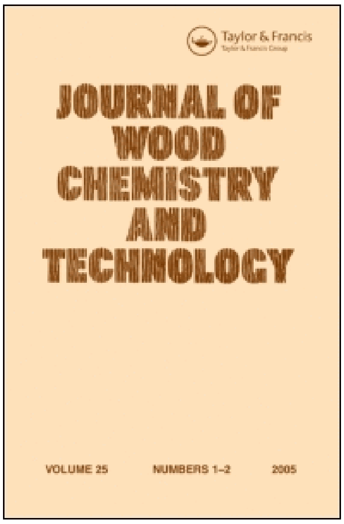

Journal of Wood Chemistry and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597282

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To cite this Article Hemingway, Richard W. and McGraw, Gerald W.(1983) 'Kinetics of Acid-Catalyzed Cleavage of Procyanidins', Journal of Wood Chemistry and Technology, 3: 4, 421 — 435
To link to this Article: DOI: 10.1080/02773818308085173
URL: http://dx.doi.org/10.1080/02773818308085173

JOURNAL OF WOOD CHEMISTRY AND TECHNOLOGY, 3(4), 421-435 (1983)

KINETICS OF ACID-CATALYZED CLEAVAGE OF PROCYANIDINS

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ABSTRACT

Comparisons of the rates of cleavage of isomeric procyanidin dimers in the presence of excess phenylmethane thiol and acetic acid showed that compounds with a C(4)-C(8) interflavanoid bond were cleaved more rapidly than their C(4)-C(6) linked isomers, that 2,3-cis isomers with an axial flavan substituent were cleaved more rapidly than a 2,3-trans isomer with an equatorial substituent, and that cleavage rate was independent of the stereochemistry in the terminal unit. Cleavage rate constants followed Arrhenius temperature dependence. The C(4)-C(8) interflavanoid bond in 2,3-cis procyanidins should be labile to cleavage at 20°C over a pH range of 3.6 to 5.4. Reaction of loblolly pine bark tannins in the presence of excess (+)-catechin with acetic acid rapidly produced oligomeric procyanidins. The ratio of the procyanidin B1 to B7 decreased from 2.9:1 after 4 hours to 1.3:1 after 50-100 hours at 90°C. The structure and reactions of condensed tannins are interpreted in light of these results.

INTRODUCTION

Acid-catalyzed cleavage of the interflavanoid bond of procyanidins is one of the most important reactions in condensed tannin chemistry. Protonation at the point of linkage to phloroglucinol rings of procyanidins results in interflavanoid bond cleavage with the generation of a flavanyl carbocation that may be oxidized to produce anthocyanidins or that can be

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captured by a nucleophile such as a thiol, or a phenol such as phloroglucinol, to form 4-substituted derviatives.[1-5] With flavan-3-ols or oligomeric procyanidins as nucleophiles, this reaction provides a convenient synthesis of procyanidins that mimics their formation in nature.[6]

Despite its importance, there has been no systematic study of the acid-catalyzed cleavage of isomeric procyanidins. Additionally, the lability of the interflavanoid bond to cleavage has not received adequate attention by chemists attempting to develop uses for condensed tannins. Clearly, more information about the reactions of the interflavanoid bond is needed to understand the behavior of these polymers during their isolation and use in products. To clarify some of the important features of acetic acid catalyzed cleavage we studied the rate of cleavage of a series of dimeric procyanidins and of a tannin isolated from loblolly pine.

RESULTS AND DISCUSSION

Previous studies of condensed tannins in southern pine bark have shown that these polymers are composed primarily of 2,3-cis procyanidin chain extenders and that (+)-catechin is the dominant terminal unit.[7-9] The procyanidin units are linked by C(4)-C(8) and C(4)-C(6) interflavanoid bonds.[8-10] The ^{13}C n.m.r. spectra of loblolly pine bark tannins can be fully accounted for as polymeric procyanidins;[8] this has also been demonstrated in condensed tannins isolated from a wide variety of plants.[11] Most of the interflavanoid bonds in condensed tannins from southern pine bark can then be represented by the dimers B1, epicatechin-(4β-8)-catechin (1); B2, epicatechin-(4β-8)-epicatechin (2); B3, catechin-(4α-8)-catechin (3); B5, epicatechin-(4β-6)-epicatechin (4); B7, epicatechin-(4β-6)-catechin (5); and the 4-benzyl sulfide derivatives of B2 (6) and B5 (7) (Fig. 1). These compounds have been prepared and characterized in earlier studies.[8-10,12] The above compounds were subjected to acid-

