RECENT ADVANCES IN THE CHEMISTRY OF CONDENSED TANNINS

R. W. HEMINGWAY and G. W. McGRAW
Southern Forest Experiment Station and Department of Chemistry, Louisiana College, Pineville, Louisiana 71360

J. J. KARCHESY
Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331

L. Y. FOO and L. J. PORTER
Chemistry Division, DSIR, Petone, New Zealand

SYNOPSIS

The presence of the procyanidins epicatechin-(4β-8)-catechin, epicatechin-(4β-6)-catechin, catechin-(4α-8)-catechin, epicatechin-(4β-8)-epicatechin-(4β-8)-catechin, epicatechin-(4β-6)-epicatechin-(4β-8)-catechin, and epicatechin-(4β-8)-epicatechin-(4β-6)catechin in the phloem of *Pinus taeda* demonstrated heterogeneity in the location of the interflavanoid bonds in oligomeric procyanidins. Partial acid-catalyzed cleavage of polymeric procyanidins from *Pinus taeda* and *Pinus palustris* barks or *Photinia glabrescens* leaves gave both the epicatechin-(4β-8)-epicatechin- and epicatechin-(4β-6)-epicatechin-benzyl or phenyl sulfides, demonstrating isomerism in the interflavanoid bond location in these polymers. Comparison of the rates of acid-catalyzed cleavage of isomeric dimers showed that those compounds with a (4β-6) interflavanoid bond were more stable than compounds with a (4β-8) bond. Also, catechin-(4α-8)-catechin was more resistant to cleavage than either epicatechin-(4β-8)-catechin or epicatechin-(4β-8)-epicatechin when heated in the presence of excess phenylmethanethiol with acetic acid. The structures of condensed tannins and the stability of these polymers under acidic conditions are discussed.

INTRODUCTION

Work by Haslam's group [1–5] suggested that condensed tannins of the procyanidin class were linear, thread-like polymers in which the catechol B-rings rotated about the central core in a right-hand helix in polymers of a (+)-catechin (*1*) or 2,3-*trans* stereochemistry and in a left-hand helix in polymers of an (−)-epicatechin (2) or 2,3-*cis* stereochemistry [6] (Fig. 1). Although isomerism in

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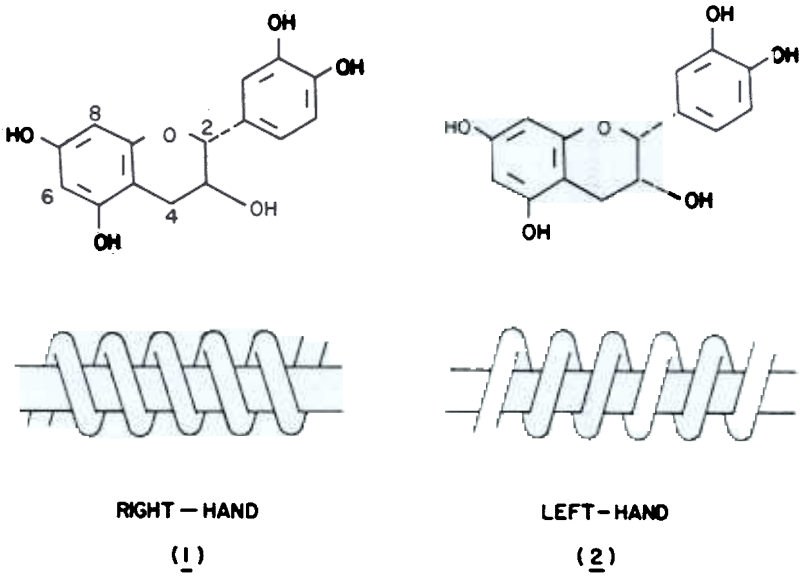

FIG. (+)-Catechin and (−)-epicatechin; basic units of procyanidins.

the location of the interflavanoid bond in dimeric procyanidins had been established, the ratio of C(4)–C(8) to C(4)–C(6) isomeric pairs was comparatively high (8: or 9 : 1) [2], and it was considered that higher-molecular-weight oligomers were largely—if not exclusively—linked by C(4)–C(8) interflavanoid bonds [4–6]. However, no proof of the location of interflavanoid bonds in polymeric procyanidins existed.

