Polymeric Proanthocyanidins. Stereochemistry, Structural Units, and Molecular Weight

By **Zofia Czochanska, Lai Yeap Foo, Roger H. Newman,** and **Lawrence J. Porter,*** Chemistry Division, Department of Scientific and Industrial Research, Petone, New Zealand

Homogeneous polymeric proanthocyanidins have been isolated from 22 plant sources and all are based on a C(4)–C(8) [or C(6)] linked polyflavan-3-ol structure. ^{13}C N.m.r. spectroscopy in $^{2}[H_6]$acetone–water is used to calculate the ratio of procyanidin to prodelphinidin monomer units, the average heterocyclic ring stereochemistry of the monomers, and the ratio of monomers to chain-terminating units. The majority of polymers isolated in this study possess monomers with predominantly a 2,3-*cis* stereochemistry [the same configuration as (−)-epicatechin]. The number-average molecular weight, calculated from monomer to terminal unit ratios, of the polymers is 1 500—5 000. The structure of the chain-terminating group is established by thiolysis degradation and g.l.c. analysis of the products.

RECENT studies have completely elucidated the structure of dimeric procyanidins,[1,2] and the existence of analogous prodelphinidin dimers has also been demonstrated.[3] However, the lower-molecular-weight proanthocyanidins are normally present in plant tissue in relatively low concentrations and the bulk of naturally occurring proanthocyanidins are oligomers and perhaps polymers. Moreover, astringency and tanning properties are associated with the higher-molecular-weight proanthocyanidins [4-6] and elucidation of their structure is therefore of importance.

The postulate that polymeric proanthocyanidins consist of linear chains of flavan-3-ol units (1) followed close on the correct proposal for the basic structure and mode of linkage of procyanidin dimers by Freudenberg and Weinges,[7] although similar structures had been proposed earlier for profisetinidin wattle tannins by Roux.[8] Prior to this other workers [9-11] had shown that the great majority of proanthocyanidins consist of procyanidins and prodelphinidins. This view has been corroborated by later work.[12-14]

Until now there has been no definitive evidence to support a structure such as (1) for intact proanthocyanidin polymers. However, such a structure seemed reasonable on the basis of the monomeric degradation products isolated from the cleavage of crude polymer preparations by thiol reagents [15-17] or by phloroglucinol [1,3,17] in dilute acid medium. Addition products (2)—(5) are formed in high yield by reaction between polymers and the latter reagent.[1,3,17]

RESULTS AND DISCUSSION

Isolation and Purification.—The method used to extract the plant material was as described previously.[3] The data reported in our earlier communication [18] were for polymers fractionated on Sephadex G-50 in acetone–water (1 : 1 v/v) by gel filtration.[19] However, this procedure has been replaced by purification on Sephadex LH-20 by adsorption chromatography [19] which yields superior preparations as judged by ^{13}C n.m.r., chiroptical, and microanalytical data. The polymers were eluted from Sephadex LH-20 with acetone–water (7 : 3 v/v) which leads to fractionation of some polymers. For example, further elution with acetone–water (1 : 1 v/v) displaces further proanthocyanidin in the case of *Cydonia*, *Ribes sanguineum* leaf, and *Vaccinium* polymers, but none (or very little) in other cases. This fractionation may be according to molecular size,[20] but could involve other factors. This matter will not be pursued further in this paper.

A still unsolved problem is the separation of polymeric proanthocyanidins from hydrolysable tannins. These commonly co-occur; *e.g.* in almost equal proportions in rose hips and strawberries, or with hydrolysable tannins preponderating in *Rubus* species.[20] The presence of hydrolysable tannins in freeze-dried polymer preparations may be deduced from a carbonyl absorption band in the i.r.[21] or ^{13}C n.m.r. spectra due to gallate or hexahydroxybiphenyl ester moieties.† All polymers described in this study lack a carbonyl function.

Criteria of Purity.—Anhydrous polymer preparations gave microanalyses consistent with a polyflavan-3-ol structure.

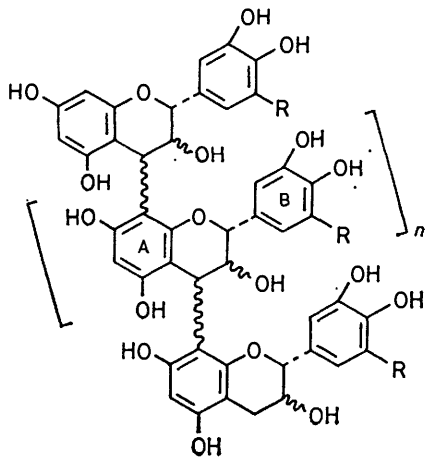
Combustion analysis also shows that freeze-dried polymer preparations contain 2.5—3 mol water of hydration per mol of monomer unit, corresponding to a 13—15% weight loss on drying.‡ All data in this paper are for freeze-dried preparations unless stated to the contrary.

The freeze-dried polymer preparations give a value of $E^{1\%}_{1cm} = 260$—280 at 500 n.m. for the vanillin addition product in HCl. This is similar to the value reported by Broadhurst and Jones,[22] and is a rapid and convenient method for estimating the purity of polymer preparations. Only those preparations attaining a minimum $E^{1\%}_{1cm}$ value of 260 were used in this study.

Structural Elucidation.—There are four criteria required to define the gross structure of a proantho-

† The situation is confused somewhat by 3-*O*-gallate esters of (—)-epicatechin and (—)-epigallocatechin occurring in such plants as *Camellia sinensis* and *Vitis vinifera*. The former compound was among thiolysis degradation products from the proanthocyanidin polymer from *Vitis* var. Siebel.[20]

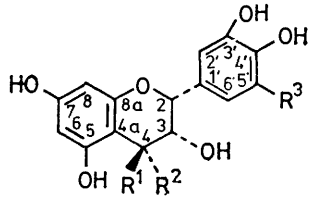
‡ The water of hydration may be removed by prolonged drying at 1 mmHg over P_2O_5 (See Experimental section).



