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Floral Procyanidins of the Forage Legume Red Clover (*Trifolium pratense* L.)

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The chemical characteristics of the purified procyanidin polymers of the flowers of the forage legume red clover (*Trifolium pratense* L.) were studied by ^{13}C NMR, acid-catalyzed degradation with benzyl mercaptan, and electrospray ionization mass spectrometry (ESI-MS). The ^{13}C NMR showed that the fraction consisted of predominantly procyanidin polymers. The thiolysis reaction products indicated a mean degree of polymerization (mDP) of 9.3 with epicatechin (81%) as the abundant flavan-3-ol extension unit and the terminating units dominated by catechin (95%). ESI-MS showed a range of oligomeric procyanidin ions (DP of 2−11). The white clover floral prodelphinidins consist of terminal units with nearly equal proportions of epigallocatechin (52%) and gallocatechin (48%) and extender units showing epigallocatechin (56%) and gallocatechin (39%). The dramatic difference in the stereochemistry of the terminal and extender units observed for the red clover floral procyanidins contrasts with the mixture of *cis* and *trans* stereochemistry observed for white clover floral prodelphinidins.

KEYWORDS: Leguminosae; red clover flowers; *Trifolium pratense*; *Trifolium repens*; proanthocyanidins; procyanidins; prodelphinidins; catechin; ^{13}C NMR; thiolysis; electrospray mass spectrometry

INTRODUCTION

Red clover (*Trifolium pratense*) has been of phytochemical interest primarily for its isoflavonoid phytoestrogens. Phytoestrogens in herbage and their metabolites in the ruminant have deleterious effects on reproduction, particularly in sheep and to a limited extent in cattle. Pastures dominated by red clover have been shown to be highly oestrogenic to ewes (*1*). Formononetin, the main isoflavone present in red clover, is implicated in these reproductive problems; it is not oestrogenic itself but is metabolized mainly to equol in the rumen, and equol is oestrogenic. Forage legumes with low concentrations of phytoestrogens have been the long-term goal of plant breeders. Grasslands G27 has been bred from the tetraploid cultivar Grasslands Pawera with selection aimed at reducing the concentration of formononetin (*2*). However, recently, there has been new interest in red clover lines with high isoflavone phytoestrogen content for use as nutriceuticals as alternative compounds for hormone replacement therapy to treat menopausal disorders (*3*−*8*). For example, in recent studies, floral extracts from red clover (*4*) have shown significant competitive binding to the oestrogen β receptor.

Isoflavones have been the subject of several investigations; however, proanthocyanidins present in red clover flowers (*9*) and at trace concentrations in the leaves (*10*, *11*) have been detected and measured but have not previously been chemically characterized. There has been increased interest recently in proanthocyanidins both as nutraceuticals (*12*) and as beneficial factors in forage (*13*), and the availability of a new creeping low-formonetin cultivar of red clover, Grasslands Broadway (*14*), prompted an examination of the floral proanthocyanidins of red clover. In this study, the chemical composition of a proanthocyanidin fraction prepared from an aqueous acetone extract of red clover flowers has been examined by a range of techniques, including ^{13}C NMR, thiolytic degradation by acid-catalyzed cleavage with benzyl mercaptan, and electrospray ionization mass spectrometry (ESI-MS). Analysis of proanthocyanidin polymers by ^{13}C NMR provides information on the mean degree of polymerization (mDP), the procyanidin to prodelphinidin unit ratio, and the nature of terminal units (*15*, *16*). Thiolytic cleavage provides evidence of the identity of the individual units that make up the proanthocyanidin polymer (*15*, *17*). Negative ESI-MS provides evidence of the compositional dispersion for oligomeric (DP of 2−10) proanthocyanidin components (*18*). In this study, we have applied these methodologies to characterize the chemical structure of the procyanidin oligomers and polymers of the flowers of the forage legume red clover. These data are compared with those for the

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floral proanthocyanidins of the major forage legume white clover (*Trifolium repens* L.), including earlier ^{13}C NMR and ESI-MS data (*19*), and new data on their products of thiolytic cleavage.