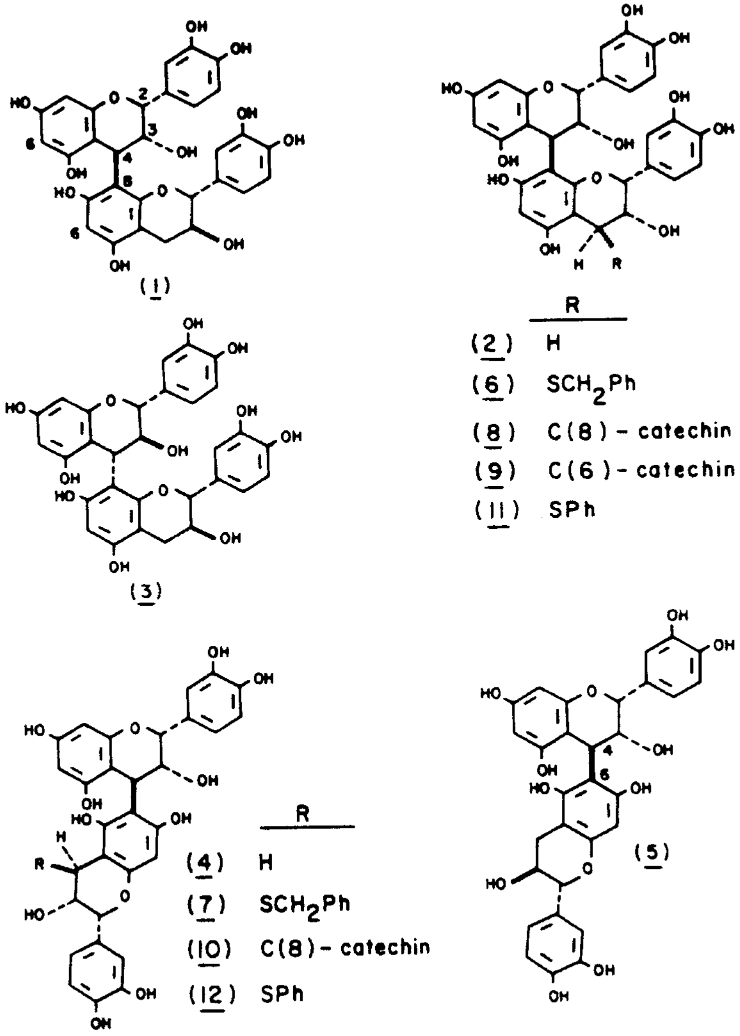


FIGURE 1. Isomeric procyanidins and derivatives related to loblolly pine bark tannins.

TABLE 1

Interflavanoid Bond Cleavage Rate Constants for Dimeric Procyanidins

		Procyanidin				
Temp °C	Acid conc.	B1 (1)	B2 (2)	B3 (3)	B5 (4)	B7 (5)
		Rate Constant (Hours^{-1})				
90	35:1	0.36	0.26	0.19	0.15	0.15
80	35:1	0.14	0.14	0.10	0.094	0.071
70	35:1	0.079	0.069	0.033	0.027	0.031
60	35:1	0.050	0.041	0.018	0.013	0.011
70	10:1	0.081	0.088	0.042	0.030	0.023
70	3.5:1	0.084	0.070	0.031	0.022	0.023
90	1:1	0.12	0.16	0.093	0.065	0.076
80	1:1	0.077	0.078	0.045	0.029	0.033
70	1:1	0.063	0.063	0.033	-----	0.022
60	1:1	0.028	0.030	0.016	0.013	0.012

catalyzed cleavage at temperatures ranging from 60° to 90°C and at acetic acid concentrations ranging from 1 to 35 moles of acetic acid in the presence of 17 moles of phenylmethane thiol per mole of procyanidin.

Plots of $\ln(C_o-C_t)$ versus reaction time were linear, showing pseudo first-order reactions with respect to procyanidin concentration (Fig. 2).[13] Linear regression on the form $\ln(k) = - EA/RT + c$ showed that these reactions closely followed Arrhenius temperature dependence, with coefficients of determination in the range of 0.95 to 0.99 (Table 2, Fig. 3).