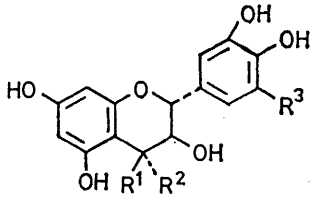
(1)

(a); R = H, procyanidin units (PC)

(b); R = OH, prodelphinidin units (PD)



(2), (3), (12), (13), (16), (17)



(4), (5), (8), (9), (10), (11), (14), (15)

(2) R^1 = 2,4,6-trihydroxyphenyl, $R^2 = R^3 = H$

(3) R^1 = 2,4,6-trihydroxyphenyl, $R^2 = H$, $R^3 = OH$

(4) $R^1 = H$, R^2 = 2,4,6-trihydroxyphenyl, $R^3 = H$

(5) $R^1 = H$, R^2 = 2,4,6-trihydroxyphenyl, $R^3 = OH$

(8) $R^1 = SCH_2Ph$, $R^2 = R^3 = H$

(9) $R^1 = R^3 = H$, $R^2 = SCH_2Ph$

(10) $R^1 = SCH_2Ph$, $R^2 = H$, $R^3 = OH$

(11) $R^1 = H$, $R^2 = SCH_2Ph$, $R^3 = OH$

(12) $R^1 = SCH_2Ph$, $R^2 = R^3 = H$

(13) $R^1 = SCH_2Ph$, $R^2 = H$, $R^3 = OH$

(14) $R^1 = R^2 = R^3 = H$, (+)-catechin

(15) $R^1 = R^2 = H$, $R^3 = OH$, (+)-gallocatechin

(16) $R^1 = R^2 = R^3 = H$, (-)-epicatechin

(17) $R^1 = R^2 = H$, $R^3 = OH$, (-)-epigallocatechin

cyanidin polymer: (*i*) The ratio of procyanidin [PC (1a)] to prodelphidin [PD (1b)] units: (*ii*) the stereochemistry of the heterocyclic ring of the monomer units: (*iii*) the structure(s) of the chain-terminating [17] flavan-3-ol unit: and (*iv*) the number-average molecular weight (M_n). All these data except (*iii*) may be deduced from the ^{13}C n.m.r. spectra of the polymers.

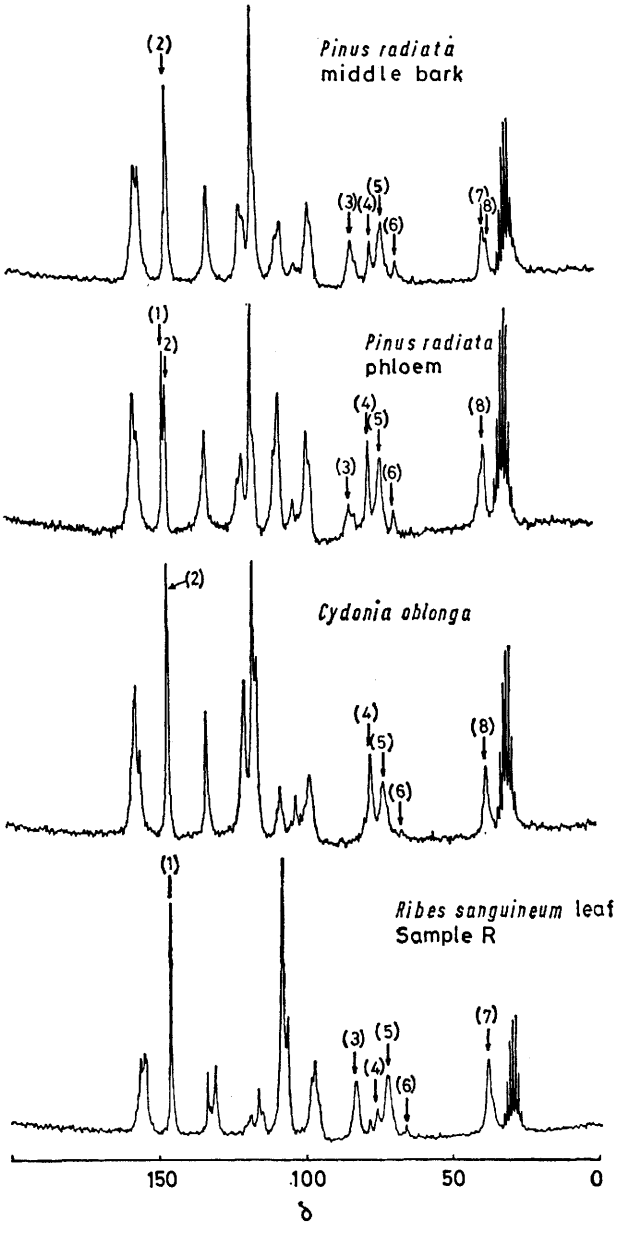


FIGURE 1 ^{13}C N.m.r. spectra of proanthocyanidin polymers from various sources: (1), C-3′ and -5′ signals of PD units; (2), C-3′ and -4′ signals of PC units; (3), C-2 of *trans*-monomer units; (4), C-2 of *cis*-monomer units; (5), C-3 of *cis*- and/or *trans*-monomer units; (6), C-3 of terminal units; (7), C-4 of *trans*-monomer units; (8), C-4 of *cis*-monomer units

Proportions of PC and PD Units.—The ratio PC : PD may be measured from the products of either of two degradation reactions, *i.e.* heating a polymer with butan-1-ol/HCl,[23] chromatographic separation of the

cyanidin and delphinidin chlorides, and estimation of their relative concentrations by visible absorption spectroscopy (method A) or degradation of a polymer with phenylmethanethiol and acetic acid in ethanol [13] and estimation of the relative concentrations of PC and PD units by g.l.c. of the trimethyl silyl (TMS) ethers [24,25] of the 4-sulphides (method B). Method B is the more accurate of the two methods, mainly due to uncertainties arising from elution of products subsequent to paper chromatography, prior to spectrophotometric analysis in method A.