In studies of the reaction of (+)-catechin with *p*- and *o*-hydroxybenzyl alcohols, we found substantial substitution at the C-6 position [7]. These results caused us to question the assumption of such a high degree of substitution at C-8 in the formation of polymeric procyanidins unless the location of condensation of flavanyl carbocations on flavan-3-ols or oligomeric procyanidin was controlled by enzymes.

The shape of the condensed tannins is important, both in regard to their biologic properties (i.e., interaction with proteins) and to their industrial application as specialty polymers (i.e., wood adhesives or dispersants). Therefore, a series of studies was undertaken to define the location of interflavanoid bonds in procyanidins. Clarification of such questions about the structure of condensed tannin polymers in conifer barks is particularly important to our concurrent efforts to develop wood adhesives using bark extracts. Details of the results of our studies of dimeric, trimeric, and polymeric procyanidins have been published [8–11]. The intent of this report is to summarize our current understanding of the chemistry of procyanidin-based condensed tannins that are major components of southern pine tree barks as well as of a broad spectrum of other plants.

RESULTS AND DISCUSSION

Our studies of the procyanidins of *Pinus taeda* (loblolly pine) bark revealed the location of the interflavanoid bonds in these compounds. While the C(4)–C(8) linkage is dominant, the C(4)–C(6) linkage occurs more frequently in the dimeric, trimeric, and polymeric procyanidins of loblolly pine phloem [8–11] than the 8–9 : 1 ratio of C(4)–C(8) to C(4)–C(6) isomeric pairs found by Fletcher et al. [2].

Dimeric Procyanidins

Three dimeric procyanidins were isolated from loblolly pine phloem in relative yields of 2.4 : 1.0 : and 0.67 [8]. The dimers present in highest and lowest proportions were shown to be epicatechin-(4β-8)-catechin (*3*) and catechin (4α-8)-catechin (*4*), respectively, by comparisons with reported data [12, 13]. The compound isolated in intermediate yield appeared to be epicatechin-(4β-6)-catechin (*5*) on the basis of its ^{13}C NMR spectrum. However, little analytical data existed for this compound [1, 2]; and, since it was isolated in such high yield relative to compound *3*, definitive proof of its structure was required. The absolute stereochemistry of the upper procyanidin unit was established by the formation of (2*R*,3*S*,4*S*)-2,3-*cis*-3,4-*trans*-3-hydroxy-3′,4′,5,7-tetramethoxy-4-phenylthioflavan (*6*) through benzenethiol degradation and methylation [14, 15]. The

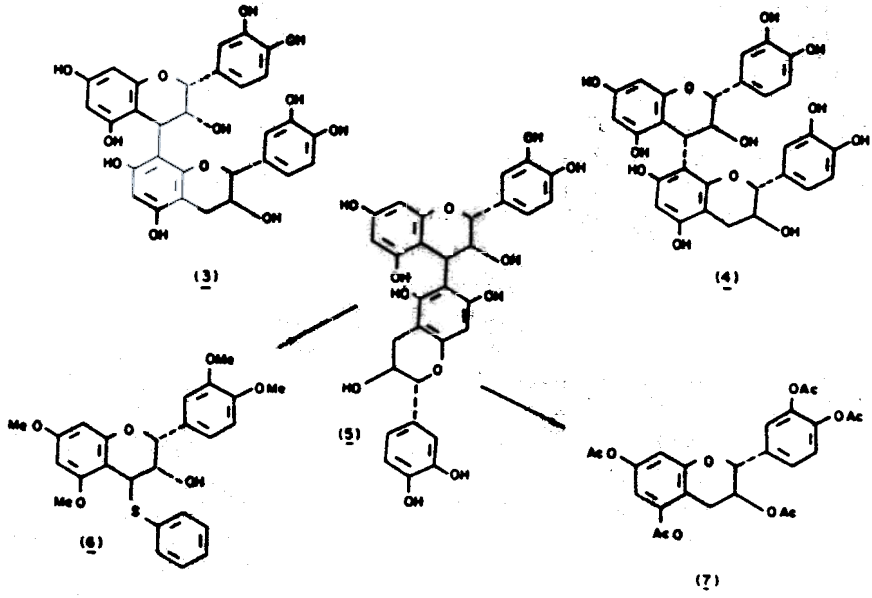

FIG. 2. Dimeric procyanidins in loblolly pine phloem and determination of absolute stereochemistry of epicatechin-(4β-6)-catechin.

