m/z* 715, and a fraction which contained both homo-prodelphinidins and hetero-dimer thiol adducts *m/z* 731 and 715, which was not characterised further. Subsequent characterisation of the thiolytic products by 1D and 2D NMR indicate the interflavanoid linkages of the *D. rectum* oligomeric proanthocyanidins to be predominantly C4 → C8 (**1**, **3** and **4**) with a small amount of C4 → C6 (**2**) as reflected by the relative proportion of the respective dimeric thiol degradation products. The ^{13}C NMR spectrum of **1** was consistent with that of a prodelphinidin dimer thio adduct. However, the upfield position of the C-2b carbon (79.2 ppm) of the flavanol unit containing the thio substituent as compared to about 83 ppm observed for the corresponding carbon (Foo and Porter, 1980), suggests some steric or γ-gauche effect was present. This can only come about if the thio subsistent is in the same spatial orientation as the H2 proton suggesting that the thio adduct has the 3,4-*cis* configuration, and **1** is therefore epigallocatechin-(4β → 8)-gallocatechin-(4β → S) benzylthioether. However, confirmation of this assignment could not be made from the ^{1}H-NMR spectrum of **1** due to poor signal resolution for proton–proton couplings determined. Corroboration for the proposed structure was made by further thiolysis of **1** as discussed below. Apparently during cleavage reactions of proanthocyanidin oligomers the production of the major 3,4-*trans* adduct is often accompanied by the small amount of the corresponding 3,4-*cis* adduct (Foo and Karchesy, 1989; Kolodziej, 1990). The interflavanoid linkage of **2** was provisionally assigned as C4 → C6 by deduction as a result of the C4 → C8 linkage assignment made to **3**. The latter C4 → C8 assignment is based on the observed long range H, C correlation between H2 (bottom) and the linked carbon (106.3 ppm).

Table 1
Comparison of extender and terminal units of proanthocyanidin, mean degree of polymerisation (mDP) and average molecular weight (MW) for the low molecular weight proanthocyanidins (LMWPA), medium molecular weight proanthocyanidins (MMWPA), high molecular weight proanthocyanidins (HMWPA) in *Dorycnium rectum* leaves by thiolysis

	LMWPA		MMWPA		HMWPA	
	Terminal (%)	Extender (%)	Terminal (%)	Extender (%)	Terminal (%)	Extender (%)
Catechin	49	1	32	1	52	1
Epicatechin	5	12	1	4	0	4
Gallocatechin	40	15	66	10	46	8
Epigallocatechin	6	72	1	85	2	87
mDP	10.3		41		127	
MW	3300		12,200		35,600	
cis:*trans*	78:22		88:12		91:09	
PC:PD	17:83		6:94		5:95	

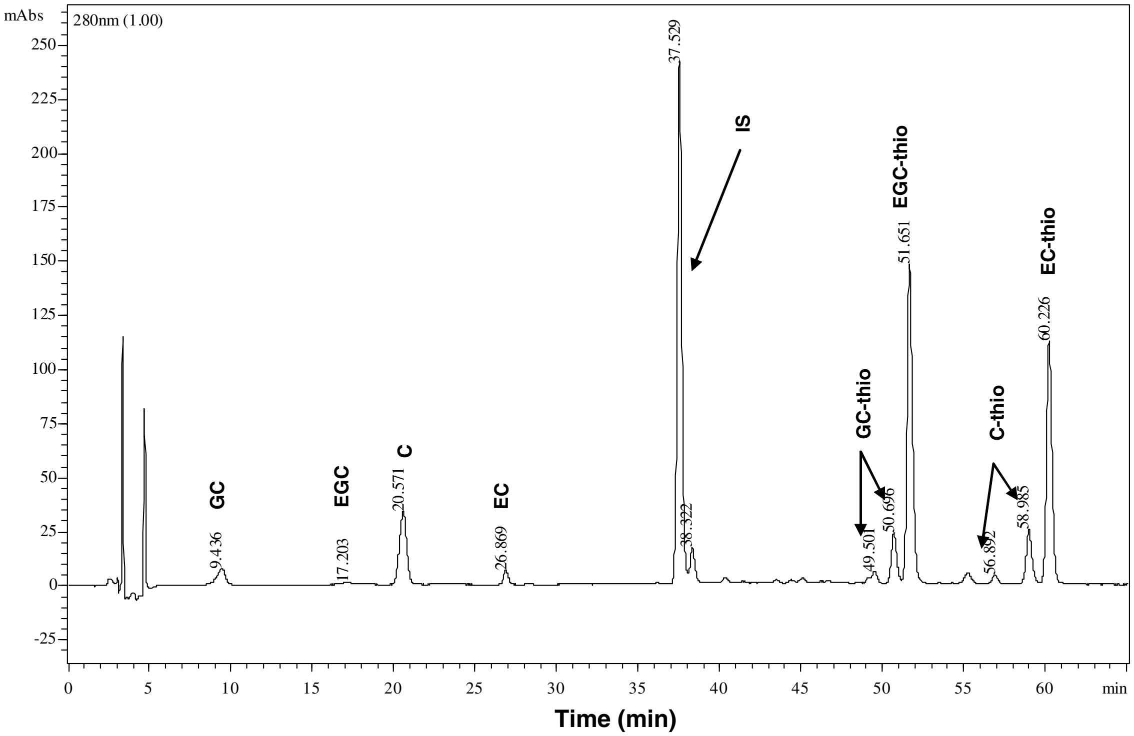

Fig. 2. Reversed phase HPLC chromatogram (absorbance at 280 nm) of thiolysis products from *Dorycnium rectum* low molecular weight proanthocyanidin (LMWPA) fraction. C, catechin; EC, epicatechin; GC, gallocatechin; EGC, epigallocatechin; IS, internal standard, dihydroquercetin; C-thio, catechin benzylthioether; EC-thio, epicatechin benzylthioether; GC-thio, gallocatechin benzylthioether; EGC-thio, epigallocatechin benzylthioether.