MATERIALS AND METHODS

Chemicals. AnalaR acetone and dichloromethane and high-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and ascorbic acid were obtained from BDH, New Zealand Ltd. Catechin, epicatechin, gallocatechin, epigallocatechin, and dihydroquercetin were obtained from Sigma, St. Louis, MO. Benzyl mercaptan was obtained from Merck, Darmstadt, Germany. Sephadex LH-20 gel was obtained from Pharmacia, Sweden.

Plant Material. Grasslands Broadway red clover (*14*) was sown in autumn in sand frames at Grasslands Research Centre, Palmerston North, New Zealand. The flowers were harvested in spring 2002, transferred to plastic bags, and frozen at −20 °C.

Extraction and Isolation. Frozen flowers (360 g) were extracted with acetone:water (7:3; 500 mL) containing ascorbic acid (1 g/L) in a Hallde VCM62 Varning blender (AB Hallde Maskiner, Kista, Sweden) for 30 min and strained through cheesecloth to remove plant material. The extract was concentrated in vacuo (40 °C) to remove acetone, and the aqueous solution was partitioned with dichloromethane (2 × 250 mL). The aqueous layer was concentrated in vacuo and subsequently freeze-dried to yield 24 g of crude proanthocyanidin extract (PAE).

Purification of Procyanidins. Freeze-dried PAE (6 g) was dissolved in 50% aqueous methanol (50 mL), filtered through a Buchner funnel using Whatman no. 40 filter paper, and centrifuged at 1000*g* for 15 min. The PAE solution was applied to a SR 25/45 Sephadex LH-20 column (Pharmacia) packed in 50% aqueous methanol connected to a Pharmacia GradiFrac system. Three fractions were eluted from the column with 50% aqueous methanol (1 L) at a flow rate of 7 mL/min; I (3.6 g), II (8 mg), and III (17 mg). Elution (7 mL/min) with acetone: water (7:3; 800 mL) yielded a proanthocyanidin fraction IV (102 mg). The fractions were concentrated in vacuo (40 °C) and freeze-dried.

White Clover Prodelphinidins. The polymer used for the thiolysis reaction was prepared as described previously by Foo et al. (*19*).

NMR Analysis. ^{13}C NMR spectra were recorded in methanol (CD_3OD) at 90 MHz using a Bruker 400 MHz instrument.

Thiolysis of the Proanthocyanidins. A method based on that described by Guyot (*20*) was utilized to perform the thiolysis. The proanthocyanidin sample [fraction IV for red clover and a similar 70% acetone fraction from LH-20 for white clover as described (*19*)] was freeze-dried and dried over P_2O_5 prior to weighting. A solution (4 mg/mL in methanol) was prepared, and a subsample (50 μL) was placed in a vial and to this was added 3.3% hydrochloric acid in methanol (50 μL) and 5% benzyl mercaptan in methanol (100 μL). The solution was heated to 40 °C for 30 min in a heating block and cooled to room temperature. An internal standard (IS), dihydroquercetin in water, was added (100 μL, 5.2×10^{-2} mg/mL solution), and the sample was analyzed immediately by reversed phase (RP)-HPLC.

RP-HPLC Analyses of Thiolysis Products. Samples were analyzed by elution of a 20 μL subsample on a 250 mm × 2.1 mm RP (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% formic acid in water (A) and 0.1% formic acid in acetonitrile (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. 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 IS. 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. (*21*).

HPLC ESI-MS Analyses of Procyanidin Oligomers. Mass spectra were collected on a Shimadzu LC-MS QP8000 alpha by ESI-MS in scan mode (*m*/*z* 250−1400) 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 nebulizing gas (N_2) flow rate of 4.5 L/min using the same chromatography conditions as outlined above for the RP-HPLC analysis of the thiolysis products.