absolute stereochemistry of the lower terminal unit was established as 2*R*,3*S* by the formation of 3,3',4',5,7-penta-*O*-acetyl-(+)-catechin (*7*) by acetylation of the thiolysis product (Fig. 2). The CD spectrum of the deca-acetate of compound *5* showed a strong positive Cotton effect for the 236-nm band, thus establishing a 4*R* stereochemistry [5, 8]. A C(4)–C(6) interflavanoid bond was shown by ^{1}H NMR of the deca-acetate of *5*, where the A-ring protons appear as a multiplet centered on δ6.6–6.7 ppm, in contrast with isomers with a C(4)–C(8) interflavanoid bond, where the A-ring protons of the upper unit are shifted upfield by about 0.5 ppm, appearing as an AB quartet centering on δ6.1 ppm [10].

The ratio of the C(4)–C(8)-linked compound *3* to its C(4)–C(6)-linked isomer *5* ranged from 1.6 : 1 to 3.3 : 1 in samples from a number of loblolly pine trees and averaged 2.4 : 1 in four separate syntheses from reaction of loblolly pine phloem tannins with (+)-catechin [8]. These proportions of C(4)–C(6)-linked dimers were far higher than the 8–9 : 1 ratio of C(4)–C(8)- to C(4)–C(6)-linked isomers reported by Fletcher et al. [2].

Trimeric Procyanidins

Trimeric procyanidins have been isolated from a wide variety of plants [1, 16–20] but until recently no evidence had been given for the locations of the interflavanoid bonds in any of these compounds. In the chromatography of ethyl acetate-soluble products from loblolly pine phloem on Sephadex LH-20, additional procyanidins are obtained after elution of the dimers, and three procyanidins were purified from these fractions by reverse-phase HPLC on Zorbax CN columns [9, 10]. All three compounds were shown to be trimeric procyanidins by MS of their methyl ethers and by GPC of their peracetate derivatives [9, 10]. Phenylmethanethiol degradation products and ^{13}C NMR spectra of the phenols and their peracetates showed that all three compounds were composed of 2,3-*cis*-procyanidin extender units with 2,3-*trans* terminal units. These trimers were present in loblolly pine phloem in proportions of about 2 : 1 : 1, and similar relative yields were obtained in syntheses from reactions of loblolly pine phloem tannins with (+)-catechin [9, 10].

The trimer present in the highest proportion was shown to be epicatechin-(4β-8)-epicatechin-(4β-8)catechin (*8*). Partial thiolytic cleavage gave compound *3* showing a (4β-8) linkage to the terminal unit and a dimer benzyl sulfide (*9*) which gave epicatechin-(4β-8)-epicatechin (*10*) on desulfurization with Raney nickel. Additional evidence for a (4β-8) linkage between the procyanidin extender units was obtained from the ^{1}H NMR spectrum of the peracetate of compound *8*; the A-ring protons of the upper unit appearing as an AB quartet centered on 6.1 ppm (Fig. 3).

A second trimer was shown to be epicatechin-(4β-6)-epicatechin-(4β-8)-catechin (*11*) by partial acid-catalyzed cleavage in the presence of excess phenylmethanethiol. As in the products of the trimer *8*, the presence of *3* showed a (4β-8) bond for the linkage to the terminal unit. However, a different dimer benzyl sulfide (*12*), which gave epicatechin-(4β-6)-epicatechin (*13*) on desul-