Further thiolysis of the isolated dimer thiol adducts (**1**, **2**, **3**, and **4**) gave two flavan thiol adducts, which were characterised by RP-HPLC. Three products were yielded upon full thiolysis of **1**, the pair of epimers of **5** (gallocatechin-(4α → S) benzylthioether and gallocatechin-(4β → S) benzylthioether) and **6**. This confirmed that **1** is epigallocatechin-(4β → 8)-gallocatechin-(4β → S) benzylthioether. Both **2** and **3** yielded **6** as the only thiolysis product confirming that these dimers are comprised of identical monomeric units but differing in their interflavanoid linkages. However, **3** is present in greater portions than **2**, in the LMWPA. Dimer **4** yielded two thiolysis products, **6** and **8**. No dimeric products containing **7** were isolated by partial thiolysis of the LMWPA, which was expected from the contribution to the extender unit composition of 1% (Table 1), but in contrast to the 49% contribution of the terminal units (Table 1).

2.4. ES–MS analysis

The oligomeric (LMWPA) fraction has been partially characterised by ES–MS in the negative ion mode using scan acquisition. For degrees of polymerisation (DP) up to 3 the singly charged ions predominate, from DP 4 to 8, the doubly charged species predominate, and at higher DP triply charged or higher multiple charges may predominate. It is thought that the longer chain length allows for multiple charges because of better charge distribution, minimizing electrostatic repulsive forces (Foo et al., 2000). Although the mass range is restricted by the limits of the quadrupole mass spectrometer (2000 amu), the practical upper limit is determined more by polymer mass and the broad chromatographic peaks for higher oligomers than by the mass limit, as the mass/charge ratio tends to compensate for this.

With the identification of molecular ions carrying a double charge (M^{2-}) we were able to interpret ES–MS spectra to distinguish oligomers ($M2^-$ to $M8^{2-}$) from polymeric proanthocyanidins. The mass spectra of the LMWPA (Fig. 4(a)) shows discrete peaks, but ions from the polymeric MMWPA and HMWPA were not detected. Thiolytic cleavage showed the LMWPA fraction comprised predominately of PD units (PD:PC 83:17; Table 1), and the ES–MS of this fraction reflected this pattern of composition with oligomers of high PD content dominant. A series of homo-oligomers were detected as the singly charged species *m*/*z* 609 $(PD)_2^-$, 913 $(PD)_3^-$, and the doubly charged species *m*/*z* 608 $(PD)_4^{2-}$, 760 $(PD)_5^{2-}$, 912 $(PD)_6^{2-}$, 1064 $(PD)_7^{2-}$, 1216 $(PD)_8^{2-}$ (Fig. 4(a)). Hetero-oligomers containing both PC and PD units (Fig. 4(a)) were detected as the singly charged species *m*/*z* 593 $(PC)(PD)^-$, 881 $(PC)_2(PD)^-$, 897 $(PC)(PD)_2^-$, and the doubly charged