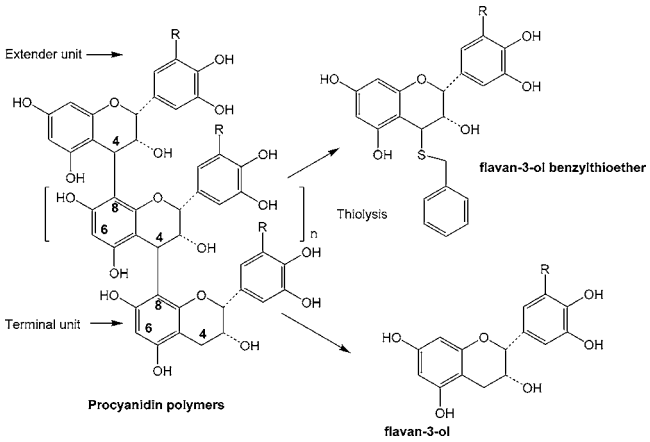

Figure 1. Thiolysis reaction of proanthocyanidin polymers consisting of procyanidin units. The *trans* stereochemistry is associated with catechin flavan-3-ol units, while *cis* stereochemistry is associated with epicatechin flavan-3-ol units. All of the terminal units in the polymer were released as flavan-3-ols, and the extender units were released as flavan-3-ol benzylthioethers.

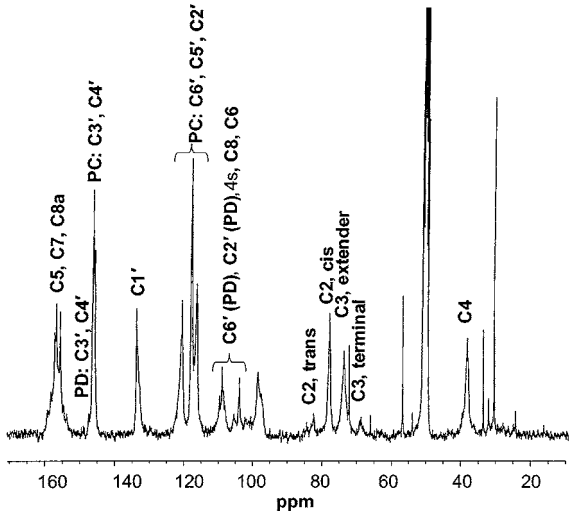

Figure 2. ^{13}C NMR spectrum of red clover floral procyanidins.

RESULTS AND DISCUSSION

^{13}C NMR Analyses of Procyanidins. Analysis of ^{13}C NMR spectra of the proanthocyanidin polymers provides valuable information on the mDP, the oxidation pattern of the B-ring of extender units (procyanidin to prodelphinidin unit ratio), and the stereochemistry of the monomers in the polymer (*15*). The proanthocyanidin LH-20 fraction from red clover flowers was determined to be predominantly of the procyanidin type (**Figure 1**). The ^{13}C NMR spectrum (**Figure 2**) shows a distinct signal at 145 ppm, which is assignable to the C3′ and C4′ carbons of the catechol B-ring of procyanidin units (catechin/epicatechin) with additional diagnostic corresponding signals at 116 (C2′), 117 (C5′), and 120 ppm (C6′). The corresponding B-ring carbon signals for the prodelphinidin units are represented by the weaker C2′ and C6′ signals at around 108−110 ppm (**Figure 2**) (*15*). However, this signal is complicated by the presence of the C6/C8 substituted phloroglucinol A-ring carbons. The region between 70 and 90 ppm is diagnostic for the 2,3-stereochemistry