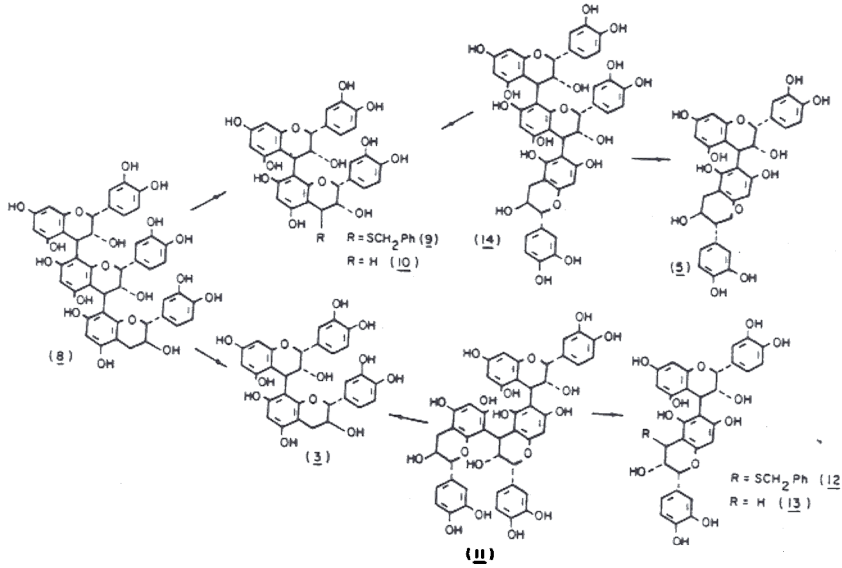

FIG. 3. Partial acid-catalyzed cleavage of trimeric procyanidins in loblolly pine phloem.

furization with Raney nickel, was obtained. This conclusion was supported by the ^{1}H NMR spectrum of the peracetate of *11* that did not show an upfield shift of the upper unit A-ring protons but a multiplet centered at about 6.6–6.8 ppm, which is characteristic of a C(4)–C(6) interflavanoid linkage [10].

The third trimer isolated from loblolly pine phloem was shown to be epicatechin-(4β-8)-epicatechin-(4β-6)-catechin (*14*). Partial acid-catalyzed thiolytic cleavage gave *5* and the dimer benzyl sulfide *9*. ^{1}H NMR of the peracetate of *14* showed an AB quartet centered on 6.1 ppm for the A-ring protons of the upper unit, consistent with an assignment of a (4β-8) bond between the procyanidin chain extenders. The ^{13}C NMR spectra of the phenol and its peracetate [11], along with the optical rotations and CD spectra of this compound as well as the trimers described above, all were consistent with the proposed structures for these compounds [10].

An all-epicatechin trimer was synthesized by reaction of the condensed tannins from horse chestnut or *Photina glabrescens* leaves with (−)-epicatechin. This compound had chromatographic and spectral properties consistent with the trimer C1 that was isolated previously by Haslam and co-workers from horse chestnut [1]. The compound was assigned an epicatechin-(4β-8)-epicatechin-(4β-8)-epicatechin structure (*15*), but no evidence was given for the location of the interflavanoid bonds. Partial acid-catalyzed thiolytic cleavage of our compound gave the dimer *10* and the dimer benzyl sulfide *9*, in addition to epicatechin and epicatechin-4-benzylsulfide, confirming the structure proposed by Thompson et al. [1] (Fig. 4).