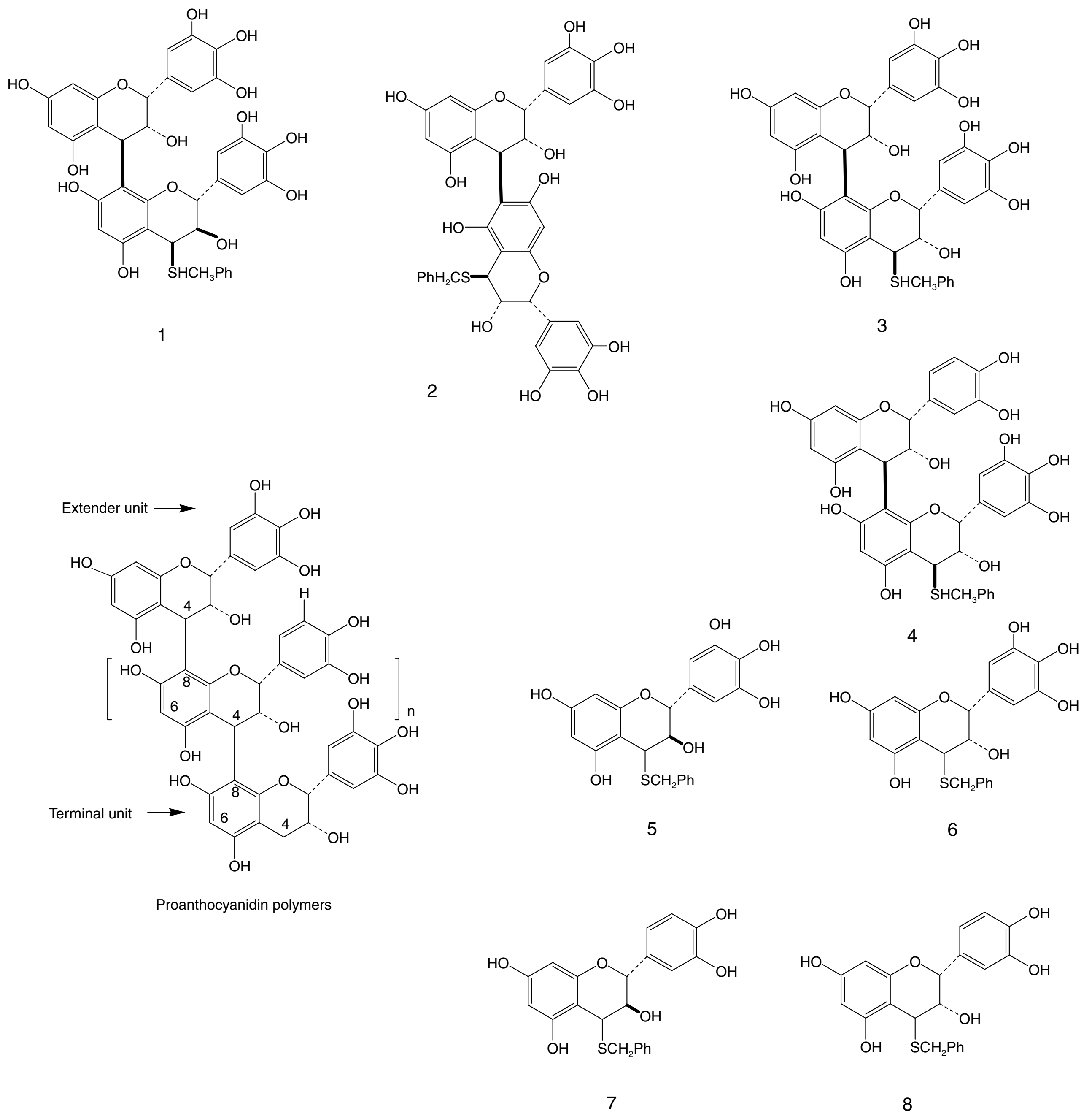

Fig. 3. Scheme showing dimer benzylthiolether products from partial thiolysis of *Dorycnium rectum*. Low molecular weight proanthocyanidin (LMWPA) fraction and their subsequent thiolysis cleavage products. Epigallocatechin-(4β → 8)-gallocatechin-(4β → S) benzylthioether (**1**); *epigallocatechin*-(4β → 6)-epigallocatechin-(4β → S) benzylthioether (**2**); epigallocatechin-(4β → 8)-epigallocatechin-(4β → S) benzylthioether (**3**); epicatechin-(4β → 8)-epigallocatechin-(4β → S) benzylthioether (**4**); gallocatechin benzylthioether (**5**); epigallocatechin benzylthioether (**6**); catechin benzylthioether (**7**); epicatechin benzylthioether (**8**).

species *m*/*z* 600 $(PC)(PD)_3^{2-}$, 744 $(PC)_2(PD)_3^{2-}$, 752 $(PC)(PD)_4^{2-}$ 1056 $(PC)(PD)_6^{2-}$, 1200 $(PC)_2(PD)_6^{2-}$. The PC homo-oligomers were detected as minor components both as singly charged species *m*/*z* 577 $(PC)_2^-$, 865 $(PC)_3^-$, 1153 $(PC)_4^-$, and doubly charged species *m*/*z* 720 $(PC)_5^{2-}$, 1008 $(PC)_6^{2-}$ were detected (Fig. 4(a)). The complete series of doubly charged pentamers ($DP5^{2-}$) (Fig. 4(b)) with C_{13} isotope ions are observed: the PC homo-oligomer *m*/*z* 720 $(PC)_5^{2-}$, all the possible hetero-oligomers *m*/*z* 728 $(PC)_4(PD)_1^{2-}$, 736 $(PC)_3(PD)_2^{2-}$, 744 $(PC)_2(PD)_3^{2-}$, 752 $(PC)(PD)_5^{2-}$, and the PD homo-oligomer *m*/*z* 760 $(PD)_5^{2-}$. The same patterns of ions are observed for dimers ($DP2^-$) through to octamers ($DP8^{2-}$) (Fig. 4(a)).

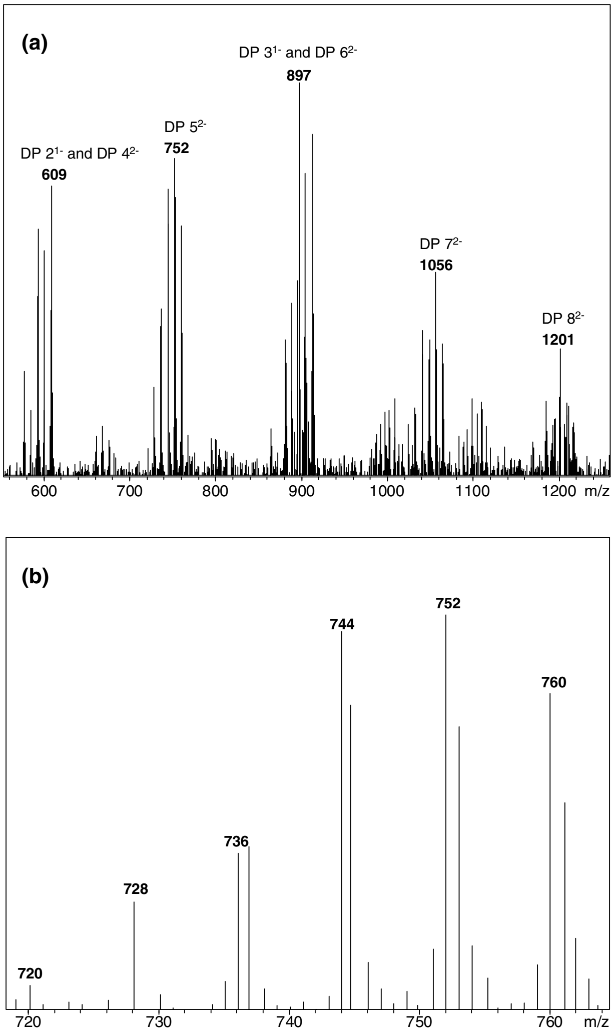

Fig. 4. Mass spectra of *Dorycnium rectum* low molecular weight proanthocyanidin (LMWPA) fraction with (a) dimer (DP2) to octamer (DP8) ions and (b) the doubly charged pentamer (DP5) series.