The same information may be obtained from the ^{13}C n.m.r. spectra of the polymers in [$^2H_6$]acetone–water. Figure 1 shows the spectra of four typical polymers. Those of *Pinus* (middle bark) and *Cydonia* are examples of almost homogeneous PC polymers, that of *Ribes sanguineum* leaf an almost homogeneous PD polymer, and that of *Pinus* phloem of a polymer containing PC and PD units in about equal proportions. Assignments for the ^{13}C n.m.r. chemical shifts encountered in the polymers are given in Figure 1 and Table 1, together with those for appropriate model compounds. The assignments were based on arguments fully elaborated elsewhere,[1,26,27] and using data from fully proton-coupled and inversion-recovery spectra where necessary.

In particular, the signals arising from the B-ring of the monomer units are indicated appropriately (see Figure 1). The signals near δ 145 which arise from quaternary C-3′ and C-4′ for a PC unit, and C-3′ and C-5′ for a PD unit, may be used to estimate the PC : PD ratio. Independent experiments, described in our earlier communication for D_2O–H_2O solutions,[18] and to be described in detail for [$^2H_6$]acetone–H_2O solutions elsewhere,[28] show that these signals have identical T_1 and η(n.O.e.) values and the PC : PD ratio may be obtained from direct integration of the signals. Peak heights may not be used, as the C-3′ and C-4′ signals of a PC unit are not coincident [see assignments for the model compound (2) where they are resolved].

The PC : PD for the various polymers, estimated by the three methods, are given in Table 2. It may be seen that the values are self-consistent. The occurrence of a pure PC or PD polymer is apparently relatively rare. Of the *ca.* 50 polymers studied in our laboratory so far, only three pure PC (and no pure PD) polymers * have been isolated.

TABLE 1

^{13}C N.m.r. chemical shifts of proanthocyanidin polymers and model compounds (δ values; $SiMe_4$ external standard, corrected for magnetic susceptibilities)

	C-2	C-3	C-4	C-4a	C-5	C-6	C-7	C-8	C-8a	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
(+)-catechin (14)	82.0	67.9	28.1	100.9	156.9	96.7	156.7	95.7	156.3	131.5	115.6	145.5	145.5	116.6	120.3
(−)-epicatechin (16)	79.1	66.8	28.6	100.3	157.1	96.8	156.7	96.0	156.7	131.8	115.4	145.0	145.2	116.4	119.6
(−)-epigallocatechin(17)	78.6	66.1	28.1	100.6	156.0	96.9	155.8	96.2	155.8	131.4	107.5	145.8	132.7	145.8	107.5
(2)	76.6	72.6	36.5	102.0	*ca.* 157	96.4	*ca.* 157	95.7	*ca.* 157	132.2	115.5	144.9	145.2	116.4	119.6
(5)	83.8	73.0	37.9	107.0	*ca.* 157	97.8	*ca.* 157	96.4	*ca.* 157	131.1	108.7	146.2	133.9	146.2	108.7
Cydonia oblonga polymer [a]	77	72	37	102	*ca.* 156	98	*ca.* 156	107	*ca.* 156	132	115	145	145	116	119
Grevillea rosmarinifolia polymer [b]	77	73	37	102	*ca.* 156	97	*ca.* 156	107	*ca.* 156	132	108	146	133	146	108
Ribes sanguineum polymer [c]	84	73	38	*ca.* 107	*ca.* 157	98	*ca.* 157	107	*ca.* 157	133	108	146	134	146	108

[a] A PC polymer with 2,3-*cis*-units. [b] A PD polymer with 2,3-*cis*-units. [c] A PD polymer with 2,3-*trans*-units.

Stereochemistry of the Monomer Units.—The PC and PD units have either of two heterocyclic ring stereochemistries: 2*R*,3*R*,4*R* (6), designated *cis* units; or 2*R*,3*S*,4*S* (7), designated *trans* units.† The ^{13}C chemical shift data in Table 1 illustrates that C-2 for a *cis* unit is at δ 77, well separated from C-2 for a *trans* unit at δ 84.[1]

TABLE 2

Ratio of procyanidin to prodelphinidin units for some proanthocyanidin polymers

	PC : PD		
	Acid degradation	Thiolysis	^{13}C N.m.r.
Actinidia chinensis [a]	90 : 10	95 : 5	88 : 12
Aesculus x *carnea* [b]	94 : 6	100 : 0	100 : 0
Aesculus hippocastanum [b]	96 : 4	100 : 0	100 : 0
Betula alba [b]	88 : 12	90 : 10	84 : 16
Cyathea dealbata [a]	59 : 41	62 : 38	60 : 40
Cydonia oblonga [b]	94 : 6	100 : 0	100 : 0
Grevillea robusta [a]	45 : 55	38 : 62	39 : 61
Grevillea rosmarinifolia [a]	30 : 70	20 : 80	18 : 82
Lotus pedunculatus [a]	23 : 77	20 : 80	18 : 82
Lotus pedunculatus [c]	38 : 62	29 : 71	23 : 77
Onobrychis viciifolia [a]	19 : 81	18 : 82	23 : 77
Photinia cv.[a,d]	97 : 3	100 : 0	100 : 0
Pinus radiata [e]	50 : 50	51 : 49	52 : 48
Pinus radiata [f]	90 : 10	94 : 6	90 : 10
Ribes nigrum [a]	32 : 68	39 : 61	37 : 63
Ribes nigrum [b]	6 : 94	4 : 96	6 : 94
Ribes rubrum [a]	9 : 91	11 : 89	8 : 92
Ribes sanguineum [b]	23 : 77	13 : 87	12 : 88
Ribes sanguineum [a]	13 : 87	10 : 90	9 : 91
Salix fragilis [a]	97 : 3	97 : 3	90 : 10
Vaccinium corymbosum [b]	100 : 0	100 : 0	100 : 0
Vitis vinifera [b,g]	80 : 20	82 : 18	87 : 13

[a] Leaf. [b] Fruit. [c] Root. [d] Full name *Photinia glabrescens* cv. *rubra* × *P. serrulata*. [e] Phloem. [f] Middle bark. [g] Cv. Gamay Beaujolais.