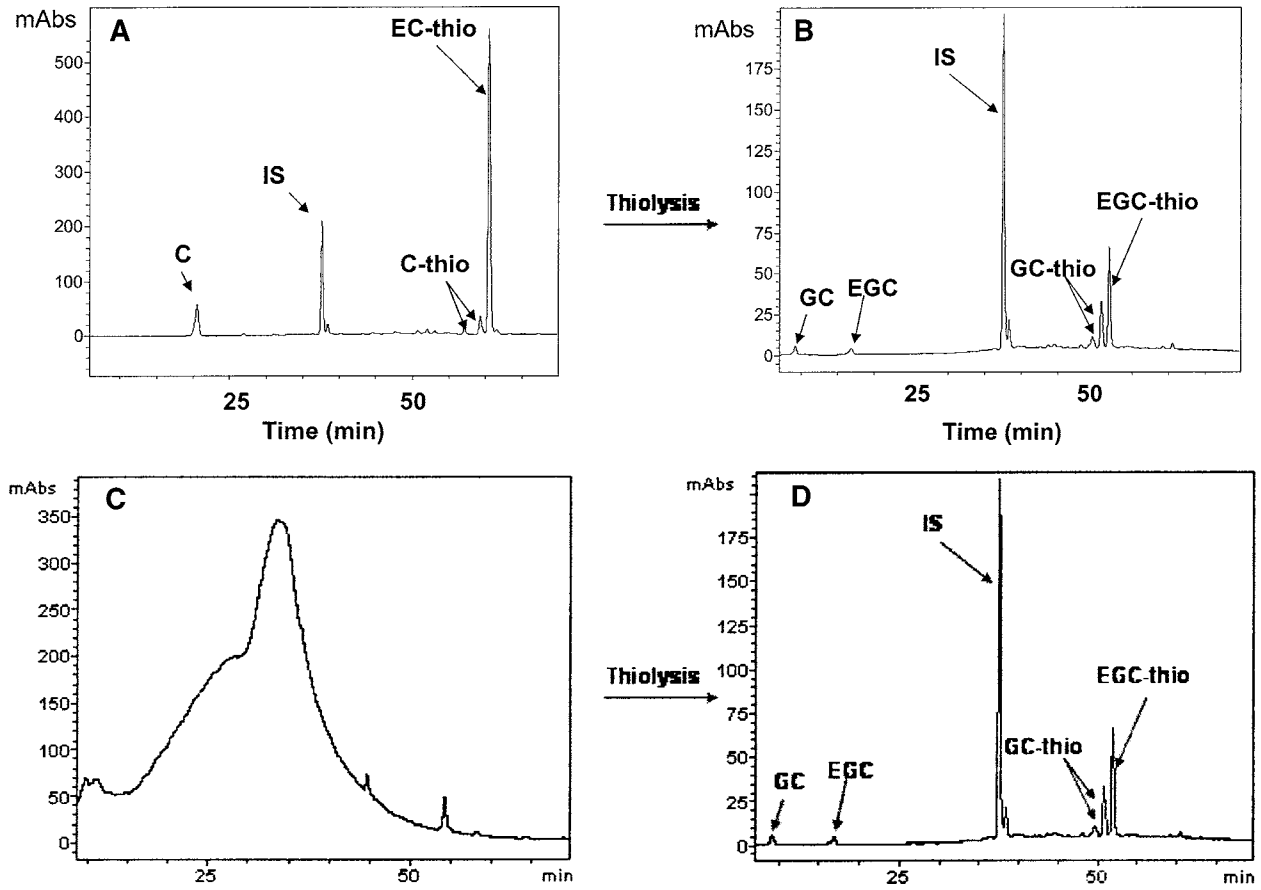

Figure 3. RP-HPLC chromatogram detected at 280 nm for (**A**) red clover procyanidin polymers after degradation by thiolysis and (**B**) white clover prodelphidinin polymers after degradation by thiolysis. Terminal units: catechin (C), gallocatechin (GC), and epigallocatechin (EGC); extender units: catechin (C-thio), epicatechin (EC-thio), gallocatechin (GC-thio), and epigallocatechin (EGC-thio) benzylthioethers; IS, dihydroquercetin.

Table 1. Thiolysis Reaction Products for Red Clover and White Clover Flower LH-20 Fractions as % Contributions of Terminal and Extender Flavan-3-ol Units (GC, Gallocatechin; EGC, Epigallocatechin; C, Catechin; and EC, Epicatechin)

		% terminal units				% extender units			
species	mDP	GC	EGC	C	EC	GC	EGC	C	EC
red clover	9.3	0	0	95	5	6	7	6	81
white clover	10.3	48	52	0	0	39	56	4	1

of the C-ring. While the signal for the extender C3 of both cis and trans isomers occurs at 73 ppm, the corresponding C2 signals for the cis and trans forms are well-resolved with the former observed at 77 ppm and the latter at 84 ppm. The relatively large signal of the 77 ppm peak as compared to that of the 84 ppm provides a clear indication of the preponderance of the cis stereochemistry among the extending units. The C2 carbon chemical shifts for the terminal flavanol units are also distinctive with the cis occurring at about 79 ppm while the trans is a little downfield at 81−82 ppm. However, in the spectrum only, the 81−82 ppm signal can be observed, indicating that the proanthocyanidin polymers are terminated almost exclusively by catechin rather than epicatechin units. In contrast, both the cis and the trans C3 terminal units generally appear upfield at around 68 ppm (*16*). From the intensity of this terminal signal relative to that of the extending C3 at 73 ppm, the mDP could be estimated to be around 6−7 DP. Similar procyanidin type polymers have previously been characterized by ^{13}C NMR from a variety of trees including *Pinus* species (*22*), the leaves of willow (*Salix alba*), and lime (*Tilia cordata*) (*23*).