2.5. Antibacterial activities of proanthocyanidin fractions

The relationship between the mDP and the antibacterial activity of the extractable proanthocyanidin fractions of the leaves of the forage legume *D. rectum* was studied with a selection of rumen bacteria. In the absence of proanthocyanidins all bacterial strains grew and reached maximum optical density (OD 600 nm) after 6–24 h of incubation at 39 °C. The proanthocyanidins decreased the in vitro growth of all strains to a significant extent (Fig. 5; significant effects of concentration by ANOVA, $P < 0.001$). However, the rumen bacteria strains responded differently to the action of each proanthocyanidin fraction. *C. aminophilum* showed resistance to all three proanthocyanidin fractions (Fig. 5(a)) with significant effects of concentration ($P < 0.001$) and mDP (fraction $P < 0.005$ and fraction × concentration $P < 0.01$) and initiated growth even at the higher concentration (300 μg/ml). At 300 μg/ml the LMWPA and MMWPA fractions were significantly more effective ($P < 0.05$) at inhibiting the growth of *C. aminophilum* at 10 h than the HMWPA fraction. At 24 h the difference between the LMWPA and MMWPA fractions at the highest concentration (300 μg/ml) becomes significant ($P < 0.005$). The inhibitory activity of all three proanthocyanidin fractions against *C. proteoclasticum* (Fig. 5(b)) was evaluated at 12 h for the significance of concentration ($P < 0.001$) and mDP (fraction $P < 0.05$ and fraction × concentration $P < 0.005$) and also at 24 h for concentration ($P < 0.001$) and mDP (fraction $P < 0.05$) but the effect of fraction × concentration was no longer significant. At a concentration of 200 μg/ml the HMWPA fraction was significantly different to both LMWPA and MMWPA fractions ($P < 0.001$) at 12 h, but at 24 h ($P < 0.05$) on the growth of *C. proteoclasticum*. *R. albus* did not initiate any growth (Fig. 5(c)) in incubations containing 200 and 300 μg/ml of any of the three fractions. There was a significant effect of concentration ($P < 0.001$) and mDP (fraction ($P < 0.001$ at 10 h and $P < 0.01$ at 24 h) and fraction × concentration $P < 0.005$ at both 10 and 24 h). Growth in the presence of all three fractions reached the same maximum OD at 100 μg/ml, but the LMWPA fraction appeared to cause a longer lag phase at 10 h ($P < 0.001$) compared to the MMWPA and HMWPA fractions. The difference at 24 h between the control and 100 μg/ml concentration was non-significant for HMWPA, but significant for LMWPA and MMWPA ($P < 0.001$). There was a significant effect of concentration ($P < 0.001$) and mDP (fraction $P < 0.05$ and fraction × concentration $P < 0.05$) on the growth of *B. fibrisolvens* CF3. In incubations containing fractions at the higher concentrations (200 and 300 μg/ml), the growth of *B. fibrisolvens* CF3 (Fig. 5(d)) at 10 h was completely inhibited. At 100 ug/ml, LMWPA and MMWPA fractions were significantly ($P < 0.001$) more effective at inhibiting the growth of *B. fibrisolvens* CF3 than the HMWPA fraction. In fact, at 100 μg/ml the HMWPA fraction stimulated the growth of *B. fibrisolvens* CF3 during the first 6 h of incubation (Fig. 5(d)). *P. anaerobius* (Fig. 5(e)) was more sensitive to the presence of proanthocyanidins than were other bacteria and it did not initiate growth in media containing proanthocyanidin fractions even at low concentrations (100 μg/ml).

P. anaerobius was particularly sensitive to all proanthocyanidin fractions regardless of the mDP, and hence no differences in the growth pattern were detected. In assays of *C. aminophilum*, *C. proteoclasticum* and *B. fibriosolvens* CF3, the LMWPA fraction with a low mDP was more effective at decreasing bacterial growth than the HMWPA fraction. As all three fractions were predominately PD the differences in activity must be as-