Although the isolation of the trimeric procyanidins *8*, *11*, and *14* from the

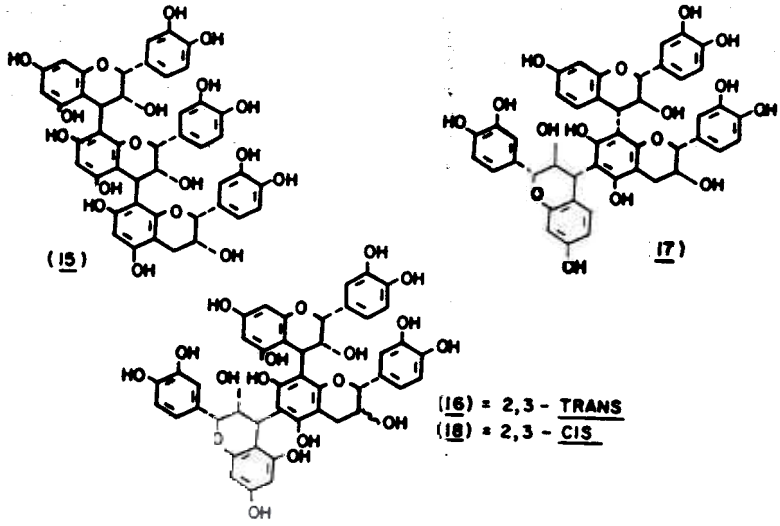

FIG. 4. Related trimeric proanthocyanidins of particular importance to interpretation of polymer structure.

phloem of loblolly pine suggests that the linear, thread-like structure proposed by Haslam and co-workers [1–6] for polymeric procyanidins may not be applicable to the polymers of pine bark, the occurrence of these compounds does not necessarily imply that the latter exist as branched-chain polymers. We have not as yet isolated the trimer epicatechin-(4β-6)-catechin-(8-4β)-epicatechin (*16*) from phloem extracts or from products of synthesis by reaction of loblolly pine phloem tannins with (+)-catechin. Botha et al [20] have synthesized the corresponding bi[(−)-fisetinidol]-catechin trimers (i.e., *17*); and here, the greater nucleophilicity of the C-6 position of the catechin unit over the resorcinolic ring would be expected to promote the formation of this "doubly substituted" product. Since the isolation and characterization of the trimers *16* and *18* is central to definitive proof of branch points in polymeric procyanidins via partial cleavage, efforts are continuing in their possible synthesis. A series of dimeric, trimeric, and tetrameric procyanidins has recently been isolated from the seeds of *Areca catechu* [21] in which the dimeric procyanidins *3* and *5* and the trimeric procyanidins *8* and *14* were also found. A doubly linked trimer analogous to *16* or *18* was not isolated from this source either.

Polymeric Procyanidins

It is known that condensed tannins can be isolated from plant tissues as homogeneous polymeric proanthocyanidins [15, 22]. Major structural variations include the oxidation states and stereochemistry of the individual C-15 units. Evidence for the location of the interflavanoid bonds in the polymers of the procyanidin class had not been obtained, however.

In earlier work on the chemistry of loblolly pine phloem tannins [15], we

isolated an octa-*O*-methyl(epicatechin)$_2$-4''-phenyl sulfide from the methylated products of partial cleavage in the presence of benzenethiol. Two products, both of which were shown to be methyl ethers of epicatechin–epicatechin dimers were formed when this isolate was treated with Raney nickel. The major product, isolated in a relative concentration of 3 : 1 over the minor product, was shown to be octa-*O*-methylepicatechin-(4β-8)-epicatechin (*19*) by comparison of its TLC R_f values, MS, and optical rotation with the product of methylation of *10* that had been synthesized by reaction of loblolly pine phloem tannins with (−)-epicatechin. The minor product was likewise shown to be octa-*O*-methylepicatechin-(4β-6)-epicatechin (*20*) by similar comparisons with *13* [8] (Fig. 5).

The dimer benzyl sulfide *9* had been partially described as a product of partial cleavage of sorghum tannins [4]. However, no evidence was given for the location of the interflavanoid linkage in this compound. Since compounds *9* and *12* were critical to the proof of structure of the trimeric procyanidins, these two compounds were synthesized in quantities sufficient for their complete characterization by reaction of tannins from *Pinus palustris* with phenylmethanethiol [9–11]. The ether-soluble products of thiolytic cleavage of this tannin were separated on Sephadex LH-20 by elution with chloroform–ethanol mixtures. Both compounds were shown to be epicatechin–epicatechin-4''-benzyl sulfides by MS of their methyl ethers and by ^{13}C NMR of their phenols and acetate derivatives [10–11]. ^{1}H NMR of their deca-acetates showed C(4)–C(8) and C(4)–C(6) interflavanoid bonds for *9* and *12*, respectively, consistent with the formation of *10* and *13* when these compounds were treated with Raney nickel [9]. The corresponding dimer phenyl sulfides *21* and *22* were also synthesized from *Pinus taeda* tannins by reaction with benzenethiol [8].