There were differences in interflavanoid bond cleavage rates associated with variations in procyanidin structure. The 2,3-*cis* procyanidins with a C(4)-C(8) interflavanoid bond (1) and (2) were cleaved about 2.6 times faster than their C(4)-C(6) linked isomers (5) and (4). Both 2,3-*cis* procyanidins with C(4)-C(8) bonds and axial substituents[5] (1) and (2) were

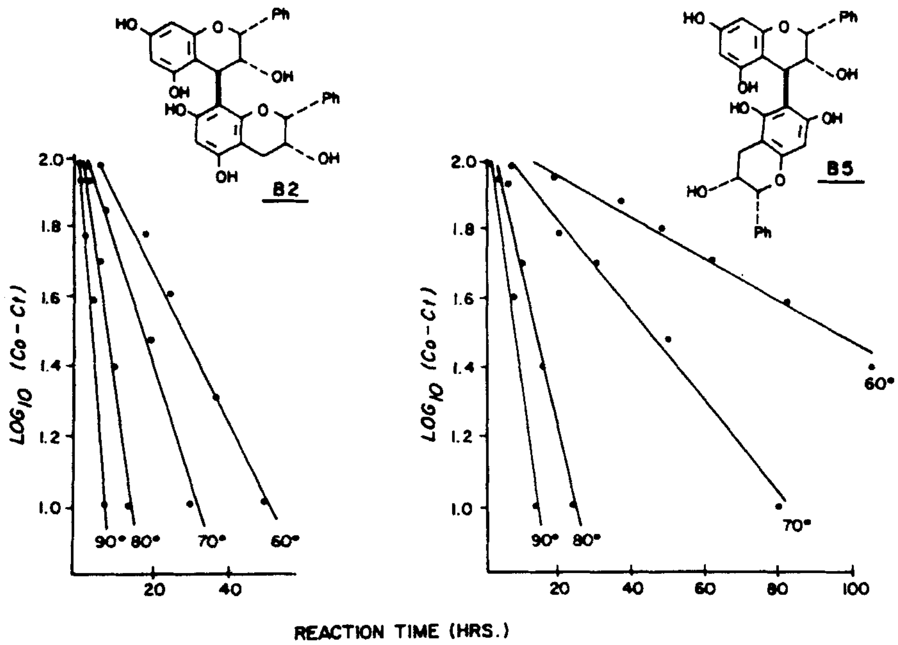
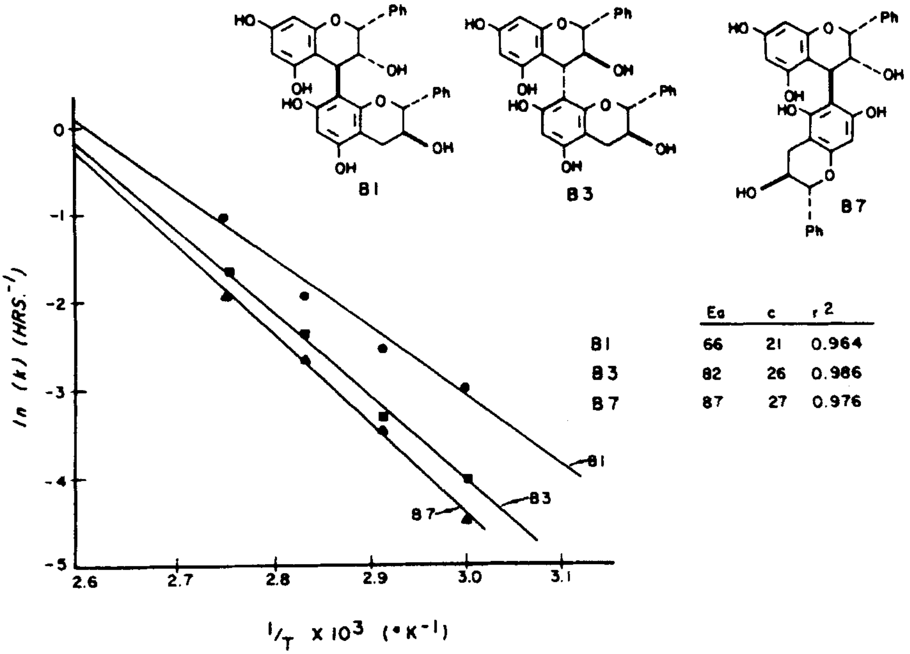


FIGURE 2. Acid-catalyzed cleavage of B2 and B5 at temperature 60-90°C.