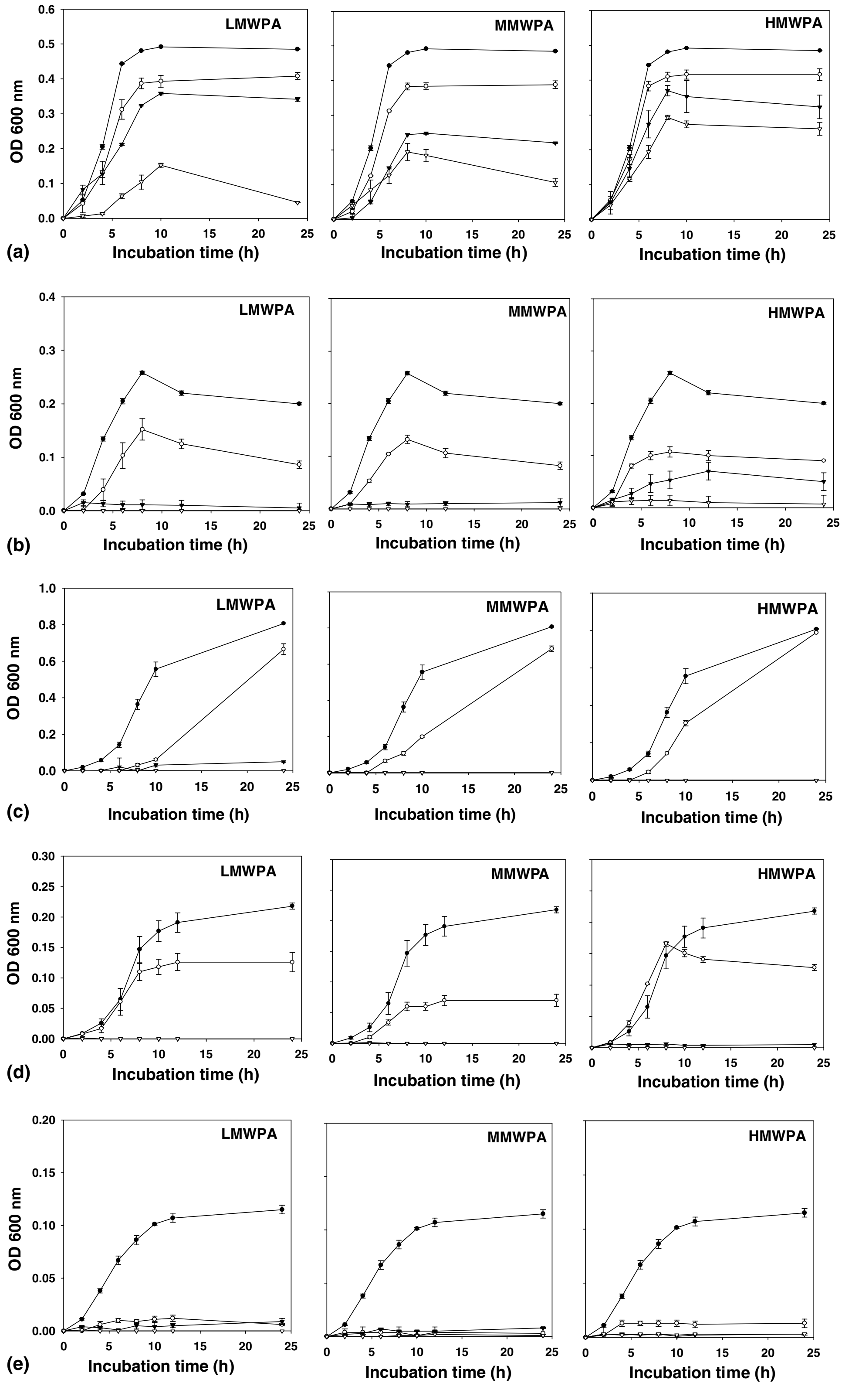

Fig. 5. In vitro activity of three proanthocyanidin fractions, low molecular weight proanthocyanidin (LMWPA) fraction, medium molecular weight proanthocyanidin (MMWPA) fraction and high molecular weight proanthocyanidin (HMWPA) fraction, from *Dorycnium rectum* leaves on the growth of the rumen bacteria (a) *Clostridium aminophilum*, (b) *Clostridium proteoclasticum*, (c) *Ruminococcus albus*, (d) *Butyrivibrio fibriosolvens* CF3, and (e) *Peptostreptococcus anaerobius* in the absence (●) and presence of 100 (○), 200 (▼), and 300 (∇) μg/ml. Values are the means of duplicate cultures and the vertical bars represent standard errors of the mean.

cribed to the polymer size (mDP) rather than polymer composition (PD:PC ratio). For the other bacteria, *R. albus* and *P. anaerobius*, there appears to be no molecular weight–activity correlation, only concentration dependence.

The effect of proanthocyanidins on rumen microbial ecology and rumen metabolism has been recently reviewed by McSweeney et al. (2001). In general, the inhibitory interactions between proanthocyanidins and rumen microorganisms and adaptive responses of the rumen microbes involved are poorly understood. The growth of proteolytic bacteria is known to be reduced by proanthocyanidins from the forage legume sainfoin (Jones et al., 1994), and from *L. corniculatus* and *L. pedunculatus* (Min et al., 2002, 2003; Molan et al., 2001), but some strains are tolerant (Jones et al., 1994; Molan et al., 2001).

Although *C. proteoclasticum* and *B. fibrisolvens* stain Gram −ve, they have Gram +ve-type cell walls. Hence the organisms in the present study are all Gram +ve bacteria and show general susceptibility to the three *D. rectum* proanthocyanidin fractions when tested in pure culture. However there was a considerable variation in response to the proanthocyanidin fractions, ranging from the very sensitive reaction shown by *R. albus* and *P. anaerobius* to the less sensitive responses of *C. aminophilum*, *C. proteoclasticum* and *B. fibrisolvens*. The effects of *D. rectum* proanthocyanidin fractions on co-cultures or mixed culture of rumen bacteria have yet to be determined.

This study has used NMR, thiolysis, and ES–MS data to provide a consistent view of the extractable proanthocyanidin fractions of *D. rectum*. They comprise a heterogenous range of polymeric species, including components of very high mDP. The terminal units are predominantly catechin and gallocatechin in approximately equal proportions. The extender units are predominantly epigallocatechin, and the higher mDP material has a higher PD content. The effects of the proanthocyanidin fractions on several rumen microbes are concentration dependent and in some cases linked to mDP.