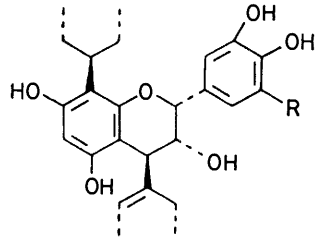
† The configuration at C-4 changes from 4*S* for a *cis*-unit, and from 4*R* for a *trans*-unit, in the model compounds (2) or (3) and (4) or (5), respectively, to the opposite configuration for a monomer unit in a polymer chain, because of the change in order of preference of the ligands. For the purpose of application of the sequence rules [29] a polymer chain may be considered to be an extended dimer, regardless of chain length. Interestingly, C-4 is in a symmetrical situation locally.

* White clover flowers are reported to contain a pure PD polymer.[19]

Measurements of T_1 and η for these resonances [18,28] on the *Pinus* phloem polymer spectrum (Figure 1), which contains both stereochemistries, shows that signals may be integrated directly to obtain average *cis* : *trans* ratios for monomers in a polymer. Data obtained in this way is listed as X, the mole-fraction of *cis* units, in Table 3.

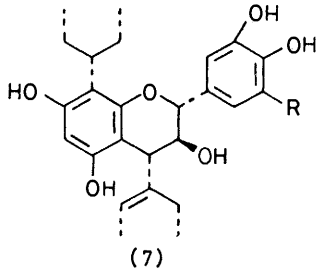
The view proposed in our earlier communication [18] that (2)—(5) are precise models for the average chiroptical properties of monomer units in a polymer chain, is optimistic. This became evident when improved ^{13}C n.m.r. spectra, resulting from narrower line widths obtained by using [$^2H_6$]acetone–H_2O rather than D_2O–H_2O as n.m.r. solvent, showed that the assumption [18] that the *Ribes sanguineum* leaf polymer contains all-*trans* units is wrong. The expanded heterocyclic ring-carbon region, δ 60—90, of the spectrum of this polymer, Figure 2, shows clearly the presence of the *cis*-isomer, represented by a small, but clearly defined resonance at δ 77.

Reinvestigation by ^{13}C n.m.r. of the original preparation of this polymer, designated here as *R. sanguineum* leaf polymer, Sample Q, showed that it contained no less than 17% *cis*-units. However, even at this level the *cis*-C-2 signal was obliterated by the wings of the C-3 signal, which is very broad in D_2O–H_2O, the



(6)

2,3 - *cis* - units



(7)

2,3 - *trans* - units

solvent used in our earlier communication.[18] In fact the *R. sanguineum* polymer displayed variable ratios of *cis* and *trans* units in the three samples isolated. Figure 2 illustrates the extreme situations encountered, of 20% *cis* in Sample R and 12% in Sample P. Whether the variability is genetic or seasonal is unknown at this stage.

The concept [18] that $[\phi]_{578}^{20}$ values for proanthocyanidin polymers obey a simple additive relationship is still valid, however. Salvadori and Ciardelli [30] have shown that, for a polymer with a high enough molecular weight to ensure independence of rotatory power and chain

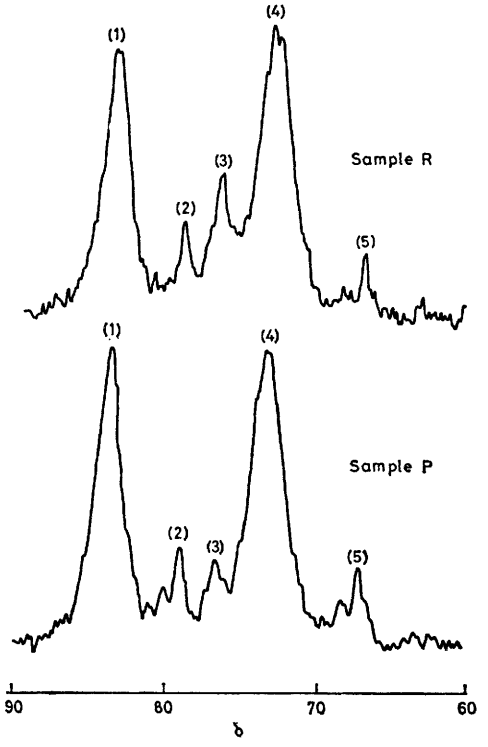


FIGURE 2 ^{13}C N.m.r. spectra of the heterocyclic ring region of *Ribes sanguineum* leaf polymers: (1), C-2 of *trans*-monomer unit; (2), C-2 of terminal unit; (3), C-2 of *cis*-monomer unit; (4), C-3 of *cis*- and *trans*-monomer units; (5), C-3 of terminal unit

length, the observed rotatory power is the average of all rotatory powers of the N species in the system, from which may be deduced equation (E1).

$$[\phi]_\lambda^T = \sum_i^N X_i[\phi]_i \qquad \text{(E1)}$$

where X_i is the mole fraction of the ith species and $[\phi] = [\alpha]^T \times M_i/100$, where M_i is the molecular weight of the repeating unit.

For proanthocyanidin polymers, $N = 2$ as only *cis* and *trans* units are possible, and M_i is effectively the same for all units as the molecular weight of PC and PD differ by only 5%. This reduces the relationship to $[\alpha]_{578}^{20}$ (polymer) $= X[\alpha]_{578}^{20}$ (*cis*) $- (1 - X)[\alpha]_{578}^{20}$ (*trans*) where X is the mole-fraction of *cis* units, and the specific rotations are for a pure *cis*- or *trans*-unit polymer, the negative sign arising from the negative specific rotation of the *trans*-units.[18] The values of $[\alpha]_{578}^{20}$ and X (Table 2) obey the expected simple linear relation, the equation of least-squares best fit being equation (E2).

$$[\alpha]_{578}^{20} \text{ (polymer)} = 174X - 320\,(1 - X) \qquad \text{(E2)}$$

Values of X calculated directly from equation (E2) are also summarised in Table 3. The fit is good except for those polymers which were judged by ^{13}C n.m.r. to contain only *cis*-units, *i.e.* $X = 1.0$. However, this disagreement is not surprising as the n.m.r. method would be expected to be unreliable at low concentrations of *trans*-isomer, due to signal-to-noise factors (for a fuller discussion see the section on molecular-weight determinations). The implications of equation (E2) were corroborated by thiobenzylation degradation experiments. In this reaction a PC *trans*-monomer-unit forms two products [(8) and (9)] with the thiobenzyl group having a quasi-axial and quasi-equatorial orientation at C-4, respectively.[13] These are formed in a ratio of 2 : 1 [confirmed independently by degradation of model compound (4)]. A PD *trans*-unit forms a similar pair of products [(10) and (11)]. On the other hand *cis*-PC or -PD units form only one product [(12) or (13)] with a quasi-axial thiobenzyl substituent.[13] The relative retention times of the TMS ethers of the minor *trans*-products [(9) and (11)] are much shorter than those of any of the other thiobenzylation products. Therefore, small amounts of *trans*-units are readily detected by g.l.c. in the presence of predominantly *cis*-units, and the relative amount can be estimated. Results from predominantly *cis*-polymers, obtained from g.l.c. data, are given in Table 3 and generally agree with values obtained from equation (E2). The g.l.c. method is less reliable for polymers containing high proportions of *trans*-units because of the lack of a satisfactory degree of separation between the quasi-axial products.