In addition to the formation of the two dimeric benzyl sulfides *9* and *12* by

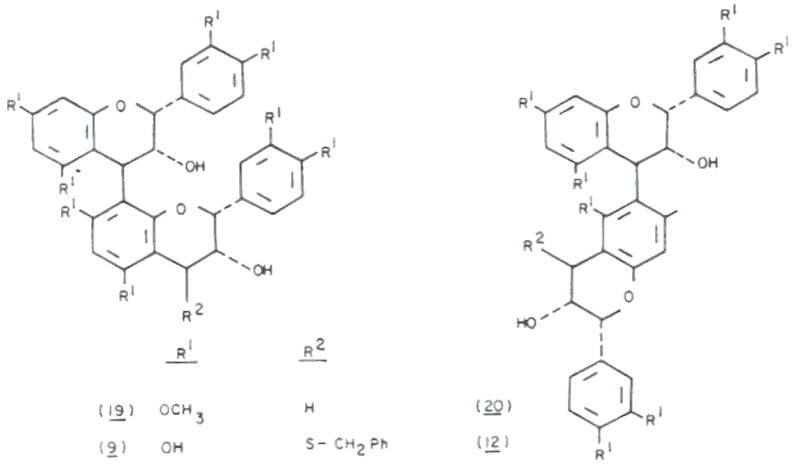

FIG. 5. Dimeric cleavage products from condensed tannins.

reaction with phenylmethanethiol, the partial acid-catalyzed cleavage of tannins from *Photina glabrescens* in the presence of excess phloroglucinol gave the corresponding phloroglucinol adducts epicatechin-(4β-8)-epicatechin-(4β)-phloroglucinol (*23*) and epicatechin-(4β-6)-epicatechin-(4β)-phloroglucinol (*24*). This demonstrated isomerism in the location of interflavanoid bonds in the tannins from these leaf tissues in addition to those of pine bark.

Not all condensed tannins exhibit such a high proportion of C(4)–C(6) interflavanoid bonds, however. Partial acid-catalyzed cleavage of tannins from *Vicia sativa* and the fruits of blueberry or horse chestnut gave small or negligible amounts of the C(4)–C(6)-linked dimer benzyl sulfide [10]. These tannins, therefore, do appear to be largely C(4)–C(8) coupled and thus fit the linear, thread-like structure proposed by Haslam [6].

Examination of the ^{13}C NMR spectra of these compounds together with the products of reaction of (−)-epicatechin with *o*-hydroxybenzyl alcohol suggest that it might be possible to detect variations in the location of the interflavanoid bonds as well as possible branch points in these polymers if higher-resolution spectra (i.e., > 20 MHz) were obtained [11]. Therefore, attention is presently being directed to high-resolution ^{13}C NMR spectra of these polymers as well as to the possible synthesis of the branch point trimers *16* and *18*.

Rates of Acid-Catalyzed Cleavage of Isomeric Procyanidins

Interpretation of the results of partial cleavage of condensed tannins and of biosynthetically patterned synthesis requires knowledge of the kinetics of acid-catalyzed cleavage of isomeric procyanidins. However, there had been no systematic study of the rate of cleavage of procyanidins differing in the location of the interflavanoid bond or in their stereochemistry. To obtain a preliminary estimate of possible variations in the rates of cleavage of isomeric procyanidins, the dimers *3*, *4*, *5*, *10*, and *13* and the benzyl sulfide adducts *9* and *12* were heated in the presence of excess phenylmethanethiol and acetic acid (i.e., HOAc–procyanidin, 35 : 1, pH 3.6). The extent of cleavage was followed by visual comparison of the relative spot intensities representing the procyanidin and its cleavage products on two-dimensional cellulose TLC plates (Schleischer and Schull, F 1440), developed with *t*-butanol–acetic acid–water 3 : 1 : 1 and 6% acetic acid) after spraying the plates with vanillin HCl. Plots of $\log_{10}(C_o - C_t)$ vs. reaction time t indicated pseudo-first-order reactions when these compounds were heated in the presence of excess phenylmethanethiol at constant acid concentrations, so rate constants were determined by the half-life method [23]. Reactions were performed at 60, 70, 80, and 90°C to permit linear regression with respect to the form

$$\ln(K) = -\frac{E_a}{R}\frac{1}{T} + C$$

to give estimates of the activation energy (E_a) and the expected half-lives ($t_{1/2}$ (Table I). These data indicated that there were substantial differences between