3. Experimental

3.1. Plant material

Dorycnium rectum leaves were harvested from Aorangi, an AgResearch farm near Palmerston North, New Zealand, in autumn 2001, transferred to plastic bags and frozen at −20 °C. The material was authenticated by Garry Waghorn, Grasslands Research Centre, Palmerston North where a voucher specimen is deposited. Dry matter calculations were performed on fresh material oven dried at 105 °C for 12 h.

3.2. Extraction

Frozen *D. rectum* leaves (1.6 kg) were extracted with Me_2CO–H_2O (7:3; 3.2 L) containing ascorbic acid (1 g/L) in a blender (Hallde VCM62 Varning, AB Hallde Maskiner, Kista, Sweden) for 30 min and strained through two layers of cheesecloth to remove plant material. The extract was concentrated in vacuo (40 °C) to remove acetone and the aq. solution partitioned with CH_2Cl_2 (4×1 L). The aq. layer was concentrated in vacuo (40 °C), and subsequently freeze-dried to yield a crude proanthocyanidin extract (51.6 g). The recovery of extractable proanthocyanidins from *D. rectum* leaves on a dry matter basis was 16% for the total proanthocyanidin extract, of which 4.25% consisted of proanthocyanidin oligomeric fractions (3.1% HMWPA, 0.54% MMWPA and 0.60% LMWPA). By comparison, Terrill et al. (1992) reported an extractable proanthocyanidin content of 8.3% dry matter for *D. rectum*, as determined by the butanol–HCl assay.

3.3. Fractionation of proanthocyanidin polymers

Freeze-dried proanthocyanidin extract (12 g) was dissolved in 50% aq. MeOH (100 ml), filtered through a Buchner funnel (Whatman No. #40) and centrifuged at 1000*g* for 15 min to remove particulate material. The proanthocyanidin extract solution (100 ml) was applied to a Sephadex LH-20 column (Pharmacia, SK 25/100) packed in 50% aq. MeOH connected to a Pharmacia GradiFrac system. Fractions were eluted from the column at a flow rate of 5 ml/min with 50% aq. MeOH; four (200 ml) fractions were collected; I (7.7 g), II (277 mg), III (147.5 mg), IV (266.5 mg). Elution (5 ml/min) with Me_2CO–H_2O (7:3) yielded four proanthocyanidin (150 ml) fractions, V (72.7 mg), VI (341 mg), VII (26.4 mg), and VIII (trace). The fractions were monitored by RP-HPLC, concentrated in vacuo (40 °C), and then freeze-dried. Fractions V to VII were combined, as RP-HPLC-PDA chromatograms revealed a series of peaks characteristic of oligomers, to yield a low molecular weight proanthocyanidin (LMWPA) fraction (440 mg). Fraction I, (2.5 g in 25 ml 50% aq. MeOH) was concentrated and applied to a Sephadex LH-20 column. The column was eluted (5 ml/min) with 50% aq. MeOH, and sixty fractions (10 ml each) were collected. Fractions four to eight were combined to yield a high molecular weight proanthocyanidin, (HMWPA) fraction (753 mg). Subsequent fractions eluted with 50% aq. MeOH contained sugars and carbohydrates. Further, elution with Me_2CO–H_2O (7:3) resulted in a medium molecular weight proanthocyanidin (MMWPA) fraction (130 mg).

3.4. NMR analysis

^{13}C NMR spectra were recorded in D_2O:Me_2CO-d_6 (1:1 v/v) at 90 MHz using a Bruker 400 MHz instrument. The calculation of mDP was performed as described by Foo and Porter (1980).

3.5. Degradation with benzyl mercaptan

A method based on that described by Guyot et al. (1998) was utilised to perform the thiolysis. All proanthocyanidin samples were freeze-dried prior to weighing. Prior to the thiolysis reaction the HMWPA was further purified to remove any co-eluting sugars on a Strata C18-E SPE column by elution with 0.5% HCO_2H.

A methanolic proanthocyanidin solution of 4 mg/ml was prepared for each of the three proanthocyanidin fractions (LMWPA, MMWPA, HMWPA) as well as each of the four thiol dimer adducts (**1**, **2**, **3** and **4**). A sub-sample (50 μl) was placed in a vial and to this was added 3.3% conc. HCl in MeOH (50 μl) and 5% benzyl mercaptan in MeOH (100 μl). The solution was heated to 40 °C for 30 min in a heating block, and cooled to room temperature. As an internal standard, dihydroquercetin in H_2O, was added (100 μl of a 0.025 mg/ml solution), and the sample analysed immediately by RP-HPLC. Single samples were analysed for the four thiol dimer proanthocyanidins. Duplicate proanthocyanidin fractions from the LH-20 column were analysed. The values for mDP reported in Table 1 are the mean of thiolysis data for the fractions from two batches (LMWPA 9.5 and 11.1; MMWPA 40 and 42; and HMWPA 117 and 138).

Concentrations of terminal flavan-3-ol units and the extender flavan-3-ol thiol adduct units were estimated by integration of chromatographic peaks detected at 280 nm relative to that for dihydroquercetin as the internal standard. Responses relative to dihydroquercetin determined from standards were 0.30 for terminal procyanidin flavan-3-ol units and 0.26 for the procyanidin benzylthioethers, and 0.06 for terminal prodelphinidin flavan-3-ol units and prodelphinidin benzylthiolethers, in accordance with published values from Gu et al. (2002).