Thiolysis of Procyanidins. The mean composition and mDP of proanthocyanidins can be determined by strong acid total thiolysis and chromatography by HPLC (HPLC-PDA-UV). The mean composition of the terminal units can be determined from the ratio of the released monomers, the mean composition of the extender units in the polymer chain from the ratio of benzylthioether adducts, and the mDP from the ratio of monomer to extender units (*24*). Thiolytic degradation of proanthocyanidins has been found to provide good yields of cleavage products (*15*, *20*, *23*−*26*), with low levels of product degradation and epimerization (*24*). The subunit composition of the red clover LH-20 fraction was estimated by chromatography (HPLC-PDA) of the thiolysis reaction products (**Table 1**). The chromatograms (monitored at 280 nm; **Figure 3A**,**B**) shows the terminal units released as monomers (eluting before 30 min) and the extender units as benzylthioether adducts (**Figure 1**) (eluting after 45 min). The response factors relative to the IS by PDA-UV detection at 280 nm were 0.30 for the flavan-3-ols catechin and epicatechin and 0.26 for their corresponding benzylthioethers. The catechin and gallocatechin benzylthioethers appear as two peaks in the chromatograms (**Figure 3A**,**B**) resulting from RP-HPLC separation of the pair of epimers. The same pattern is not observed for epicatechin and epigallocatechin benzylthioethers with very little formation of epimers during thiolysis (*17*). The thiolysis data show the red clover floral proanthocyanidins to be a predominantly procyanidin polymer mixture with a mDP of 9.3, a value that

is slightly higher than that estimated by ^{13}C NMR. Such variation is not unexpected as the thiolysis reaction is not known to yield quantitative products. The terminal units were found to consist predominately of catechin (95%), with a minor epicatechin component (5%), which is consistent with the ^{13}C NMR data. The cleavage products of the extender units are dominated by epicatechin benzylthioether (81%), and components of catechin, gallocatechin, and epigallocatechin benzylthioethers (6%) were also detected. Thus, the predominant cis stereochemistry (epicatechin) of the extender units differs from that of the predominantly trans stereochemistry (catechin) of the terminal units (**Table 1**).

Procyanidins from a variety of plant species have been characterized by thiolysis, including grape seeds (DP of 2−15) (*26*), apple (DP of 2−190) (*25*), cocoa (DP of 14), brown sorghum bran (DP of 14), lowbush blueberry (DP of 39), and cranberry (DP of 15) (*21*). Red clover flowers are comparable in their flavan-3-ol composition to brown sorghum bran, which is dominated by catechin (88%) terminal units and epicatechin extension units (*21*).

For comparison, an LH-20 proanthocyanidin fraction from white clover flowers was examined by thiolysis. The response factors by PDA-UV detection at 280 nm were 0.06 for the flavan-3-ols gallocatechin and epigallocatechin and for their corresponding benzylthioethers (*17*). The white clover floral proanthocyanidins were shown to comprise largely prodelphinidins (**Figure 3B**) with terminal units consisting of nearly equal proportions of epigallocatechin (52%) and gallocatechin (48%) and extender units showing greater proportions of epigallocatechin benzylthioether (56%) than gallocatechin benzylthioether (39%) (**Table 1**). These data are in good agreement with the earlier ^{13}C NMR analysis (*19*). Thus, in contrast to the red clover floral procyanidins, the extender and terminal units have similar proportions of the 2,3-cis stereoisomers (epigallocatechin), and the stereochemical composition of the terminal groups does not differ greatly from that of the extender units.