TABLE 2

Arrhenius Temperature Dependence Parameters for Cleavage of Dimeric Procyanidins

	Procyanidin				
	B1 (1)	B2 (2)	B3 (3)	B5 (4)	B7 (5)
Activation Energy			pH 3.6		
(KJ·$^{o}K^{-1}$·mole^{-1})	66	63	82	87	87
Reaction Constant	21	20	26	27	27
Coefficient of Determination r^2	0.964	0.994	0.986	0.996	0.976
Activation Energy			pH 5.4		
(KJ·$^{o}K^{-1}$·mole^{-1})	47	52	56	52	60
Reaction Constant	13	15	16	14	17
Coefficient of Determination r^2	0.949	0.964	0.978	0.955	0.976



	Ea	c	r2
B1	66	21	0.964
B3	82	26	0.986
B7	87	27	0.976

FIGURE 3. Arrhenius relationships for B1, B3, and B7.

cleaved 1.9 times faster than was a 2,3-*trans* isomer with a C(4)-C(8) linkage and an equatorial substituent[5] (3). As might be expected, variation in the stereochemistry in the terminal unit had no detectable influence on the cleavage rate [compare (1) with (2) and (4) with (5)]. Cleavage of the dimers (2) and (4) was about 1.4 times faster than that of their corresponding 4-benzyl sulfide derivatives (6) and (7). As was found in the dimeric procyanidins, the C(4)-C(8) linked isomer (6) was cleaved much more rapidly than was the C(4)-C(6) linked pair (7).

Projections from Arrhenius equations were made to provide estimates of probable cleavage rates for these compounds at 20°C over a pH range of 3.6-5.4. Although there is considerable variability, these results suggested that the 2,3-*cis* procyanidins linked C(4)-C(8) have half-lives in the neighborhood of 200-400 hours while the C(4)-C(6) linked isomers are more resistant to cleavage with half-lives in the range of 1,000-4,000 hours. Since most of the interflavanoid bonds in southern pine bark tannins can be represented by (2), these polymers should be expected to undergo interflavanoid bond cleavage in aqueous solution under ambient conditions. When the condensed tannins are extracted from bark and concentrated in industrial conditions, it is clear that the lability of the interflavanoid bond to cleavage must be considered to obtain extracts of uniform properties.

To assess how the cleavage rate constants obtained for the dimeric procyanidins could be related to interflavanoid bond cleavage in natural condensed tannin polymers, loblolly pine bark tannins were reacted with (+)-catechin and acetic acid at 90°C over reaction periods of 4 to 100 hours. The products obtained from these reactions (Table 3) indicated that about 50 percent of the water soluble tannin was converted to ethyl acetate soluble oligomers after only 4 hours of reaction. The amount of water soluble material decreased slowly thereafter, but the ^{13}C n.m.r. spectra of the residual water soluble material (Fig. 4) and VPO of their peracetates (Table 3) showed that residual water soluble materials consisted mainly of low molecular weight oligomers after 4 hours.

The number average molecular weight of condensed tannins from loblolly pine bark generally centers in the region of 2,500-3,000.[8-14] In the reaction of this tannin ($\bar{Mn}$ peracetate 3437) with (+)-catechin, at a weight ratio of 1:2, the yield of catechin recovered would decrease to 60.6 percent of that added had the polymer been converted completely to dimeric procyanidins. If losses in sample work-up amounted to about 18 percent as in the 50 hour reaction, we would estimate that the number average molecular weight of the total product would be about 700. Yields of the dimeric procyanidins (1) and (5), the trimer (8), and consideration of the ^{13}C n.m.r. spectra of the oligomeric procyanidin fractions (Fig. 4) suggest that the number average