3.6. High pressure liquid-chromatography (HPLC)

Samples were analysed by elution of a 10 μl sub-sample on a 250 × 2.1 mm reversed-phase (C-18) Alltima column (Alltech) in a Shimadzu LC-MS QP8000 alpha equipped with a Shimadzu SPD-M10A VP PDA detector. The elution solvents were 0.1% HCO_2H in H_2O (A), and 0.1% HCO_2H in CH_3CN (B), with the following gradient: initial concentration 10% B, to 7 min; 18% B at 23 min; 23% B at 28 min; 41% B at 64 min; 95% B at 67 min; 95% B at 77 min; 10% B at 80 min; 10% B at 95 min, and a flow rate of 0.2 ml/min.

3.7. LC-MS

Mass spectra were collected on a Shimadzu LC-MS QP8000 alpha by electrospray-ionisation mass spectrometry in scan mode (*m/z* 250 to 1400) and detection in negative ion mode with the curved desolvation line at 250 °C, scan speed of 2000 amu/s, source voltage at −3.5 kV, detector voltage at 1.6 kV and a nebulising gas (N_2) flow rate of 4.5 L/min. The elution solvents were 0.1% HCO_2H in H_2O (A), and 0.1% HCO_2H in CH_3CN (B), with the following gradient: initial concentration 10% B, to 7 min; 18% B at 23 min; 23% B at 28 min; 31% B at 44 min; 80% B at 47 min, 80% B at 53 min; 10% B at 56 min; 10% B at 70 min, and a flow rate of 0.2 ml/min.

3.8. Partial thiolysis and purification of dimer thiol products

The LMWPA fraction was subjected to partial thiolysis with HOAc (1 ml) and benzyl mercaptan (1 ml), which were added to a screw top tube containing the proanthocyanidin sample (100 mg in 2 ml MeOH). Tubes (9 replicates) were sealed and placed in a heating block at 40 °C for 48 h. The combined reaction mixture was concentrated in vacuo. The residue was dissolved in water (25 ml) extracted with Et_2O (3 × 25 ml) to remove monomeric thiol adducts and flavanols which was discarded and the aq. layer freeze dried (606 mg). The thiol dimer adduct mixture was dissolved in 50% aq. MeOH (10 ml) loaded on a Sephadex LH-20 column (Pharmacia, SK 25/100) and eluted with a 50% aq. MeOH to 100% MeOH gradient and then Me_2CO–H_2O (7:3). The thiol dimer products were further purified by RP-HPLC on a Bio-Rad model AS-48 gradient module HPLC equipped with a Shimadzu SPD-M10A PDA detector, and connected to a Phenomenex reversed phase (C-18) Prodigy 5 μ column (250 × 10 mm) with a BioRad model 2128 fraction collector. The solvents used were 100% H_2O (A) and 100% MeOH (B), with the following gradient: initial concentration of 30% B, to 5 min; 50% B at 55 min; 100% B at 60 min; and a flow rate of 2.5 ml/min, with 400 μl injections (3.75×10^{-2} mg/ml) and 1 min fractions collected. Fractions were screened by RP-HPLC-PDA and combined to give 5 dimer thiol adduct fractions (**1**, 6.5 mg; **2**, 8.5 mg; **3**, 24.14 mg; mixed adducts, 10.6 mg; and **4**, 4.9 mg) with subsequent analyses by ^{13}C NMR (^{1}H, ^{13}C, HMBC and HMQC) and LC-MS as detailed above.

3.9. Epigallocatechin-(4β → 8)-gallocatechin-(4β → S) benzylthioether (**1**)

Freeze-dried solid (6.5 mg). ES–MS: [M − H]$^-$: *m/z* 731; RT: 40.86 min. ^{13}C NMR (75 MHz, D_2O–acetone-d_6) δ 36.4 (C-4t), 39.1 (C-SCH_2), 44.6 (C-4b), 72.3 (C-3t), 72.7 (C-3b),76.9 (C-2t), 79.2 (C-2b), 95.6–96.1 (C-6t, 8t, 6b), 97.3 (C-4at), 102.4 (C-4ab), 108 and 106.6–107.5 (C-2′t, 6′t, 2′b, 6′b), 108.1 (C-8b), 130–127.5 (benzene C-2-6), 131.6 (C-1′t, C-1′b), 132.7–133.4 (C-4′t, C-4′b), 140.1 (benzene C-1), 146–145.9 (C-3′t, 5′t, 3′b, 5′b), 157.6–155.2 (C-5t, 7t, 8at, 5b, 7b, 8ab). Thiolysis with HCl (as described above) yielded three products; gallocatechin-(4α/β-S) benzylthioethers (**5**), RT 49.6 and 50.8 min, epigallocatechin-(4β-S) benzylthioether (**6**), RT 51.8 min.

3.10. Epigallocatechin-(4β → 6)-epigallocatechin-(4β → S) benzylthioether (2)

Freeze-dried solid (8.5 mg). ES–MS: [M − H]$^-$: *m/z* 731; RT: 42.96 min; ^{13}C NMR (75 MHz, D_2O–acetone-d_6) δ 36.3 (C-4t), 37.3 (C-SCH_2), 43.5 (C-4b), 70.5 (C-3b), 72.1 (C-3t) 75.3 (C-2b), 76.8 (C-2t),), 97.8–95.6 (C-6t, 8t, 6b), 100.3 (C-4ab), 102.4 (C-4at), 106.6–106.3 (C-2′t, 6′t, 2′b, 6′b), 107.1 (C-8b), 129.8–127.7 (benzene C-2-6), 131.1 (C-1′t, C-1′b), 132.7–132.6 (C-4′t, C-4′b), 139.8 (benzene C-1), 146.2–146.1 (C-3′t, 5′t, 3′b, 5′b), 157.3–153.9 (C-5t, 7t, 8at, 5b, 7b, 8ab). Thiolysis with HCl (as described above) yielded one product; epigallocatechin-(4β-S) benzylthioether (**6**), RT 51.9 min.