TABLE I
Arrhenius Temperature Dependence Parameters for Acid-Catalyzed Cleavage of Procyanidins

Parameter	Procyanidin (3)	(10)	(4)	(5)	(13)
	65.7	62.9	82.2	86.6	87.3
	15.7	15.0	19.7	20.7	20.9
Constant C	20.6	19.5	25.6	26.8	27.1
Regression coefficient	0.982	0.997	0.993	0.998	9.988
Half-life ($t_{1/2}$ hours) at °C					
100	1.2	1.5	1.7	2.1	1.9
90	2.2	2.7	3.2	4.5	4.1
80	4.1	4.9	7.8	10.2	9.2
70	7.8	9.2	17.6	23.9	21.9
60	15.7	17.9	42.1	59.6	55.1
50	32.1	35.7	103	154	143
30	162	167	780	1290	1220
20	393	392	2370	4150	3970

isomeric procyanidin dimers in their lability to acid-catalyzed cleavage. Procyanidins with a C(4)–C(6) interflavanoid bond, epicatechin-(4β-6)-catechin (*5*) and epicatechin-(4β-6)-epicatechin (*13*), were much more resistant to cleavage than their C(4)–C(8) pairs epicatechin-(4β-8)-catechin (*3*) and epicatechin-(4β-8)-epicatechin (*10*). Catechin-(4α-8)-catechin (*4*) was more resistant to cleavage than either *3* or *10*, which were cleaved at similar rates.

Differences in the rates of cleavage of the procyanidins at different temperatures were large enough to permit comparisons between them (see Table I for the regression coefficients for activation energies). However, cleavage rate constants were comparatively insensitive to variations in acid concentration over a range of acetic acid–procyanidin from 35 : 1 to 1 : 1 (i.e., pH 3.6 to 5.4). Other approaches to measuring the extent of cleavage of these compounds will be required to define these relationships at varying acid concentrations.

The results help to explain the products of partial acid-catalyzed cleavage of polymers over varying periods of time. For example, the degradation of longleaf pine bark tannins led initially to the rapid formation of *9*, reaching a maximum concentration in 3–4 h, while the C(4)–C(6)-linked isomer *12* was initially formed in low yield and reached a maximum concentration after 18–20 h. These observations are partly explained by the fact that the C(4)–C(6) bond is cleaved at a slower rate than the C(4)–C(8) bond in acid solution, but they fail to explain

the increase in the absolute concentration of *12* after longer reaction periods. This behavior may be rationalized if it is assumed that the C(4)–C(6) linkages are initially inaccessible to attack.

These observations suggest that the polymers could be of two types: The first group would include those of horse chestnut, etc., where the interflavanoid linkages appear to be almost exclusively C(4)–C(8), and are linear polymers that are rapidly cleaved in acidic phenylmethanethiol. The second group would include the tannins of pine bark and *Photina glabrescens* that contain a comparatively high proportion of C(4)–C(6) interflavanoid bonds and may be highly branched structures. Thiolytic degradation of the latter polymers is much slower [15] and proceeds initially by cleavage of the peripheral units, mostly C(4)–C(8) linked, before the internal C(4)–C(6) bonds become accessible to attack. Further experiments such as high-field ^{13}C NMR are in progress to explore this proposal. Resolution of this question is important, since it implies that the biosynthesis of one class of tannins is under enzymic control, both in the formation of the flavanyl carbocation and in the location of its condensation on flavan-3-ols or oligomeric procyanidins. Others may be under enzymic control only in the formation of the flavanyl carbocation. Additionally, many important properties of these two types of tannins would be expected to differ depending on whether they existed as linear, thread-like chains or were rather more globular, branched-chain structures.

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