TABLE 3

Loblolly Pine Bark Tannins Reacted with (+)-Catechin

	Reaction Time at 90° (Hours)				
	4	8	24	50	100
Partition H_2O/EtOAc					
H_2O-Soluble (g)	0.78	0.69	0.61	0.50	0.52
EtOAc-Soluble (g)	3.4	3.5	3.6	3.8	4.1
Recovery (%)	94	92	94	95	100
Peracetate of H_2O-Soluble Residue					
$M\bar{n}$ (VPO)	1800	1900	1600	1800	1700
Analysis C	59.4	59.4	59.5	59.3	59.6
H	4.6	4.5	4.7	4.6	4.7
Fractionation of EtOAc on Sephadex LH-20[15]					
Catechin (%)	53	51	53	48	43
B1 (1)	7.8	8.5	9.5	9.9	8.4
B7 (5)	4.5	5.9	7.9	7.4	7.3
Trimer (8)	0.95	2.4	2.5	2.3	2.7
EtOH Oligomers	3.9	5.4	4.0	5.3	4.0
Acetone Oligomers	9.4	19	17	14	29
Total Recovered	80	94	93	87	93
Purity of Dimers by H.P.L.C.					
B1 (1)	77	62	80	63	59
B7 (5)	45	45	59	64	52
Corrected Ratio B1/B7	2.9:1	2.0:1	1.6:1	1.3:1	1.3:1

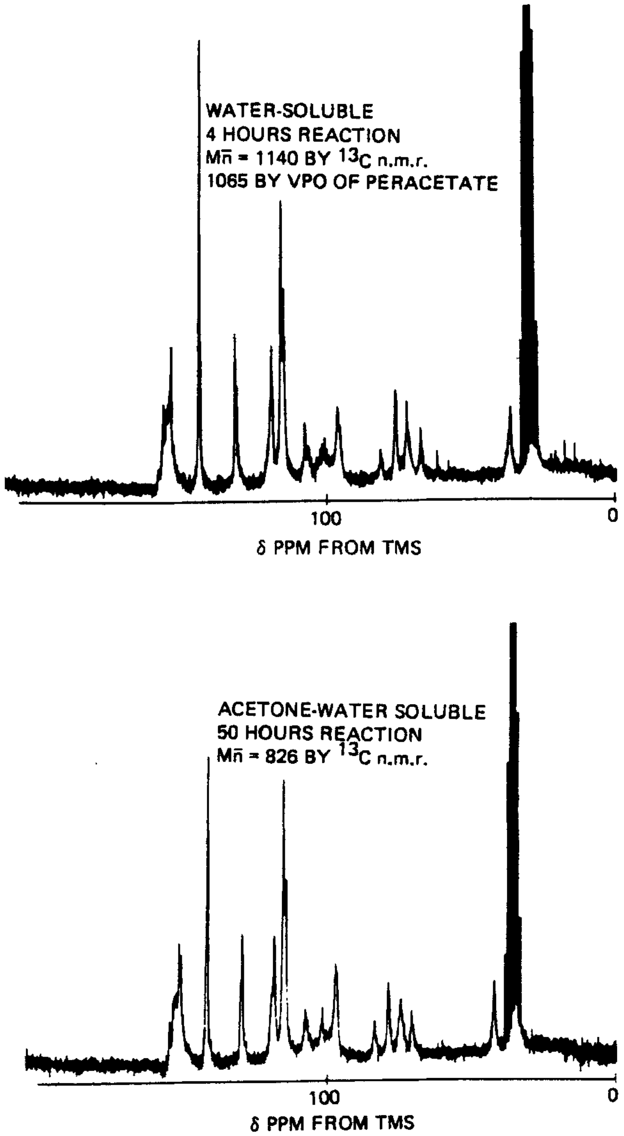
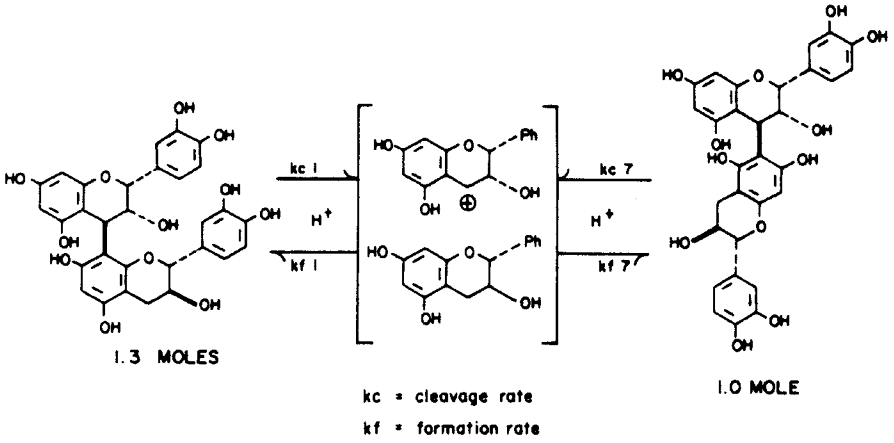