3.11. Epigallocatechin-(4β → 8)-epigallocatechin(4β → S) benzylthioether (3)

Freeze-dried solid (24.14 mg). ES–MS: [M − H]$^-$: *m/z* 731; RT: 43.32 min. ^{13}C NMR (75 MHz, D_2O–acetone-d_6) δ 36.6 (C-4t), 37.3 (C-SCH_2), 43.6, (C-4b), 70.6 (C-3b), 72.8 (C-3t), 75.3 (C-2b), 76.4 (C-2t), 95.8–97.5 (C-6t, 8t), 96.4 (C-8b),100.4 (C-4at, 4ab), 106.6–106.3 (C-6b, 2′t, 6′t, 2′b, 6′b, 8b), 127.7–129.8 (benzene C-2-6), 131.4 (C-1′t, C-1′b), 132.7–132.8 (C-4′t, C-4′b), 139.8 (benzene C-1), 146.1–146.2 (C-3′t, 5′t, 3′b, 5′b), 156.6–157.5 (C-5t, 7t, 8at, 5b, 7b, 8ab). Thiolysis with HCl (as described above) yielded one product; epigallocatechin-(4β-S) benzylthioether (**6**), RT 51.9 min.

3.12. Epicatechin-(4β-8)-epigallocatechin-(4β→S) benzylthioether (4)

Freeze-dried solid (4.9 mg). ES–MS: [M-1]$^{-1}$ 715; RT: 49.28 min. ^{13}C NMR (75 MHz, D_2O–acetone-d_6) δ 36.8 (C-4t), 37.2 (C-SCH_2), 43.5, (C-4b), 70.7 (C-3b), 72.8 (C-3t), 75.4 (C-2b), 76.8 (C-2t), 95.8–97.5 (C-6t, 8t, C-6b),100.4 (C-4at, 4ab), 106.4 (C-2′b, 6′b, 8b), 115.1–115.5 (C-2′t, 5′t), 119.2 (6′t), 127.7–129.8 (benzene C-2-6), 131 (C-1′t), 132.2–132.7 (C-1′b, 4′b), 139.8 (benzene C-1), 145.2–146.2 (C-3′t, 3′b, 4′t, 5′b), 156.6–158.5 (C-5t, 7t, 8at, 5b, 7b, 8ab). Thiolysis with HCl (as described above) yielded two products; epigallocatechin-(4β-S) benzylthioether, RT 51.9 min (**6**) and epicatechin-(4β-S) benzylthioether (**8**), RT 60.5 min.

3.13. Rumen bacterial strains

The bacterial strains used in this study were obtained from the Rumen Microbiology Culture Collection, AgResearch Ltd., Palmerston North, New Zealand. The bacterial strains were *Clostridium aminophilum*, *C. proteoclasticum*, *Ruminococcus albus*, *Butyrivibrio fibriosolvens* CF3 and *Peptostreptococcus anaerobius*. Cultures of *C. aminophilum* were grown anaerobically in HAP medium (Attwood et al., 1998). Vitamins and cystein–HCl were added after autoclaving. Cultures of *R. albus* were grown anaerobically in medium 436 (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) while *C. proteoclasticum*, *B. fibriosolvens* CF3 and *P. anaerobius* were grown in L10 medium (Beard et al., 1995). Anaerobic conditions were obtained by boiling the media for 2 min and then sparging with CO_2 for 15 min in an ice bath. The preparation, distribution and inoculation of the media were carried out in an anaerobic chamber with a 95% CO_2/5% H_2 atmosphere (Coy Laboratory Products Inc., MI, USA).

3.14. In vitro incubations

A series of in vitro experiments were undertaken to determine the effect of three *D. rectum* proanthocyanidin fractions at three different concentrations on the growth of five strains of rumen bacteria. Stock solutions of the fractions were dissolved in CO_2-saturated distilled water and filter sterilised. Single bacterial strains were investigated in separate experiments. The three (fractions) of three (concentrations) plus control were analysed as a batch of 10 treatments in duplicate at 6 time points. The fractions were added to culture media in Hungate tubes to final concentrations of 100, 200 or 300 μg/ml immediately before the addition of the bacterial inocula, and all cultures were incubated at 39 °C. Cell growth was initiated with a 5% inoculum of a 18 h culture. Growth of the cultures was measured spectrophotometrically by measuring optical density at 600 nm against blanks of uninoculated media containing proanthocyanidins at 0, 100, 200 and 300 μg/ml. Initial absorbance values after inoculations (0 h) were subtracted from these readings to give adjusted optical density readings. Each tube was measured every 2 h (at 2, 4, 6, 8, 10 h) and then a final measurement at 24 h.

3.15. Statistical analyses

Univariate tests were used for analysis of the maximum growth time for proanthocyanidin fraction treatments all bacteria., The single time-point at which maximum recorded growth (OD) occurred, but before the growth curves flattened off or decreased, was analysed for *C. aminophilus* (10 h), *B. fibrisolvens* (10 h) and *P. anaerobius* (12 h). For *R. albus* and *C. proteoclasticum*, both this time point and the 24 h time point were analysed. The effects of concentration and fraction (mDP), both fitted as factors, were tested using ANOVA (proc GLM, SAS v8). Tubes were used as blocks when two time points were analysed (*R. albus* and *C. proteoclasticum*) (GenStat, v7). When necessary, the data were square-root transformed, to ensure that the variance was homogeneous over all treatments. *t* tests were used to compare pairs of treatments of particular interest.

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