The specific rotations were all obtained on freeze-dried samples and the true specific rotation must be calculated with allowance for the water of hydration. As discussed earlier the polymers contain 13—15% water, which implies a value of about +200° for all-*cis*-polymer. Redetermination of $[\alpha]_{578}^{20}$ for the *Actinidia* polymer, after drying to constant weight over P_2O_5, increased $[\alpha]_{578}^{20}$ from +161 to +183°, close to the value theoretically predicted (+186°) from the 13% weight loss on drying of the polymer. Other polymers gave similar results, yielding rotations close to that predicted from weight loss on drying.

Equation (E2) gives values for the molar rotation of monomer units in a polymer, allowing for water of hydration, of $[\phi]_{cis} = ca.$ +610 and $[\phi]_{trans} = ca.$ −1 120°. Both these values are higher than those measured for the pure model compounds (2) and (5), which yield values of $[\phi]_{cis} = +516$ and $[\phi]_{trans} = -897°$. The values for the molar rotations of the model compounds are higher than those given previously[18] as we were able, subsequently, to prepare compounds of high optical purity by chromatography on a reversed-phase h.p.l.c. column, following Sephadex LH-20 chromatography. The model compounds (2) and (5) were judged to be at least 99% sterically homogeneous by ^{13}C n.m.r.

Although the model compounds possess molar rotations of the same order of magnitude as the monomer units in the polymer, they are not the completely accurate models we suggested earlier.[18] The difference between the molar rotations of the models and the poly-

TABLE 3

Relative proportion of monomer units with *cis*-stereochemistry in some proanthocyanidin polymers

Polymer	$[\alpha]_{578}^{20}$ [a]	Concentration [b]	^{13}C n.m.r.	X [c] Calculated	Thiolysis
Actinidia chinensis (leaf)	+161	0.33	1.0	0.97	0.95
Aesculus × *carnea* (fruit)	+141	1.00	0.96	0.93	0.91
Aesculus hippocastanum (fruit)	+162	0.49	1.0	0.97	0.96
Betula alba (fruit)	+84	0.50	0.81	0.82	0.81
Cyathea dealbata (leaf)	+155	0.29	1.0	0.96	
Cydonia oblonga (fruit)	+151	0.29	0.96	0.95	0.96
Grevillea robusta (leaf)	+35	0.31	0.67	0.72	0.77
Grevillea rosmarinifolia (leaf)	+123	0.34	0.90	0.90	0.90
Lotus pedunculatus (leaf)	+85	0.23	0.75	0.80	
Lotus pedunculatus (root	+43	0.70	0.72	0.73	0.76
Onobrychis viciifolia (leaf)	+112	0.23	0.88	0.87	
Photinia var. (leaf) [d]	+161	0.27	1.0	0.97	0.98
Pinus radiata (phloem)	+59	0.41	0.76	0.77	
Pinus radiata (middle bark)	−113	0.57	0.43	0.42	
Ribes nigrum (fruit)	+52	0.26	0.76	0.75	
Ribes nigrum (leaf)	−258	0.27	0.14	0.13	
Ribes rubrum (leaf)	+137	0.27	0.90	0.92	0.90
Ribes sanguineum (fruit)	+38	0.25	0.72	0.72	
Ribes sanguineum (leaf) [e]	−266	0.49	0.12	0.11	
Ribes sanguineum (leaf) [f]	−230	0.34	0.17	0.18	
Ribes sanguineum (leaf) [g]	−216	0.32	0.20	0.21	
Salix fragilis (leaf)	+111	0.30	0.90	0.87	0.87
Vaccinium corymbosum (fruit)	+144	0.18	1.0	0.94	0.95
Vitis vinifera (fruit)	+134	0.29	0.91	0.92	
A.h.: R.s = 0.545 : 0.455 [h]	−32	0.49	0.59	0.58	
A.h.: R.s = 0.175 : 0.825 [h]	−176	0.46	0.27	0.29	

[a] Shown to be constant over the concentration range used by independent experiments. [b] In water, g per 100 ml. [c] Mole fraction of *cis*-monomer. [d] *Photinia glabrescens* var. *rubra* × *P. serrulata*. [e] Sample P. [f] Sample Q. [g] Sample R. [h] Weight proportions of the two polymers *Aesculus hippocastanum* (A.h.) and *Ribes sanguineum* (R.s.), sample P.

mers is not surprising as the symmetries of the aromatic chromophores bonded to the chiral centre at C-4 in a polymer, whose absolute configuration largely determines the shape of the o.r.d. curve,[31] are quite different from those in the model compounds. The rotatory strength of transitions associated with aromatic chromophores is largely controlled by their oscillator strength, and hence the substitution pattern on the aromatic rings.[32]

Ultraviolet Spectra.—The polymer u.v. spectra in water consist of maxima at λ 205 (^1B band), around 240 (shoulder, ^1L_a band), and 270—280 n.m. (^1L_b band) (Figure 3). The observed spectral bands arise from the