FIGURE 4. ^{13}C nmr of products from reaction of tannin (+)-catechin.

molecular weight of the total product should be about that of a trimeric procyanidin. The fact that we found a mixture of oligomeric procyanidins rather than a high proportion of the dimers (1) and (5) indicates that the phloroglucinol A-ring of the oligomeric products competes well with the (+)-catechin in capturing the generated carbocations.

There is no appropriate estimate of the relative rate of condensation of flavan carbocations at the C-6 and C-8 positions of catechin since any suitable model will concurrently suffer interflavanoid bond cleavage. It is possible to calculate the relative condensation rate from the product of the relative cleavage rate of the C(4)-C(8) and C(4)-C(6) interflavanoid bonds (i.e., 2.6 and 1) and the ratio of the C(4)-C(8) and C(4)-C(6) linked dimers at equilibrium. In reactions of loblolly pine bark tannins with (+)-catechin, the ratio of (1) to (5) converged on 1.3 to 1 after 50 hours of heating (Table 3), so one would estimate the relative rate of condensation at the C-8 and C-6 positions of catechin to be approximately 3.3 and 1 (Fig. 5). This calculated ratio of C(4)-C(8) and C(4)-C(6) linked isomers is in reasonable agreement with the observed relative proportions of dimeric procyanidins (1) and (5) isolated from the phloem of loblolly pine (i.e., 2.4 and 1),[9] the relative yield of (1) and (5) found after only 4 hours of reaction of tannins with (+)-catechin (i.e., 2.9 and 1), the relative yields of the three isomeric trimers (8), (9), and (10) isolated from loblolly pine phloem (i.e., 3 and 1 and 1),[10] and the relative yields of the dimeric phenylsulfides (6) and (7) obtained after a 20-hour partial thiolytic cleavage of loblolly pine bark tannins (i.e., 3.6 and 1).[9]

The differences in cleavage rate observed among the isomeric dimers clearly show that it is not possible to determine the *in-vivo* proportions of C(4)-C(8) and C(4)-C(6) bonds in polymeric procyanidins directly by partial thiolytic cleavage. As has been observed in previous studies,[9,10] the relative yield of the C(4)-C(6) linked dimer sulfides (7) and (12) can be expected to increase with increasing reaction time because of differences



$$\frac{1.3}{1.0} = \text{EQUILIBRIUM } \frac{B1}{B7} = \frac{\dfrac{kf\ 1}{kf\ 7}}{\dfrac{kc\ 1}{kc\ 7}} = \frac{\dfrac{X}{1.0}}{\dfrac{2.6}{1.0}} \qquad \underline{X = 3.3}$$

FIGURE 5. Calculation of reactivity of C-6 and C-8 from kinetics data.

in cleavage rate rather than because of differences due to accessibility in the polymer as had been proposed earlier.[10]

CONCLUSIONS

The rate of cleavage of procyanidin interflavanoid bonds is dependent on the bond location and on the stereochemistry of the upper unit. The 2,3-cis procyanidins with a C(4)-C(8) interflavanoid bond are most susceptible and should be expected to undergo interflavanoid bond cleavage in aqueous solutions under ambient conditions. Reactions of condensed tannins from loblolly pine bark with (+)-catechin also demonstrated rapid interflavanoid bond cleavage with the formation of low molecular weight oligomers after only 4 hours of reaction at 90°C. The ratio of the C(4)-C(8) and C(4)-C(6) linked isomers B1 and B7 converged to an equilibrium of about 1.3:1 after 50 hours of heating. Since the relative rate of cleavage of their interflavanoid bonds is about 2.6:1, the relative rate of condensation at the C-8 and C-6 positions would be approximately 3.3:1, a ratio consistent with that of the C(4)-C(8) and C(4)-C(6) interflavanoid bonds found in natural procyanidins from loblolly pine bark. The facile cleavage of interflavanoid bonds in polymeric procyanidins clearly needs particular attention when developing uses for these abundant natural polymers.