ESI-MS of Procyanidin Oligomers. Mass spectrometry can provide useful information on the composition of proanthocyanidin oligomer mixtures in the form of ion masses corresponding to sets of oligomers of the same DP and composition (*18*). Procyanidins in cocoa (*27*) and blueberry (*21*) have been identified using normal-phase HPLC-ESI-MS. RP-HPLC-ESI-MS in the negative ion mode has been used to characterize procyanidins in cider apple skin and pulp (*18*) and the leaves and flowers of *Crataegus* spp. (*28*) and procyanidin gallates in grape products (*29*).

ESI-MS of the red clover floral procyanidin LH-20 fraction was carried out in negative ion mode under weakly acidic conditions. Ions from a series of oligomers of DP of 2−11 were detected (**Figure 4**), broadly consistent with the mDP estimated by thiolysis of 9.3 and by ^{13}C NMR to be around 6−7. The components were predominantly homooligomers comprising procyanidin units only, consistent with the mean compositions determined by thiolysis (**Table 1**).

A wide molecular weight range of ions was observed from (singly charged) dimers to (doubly charged) heptamers and (triply charged) undecamers. In each mass range, the dominant species observed was derived from procyanidin homooligomers, namely, singly charged dimer and trimer species (*m*/*z* 577 and 865), doubly charged tetramer to nonamer species (*m*/*z* 576, 720, 864, 1008, 1152, and 1296), and triply charged decamer and undecamer species (*m*/*z* 960 and 1056) (**Figure 4**). All of the major signals were consistent with molecular ion masses of oligomers of the procyanidin type.

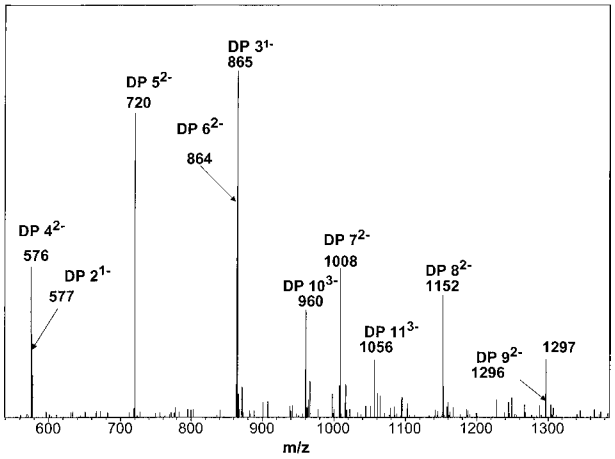

Figure 4. ESI-MS spectrum of procyanidin oligomers from red clover flowers in negative ion mode with dimer to undecamer ions present (DP range of 2–11 by ESI-MS and mDP of 9.3 and 6–7 determined by thiolysis and ^{13}C NMR, respectively).

Structure, Stereochemistry, and Biosynthesis. The NMR, thiolysis, and ESI-MS data thus provide a consistent view of the procyanidins of red clover flowers. They comprise a homogeneous range of polymeric species, where the terminal units are dominated by catechin and the extender units are predominantly epicatechin. The relationship between these structural features and the biological activity of the procyanidin polymers of red clover flowers is the subject of continuing study.

The stereochemical findings are of interest in the light of recently reported discoveries in flavan-3-ol biosynthesis. The dramatic difference in the predominant 2,3-trans and cis stereochemistry of the terminal and extender units observed for the red clover floral proanthocyanidins has also been observed for brown sorghum bran proanthocyanidins (*21*). This is in contrast to the white clover floral proanthocyanidins, for which both extender and terminal units show a similar mixture of cis and trans (prodelphinidin) stereochemistry and the proanthocyanidins of the model plant *Arabidopsis thaliana* for which both terminal and extender units have recently been shown to be of exclusively cis (procyanidin) stereochemistry (*30*).

Recent reports (*31*, *32*) have established that the flavan-3-ol stereoisomers catechin and epicatechin are the products of different biosynthetic conversion processes from the common precursor leucocyanidin. However, the molecular species involved in chain extension and the determinants of their stereochemistry remain a matter of conjecture (*31*, *33*). The pattern of observations of proanthocyanidin structures observed here and elsewhere suggests that there is no intrinsic linkage between the stereochemistry of the polymer extension units and that of the terminal units and that their biosynthesis is independently regulated.

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