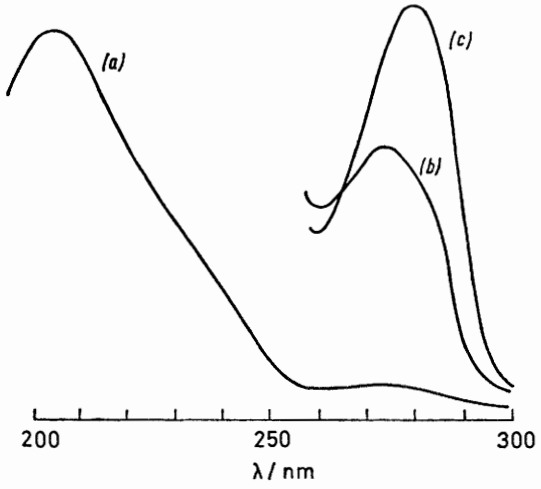


FIGURE 3 U.v. spectra of polymers in water. *Ribes sanguineum* leaf, sample P, (*a*) at high dilution; (*b*) showing the shape of the ^1L_b band at higher concentration; (*c*) *Photinia* polymer ^1L_b band

contribution of two phenolic chromophores: ring A [see (1) for assignments], common to both PC and PD units, approximating to a phloroglucinol ring and forming part of the polymer backbone; and ring B which differentiates the PC and PD units. Ring B of PC units approximates to a pyrocatechol ring whose ^1L_b band is about three times more intense than that of the PD unit chromophore, which approximates to a pyrogallol ring.*

Consideration of the longer wavelength region of the spectrum (Figure 3) of the *Ribes sanguineum* leaf polymer, which contains almost wholly PD units, shows that the ^1L_b band is partly resolved into two peaks, the less intense band due to the ring-A chromophore appearing at λ 278 nm. In contrast, the ^1L_b band of *Photinia* polymer, Figure 3, which contains only PC units, is a symmetrical band at λ 279 nm, implying the overlap of two bands of similar $\lambda_{max.}$.

If the ring A and B chromophores behave as a set of uncoupled oscillators (*i.e.* exhibit no conjugation) the observed $\lambda_{max.}$ for the ^1L_b band should always lie between 270 and 280 nm (its actual value depending on the ratio of PC to PD) and a plot of $E^{1\%}_{1cm}$ *vs.* mole fraction of PC (or PD) should be a simple straight line. Figure 4 shows that such a relationship is obeyed and yields values of $E^{1\%}_{1cm}$ of 130 and 62 for the $\lambda_{max.}$ of the ^1L_b band for pure PC and PD polymers, respectively.

Chain-terminating Flavan-3-ol Unit.—On current evidence a proanthocyanidin polymer chain is terminated by a flavan-3-ol unit. There are four possibilities for the structure of these units: (+)-catechin (14), (−)-epicatechin (16), (+)-gallocatechin (15), and (−)-epigallocatechin (17). Although the heterocyclic ^{13}C resonances of the terminal unit are usually observed in the n.m.r. spectrum, it is not possible to deduce information relating to their ring-B-oxidation pattern.

The structure of the chain-terminating unit may be obtained from the degradation products (see Experimental section), the average composition of the flavan-3-ol units being obtained directly from g.l.c. analysis. Some terminal group compositions are given in Table 4.

As has been observed in the chemistry of procyanidin dimers [1,6] there is little correlation between the stereochemistry of the terminal flavan-3-ol unit and the proanthocyanidin units. Thus in essentially all-*cis*-polymers as found in *Actinidia*, *Aesculus hippocastanum*, and

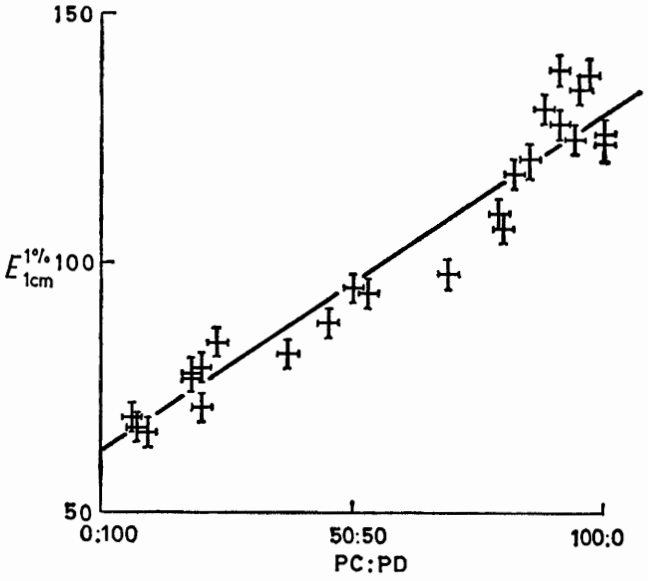


FIGURE 4 Plot of u.v. absorbance *vs.* mole fraction PC

Photinia, only the latter has a single terminal unit of matching stereochemistry. Therefore, the biosynthesis of the polymers appears to be fully consistent with the intermediacy of the flav-3-en-3-ol, or its biological equivalent, which has been proposed on the basis of data exclusively derived from dimers.[6,34]

There is more of a pattern to ring-B hydroxylation. Those polymers containing largely PC units always have (+)-catechin or (−)-epicatechin as terminal units, and (−)-epigallocatechin only occurs in polymers with

* The $\lambda_{max.}$ (ε) values for the ^1L_b band of suitable model compounds are: [33] C-methylphloroglucinol 278 (800) nm, ring A; 3,4-dihydroxytoluene 283 (2800) nm, PC ring B; pyrogallol 271 (750)nm PD ring B. The extinction coefficients are of approximately the correct relative magnitude, compared with those observed for the polymers.

reasonably high PD-unit ratios. No polymer has been isolated so far with (+)-gallocatechin (15) as a terminal unit.

Number-average Molecular Weight (M_n).—The C-3 signal of the chain-terminating flavan-3-ol unit can often be clearly discerned at δ 67—68 in the ^{13}C n.m.r. spectrum of a polymer, well separated from that for the proanthocyanidin monomer units at δ 72—73 (see Table 1) as displayed by all the spectra in Figure 1. In favourable cases the monomer and terminal group C-3 signals may be integrated to obtain the ratio of the average numbers of monomers (M) to the terminal group (T). Measurements of T_1 and η once more show that this is a valid procedure.[18,28] The obvious limitation to an estimation of M_n by this method is whether or not integration of the terminal unit C-3 resonance may be carried out with reasonable accuracy (*i.e.* whether or not the signal is significantly above background noise).