EXPERIMENTAL

Each of the dimeric procyanidins (1) through (5) or the benzyl sulfides (6) or (7) was dissolved in ethanol (2 ml), and phenylmethane thiol (10 μl) was added while the solution was purged with argon. Acetic acid (1, 3, or 10 μl of glacial or a 10% solution in ethanol) was added and the reaction vial sealed. The samples were heated in a Haake[15] constant temperature water bath at temperatures of 60°, 70°, 80°, or 90°C. After appropriate reaction times, samples (5 μl) were withdrawn by syringe and applied to two dimensional cellulose t.l.c. plates (Schleicher and Schuell, F-1440, cut to 10 x 10 cm). The plates were developed in the first dimension with t-butanol-acetic acid-water 3:1:1 v/v/v and, after drying, in the second dimension with 6% acetic acid. The plates were then sprayed with vanillin-HCl and heated to obtain red colored spots for the flavan derivatives. The extent of cleavage was estimated by a visual comparison of the relative spot intensity of the starting compound and the flavan-3-ol and flavan-4-benzyl sulfide cleavage products. At least four plates were developed through the course of cleavage of each compound under each reaction condition.

Plots of $\log_{10}(C_o - C_t)$ versus reaction time were made to estimate the cleavage rate constant. Linear regression of ln(k) versus the reaction temperature were made to estimate the activation energy and the reaction constant.

In preliminary experiments we examined the possiblity of using g.l.c. of trimethylsilyl ethers and acetate derivatives, but we were not able to perfect a satisfactory analytical g.l.c. method. We also attempted to obtain analytical data by reverse phase h.p.l.c. of the phenols on Zorbax CN and C-18 Microbondapak columns. We were not able to find a solvent that would permit isocratic separation of the dimeric procyanidin and its flavan-3-ol and flavan-4-benzyl sulfide reaction products. Differences in cleavage rates among the isomeric procyanidins at the different reaction temperatures examined were so large that it was judged that useful information could be obtained using t.l.c.

Condensed tannins were isolated from loblolly pine bark and purified by separation on Sephadex LH-20 as described earlier.[11] The freeze-dried tannin (1.5 g) was combined with (+)-catechin (3.0 g), dissolved in 50 ml of ethanol, and after purging the reaction vials with argon, acetic acid (3 ml) was added and the vials were sealed. Five such samples were prepared and then heated in a constant temperature water bath at 90°C for time periods of 4, 8, 24, 50, and 100 hours.

Products were added to water (250 ml) and extracted six times with ethyl acetate (150 ml). Residual water soluble products were acetylated, after which their constitution was determined by elemental analysis and their molecular weight determined by vapor pressure osmometry. Ethyl acetate soluble products were separated on a Sephadex LH-20 column (2.5 x 80 cm) by elution with ethanol where 15 ml fractions were collected up to about 250 fractions. Residual product on the column was then eluted with acetone-water 1:1. Elution of the products from the column was monitored by one-dimensional cellulose t.l.c. (6% HOAc). Fractions containing (+)-catechin, procyanidin B1, procyanidin B7, the trimer (8), and a mixture of oligomeric procyanidins eluted with ethanol were combined in these groups and yields of crude isolates were determined after freeze-drying.

The crude isolates of the procyanidins B1 and B7 were separated by reverse phase h.p.l.c. on a Supelco LC-8 column (10 mm x 2.4 cm) by elution with methanol-water 25:75 at 1.5 ml/min. The purity of each isolate of procyanidin B1 and B7 was then determined by measuring the weight of the dimer peak as compared with the weight of all peaks recorded with a 254 nm UV detector. The yields of the dimers B1 and B7 obtained from the Sephadex column were then corrected by the estimated purity and the ratio of these two products compared after different reaction times.

The ^{13}C n.m.r. spectra of the oligomeric fractions eluted from Sephadex LH-20 columns with ethanol and with acetone-water as well as spectra of the residual water soluble products were recorded in D_2O-d_6-acetone 1:1 v/v at 20 MHz with a Varian FT-80A spectrometer.

ACKNOWLEDGMENTS

Dr. Lai Yeap Foo, Chemistry Division, Department of Scientific Research, Petone, New Zealand, contributed suggestions helpful to the completion of this work.

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