The success of the procedure depends on a number of factors. Obviously M_n will limit the relative intensity of the terminal C-3 signal and there must be an upper limit where it can no longer be measured, regardless of other factors. Secondly, the signal-to-noise (S/N) ratio, assuming constant machine performance, depends on two factors: the size of the sample and the total number of transients (acquisition time) accumulated for any one sample. There are obvious practical limitations on both these latter factors, so that only those values of M_n derived from ^{13}C n.m.r. are included in Table 4 which have attained a satisfactory standard error, defined as 30%.

The standard error for an experiment may be estimated from the relation: standard error = $0.2(M_n - 300)/(S/N)$ where 300 is taken as an average molecular-weight value for a monomer unit, and the signal-to-noise ratio is measured on the C-3 signal. This relation is based on measured effects of random noise on integral reproducibilities for selected spectra. It is estimated that for the best S/N ratios obtained in these experiments (S/N *ca.* 50) the highest M_n that may be measured with a standard error of 30% is *ca.* 7 500.

An alternative method proposed[17,18] for obtaining M_n is measurement of M : T by the ratio of products from the thiolysis reaction (Scheme B). However, data based on this method are not included in this paper as more detailed studies have shown that M : T ratios obtained by this method on the same sample are extremely variable, even under carefully controlled experimental conditions. The origin of the irreproducibility appears to lie in the occurrence of a number of yet uninvestigated side-reactions, whose products are readily detected by g.l.c. We are currently re-investigating this reaction with a view to identifying these reaction products, and ultimately perfecting the method as a viable analytical procedure for M : T ratios.

The order of magnitude of values of M_n obtained by ^{13}C n.m.r. are supported by values obtained in preliminary experiments by vapour-pressure osmometry in methanol, which gave values of 2 200 for the *P. radiata* phloem polymer and 2 300 for the *R. sanguineum*, (sample P) polymer. These may be compared with values of 2 300 and 3 300, respectively, obtained by ^{13}C n.m.r. for the same samples (Table 4).

TABLE 4

Terminal group compositions and M_n for some proanthocyanidin polymers

Polymer	Terminal group(s) [a]	M_n	Standard error (in units of M_n)
Actinidia chinensis (leaf)	e : c = 78 : 22	2 100	190
Aesculus × *carnea* (fruit)	e : c = 80 : 20	2 200	420
Aesculus × *hippocastanum* (fruit)	e : c = 85 : 15	1 750	280
Cydonia oblonga (fruit)	e : c = 60 : 40	3 000	660
Grevillea rosmarinifolia (leaf)	e : c : egc = 6 : 13 : 81	3 300	690
Pinus radiata (phloem)	c : egc = 60 : 40	2 300	320
Pinus radiata (middle bark)	c : egc = 65 : 35	1 740	240
Ribes nigrum (leaf)	e : c : egc = 3 : 11 : 86	4 000	600
Ribes sanguineum (fruit)	c : egc = 9 : 81	2 700	350
Ribes sanguineum (leaf) [b]	e : c : egc = 5 : 5 : 90	3 300	260
Vaccinium corymbosum (fruit)	e : c = 30 : 70	3 500	1 050

[a] e = epicatechin; c = catechin; egc = epigallocatechin. [b] Sample R.

EXPERIMENTAL

Optical rotations and c.d. spectra were recorded in water on Perkin-Elmer 241 and JASCO J-20 spectropolarimeter instruments, respectively. High-performance liquid chromatography (h.p.l.c.) and g.l.c. were carried out, respectively, on a Waters μ-Bondapak C-18 column using a Waters Associates ALCGPC 244 unit and a Pye GCV instrument. ^{13}C N.m.r. were recorded in D_2O–H_2O or[$^2H_6$]acetone–H_2O (1 : 1 v/v) at 20 MHz using a Varian FT-80A instrument.

Isolation of Proanthocyanidin Polymers.—Fresh plant material was extracted as described previously [3] to yield a crude, dialysed, aqueous solution of polymer. An equal volume of methanol was added to this solution and applied to a column of Sephadex LH-20, pre-swollen in methanol–water (1 : 1 v/v). The adsorbed proanthocyanidin was washed with 1 000—2 000 ml of the same solvent and the purified polymer eluted as a discrete, visible, band with 100—200 ml of acetone–water (7 : 3, v/v). The acetone was removed from the eluted polymer solution *in vacuo* at <40 °C and the water removed by freeze-drying to yield the purified proanthocyanidin polymer hydrate as a light tan fluffy solid which readily redissolves in water. Microanalyses of some freeze-dried preparations are as follows: *

* The stoicheiometry of the average proanthocyanidin unit is calculated from the PC : PD ratios in Table 2.

Aesculus hippocastanum polymer (Found: C, 52.6; H, 5.3. $C_{15}H_{12}O_6 \cdot 3H_2O$ requires C, 52.6; H, 5.3%); *Betula alba* polymer (Found: C, 51.7; H, 5.1. $C_{15}H_{12}O_{6.2} \cdot 3H_2O$ requires C, 52.1; H, 5.3%); *Cyathea dealbata* polymer (Found: C, 51.8; H, 5.4. $C_{15}H_{12}O_{6.4} \cdot 3H_2O$ requires C, 51.7; H, 5.2%); *Pinus radiata* phloem polymer (Found: C, 52.8; H, 4.8%. $C_{15}H_{12}O_{6.5} \cdot 2.5H_2O$ requires C, 52.8; H, 4.8%); *Ribes sanguineum* leaf polymer (Found: C, 52.4; H, 4.8; $C_{15}H_{12}O_{6.9} \cdot 2.5H_2O$ requires C, 51.7; H, 4.9%). The water of hydration may be removed by prolonged drying at room temperature at 1 mmHg pressure over P_2O_5.* Some microanalyses of anhydrous polymers are as follows: *Aesculus carnea* (Found: C, 62.6; H, 4.3. $C_{15}H_{12}O_{6.0}$ requires C, 62.5; H, 4.2%); *Aesculus hippocastanum* (Found: C, 62.6; H, 4.3. $C_{15}H_{12}O_{6.0}$ requires C, 62.5; H, 4.2%); *Betula alba* (Found: C, 61.6; H, 4.5. $C_{15}H_{12}O_{6.2}$ requires C, 62.0; H, 4.2%); *Cydonia oblonga* (Found: C, 62.4; H, 4.6. $C_{15}H_{12}O_{6.0}$ requires C, 62.5; H, 4.2%); *Grevillea robusta* (Found: C, 60.0; H, 4.4. $C_{15}H_{12}O_{6.6}$ requires C, 60.5; H, 4.1%); *Grevillea rosmarinifolia* (Found: C, 60.0; H, 4.5; $C_{15}H_{12}O_{6.8}$ requires C, 59.8; H, 4.0%); *Lotus pedunculatus* leaf (Found: C, 59.3; H, 4.6. $C_{15}H_{12}O_{6.8}$ requires C, 59.8; H, 4.0%); *Lotus pedunculatus* root (Found: C, 60.6; H, 4.5; $C_{15}H_{12}O_{6.7}$ requires C, 60.2; H, 4.0%); *Pinus radiata* phloem. Found: C, 61.1; H, 4.2. $C_{15}H_{12}O_{6.5}$ requires C, 60.8; H, 4.1%. *Pinus radiata* middle bark, (Found: C, 62.4; H, 4.9. $C_{15}H_{12}O_{6.1}$ requires C, 62.2; H, 4.2%); *Ribes nigrum* leaf (Found: C, 60.0; H, 4.3. $C_{15}H_{12}O_{6.9}$ requires C, 59.5; H, 4.0%); *Ribes sanguineum* fruit (Found: C, 59.6; H, 4.4. $C_{15}H_{12}O_{6.8}$ requires C, 59.8; H, 4.0%); *Ribes sanguineum* leaf, Sample P (Found: C, 59.5; H, 4.3; $C_{15}H_{12}O_{6.9}$ requires C, 59.5; H, 4.0%); *Ribes sanguineum* leaf, Sample R (Found: C, 59.9; H, 4.1. $C_{15}H_{12}O_{6.9}$ requires C, 59.5; H, 4.0%); *Salix fragilis* (Found: C, 62.4; H, 4.6. $C_{15}H_{12}O_{6.0}$ requires C, 62.5; H, 4.2%); *Vaccinium corymbosum* (Found: C, 62.3; H, 4.8. $C_{15}H_{12}O_{6.0}$ requires C, 62.5; H, 4.2%).

Anthocyanidin Estimation.—The production and estimation of cyanidin and delphinidin chlorides was carried out spectrophotometrically as described previously.[3]

Polymer Cleavage with Thiol Reagent.—The polymer—(20 mg) was dissolved in absolute ethanol (5 ml) and phenylmethanethiol (0.2 ml) in a vial and purged with O_2-free nitrogen for 10 min, and acetic acid (0.5 ml) was then added while continuing to purge; the vial contents were then frozen in liquid nitrogen and the vial sealed. The vial was heated in an oven at 100—105 °C for 18 h. The vial was cooled, the contents re-frozen, and the solvents removed from the open vial by freeze-drying. The resulting reaction products were shown by t.l.c. on cellulose to contain no polymeric vanillin-reactive products. The mixture of 4-sulphides and catechins were analysed by g.l.c. on a column of JXR silicone as previously described [25] as their trimethylsilyl (TMS) ethers, prepared by the method of Collier and Mallows.[24] The relative retention times of the TMS ethers of the various flavan-3-ols are: (−)-epicatechin (16) 1.00; (+)-catechin (14) 1.02; (−)-epigallocatechin (17) 1.05; (+)-gallocatechin (15) 1.22; (2*R*,3*S*,4*R*)-4-(benzylthio)flavan-3,3′,4′,5,7-pentaol (12) 1.44; (2*R*,3*S*,4*R*)-4-(benzylthio)flavan-3,3′,4′,5,5′,7-hexaol (13) 1.50; (2*R*,3*R*,4*R*)-4-(benzylthio)flavan-3,3′,4′,5,7-pentaol (9) 1.37; (2*R*,3*R*,4*R*)-4-(benzylthio)flavan-3,3′,4′,5,5′,7-hexaol (11) 1.39; (2*R*,3*R*,4*S*)-4-(benzylthio)flavan-3,3′,4′,5,7-pentaol (8) 1.47; (2*R*,3*R*,4*S*)-4-(benzylthio)flavan-3,3′,4′,5,5′,7-hexaol (10) 1.49.

(2R,3R,4S)-4-(2,4,6-*Trihydroxyphenyl*)*flavan*-3,3′,4′,5,7-*pentaol*.—The polymer from *Photinia* leaves was treated with phloroglucinol hydrate as described previously.[3] The product was chromatographed twice on Sephadex LH-20 in ethanol [13] and further purified by h.p.l.c. in 10% methanol–water to yield a colourless *solid* after freeze-drying (Found: C, 59.7; H, 4.7. $C_{21}H_{18}O_9 \cdot 0.5H_2O$ requires C, 59.6; H, 4.5%); $[\alpha]_{578}^{20}$ +122° (*c* 0.28, water dried sample); $\lambda_{max.}$ (water) 277 nm (ε 3 900).

(2R,3S,4R)-4-(2,4,6-*Trihydroxyphenyl*)*flavan*-3,3′,4′,5′,7-*hexaol*.—This was prepared from *Ribes sanguineum* leaf polymer as described for the *Photinia* product. Purification by h.p.l.c. in 5% methanol–water yielded a colourless *solid* (Found: C, 58.5; H, 4.4. $C_{21}H_{18}O_{10}$ requires C, 58.6; H, 4.2%); $[\alpha]_{578}^{20} = -204°$ (*c* 0.34, water, dried sample); $\lambda_{max.}$ (water) 270 nm (ε 2 875).

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* The anhydrous polymers are extremely hygroscopic. They may be conveniently dried in Al-foil capsules which allow the water to diffuse out at reduced pressure, but not back in at atmospheric pressure (Professor A. D. Campbell, personal communication).

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