

Variations in the Heterogeneity of the Decay of the Fluorescence in Six Procyanidin Dimers

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Abstract: The decay of the fluorescence has been measured in 1,4-dioxane for six dimers of (2*R*,3*R*)-(-)-epicatechin and (2*R*,3*S*)-(+)-catechin, hereafter denoted simply epicatechin and catechin. The dimers are epicatechin-(4β→8)-catechin, epicatechin-(4β→8)-epicatechin, catechin-(4α→8)-catechin, catechin-(4α→8)-epicatechin, epicatechin-(4β→6)-epicatechin, and epicatechin-(4β→8;2β→0→7)-epicatechin. The monomers and the bridged dimer have a fluorescence that decays as a single exponential. The remaining five dimers have a heterogeneous decay that can be described by the sum of two exponentials. The heterogeneity is most apparent in the two dimers with 4α→8 interflavan bonds. In view of the molecular origin of the heterogeneous decay in the presence of two rotational isomers at the interflavan bond, polymeric procyanidins with predominantly α stereochemistry for the interflavan bond at C(4) should be more disordered and more compact than those with predominantly β stereochemistry.

The most common naturally occurring polymeric procyanidins are polymers of two 2,3-flavan-3-ols, (2*R*,3*R*)-(-)-epicatechin and (2*R*,3*S*)-(+)-catechin, hereafter referred to simply as epicatechin and catechin. These polymers are found in the leaves, fruits, and barks of many woody and herbaceous plants.¹ Interest in these polymers is increasing because of their potential as a renewable source of useful chemicals,¹ their probable use by plants as a defense mechanism,² and their formation of complexes with a variety of naturally occurring and synthetic polymers.³⁻⁷

An interflavan bond from C(4) of one monomer unit to C(8) of its neighbor is the most common linkage between monomer units in the naturally occurring polymers.⁸ Interflavan bonds from C(4) to C(6) also occur. Figure 1 depicts the covalent structures of six dimers of catechin and/or epicatechin. The trivial nomenclature of the six unbridged dimers is procyanidin B1, B2, B3, B4, B5, and B7. The full nomenclature described by Hemingway et al.⁹ is epicatechin-(4β→8)-catechin, epicatechin-(4β→8)-epicatechin, catechin-(4α→8)-catechin, catechin-(4α→8)-epicatechin, epicatechin-(4β→6)-epicatechin, and epicatechin-(4β→6)-catechin, respectively. These names are based on the constituent monomer units and the location and stereochemistry of the interflavan bond between monomer units. Figure 2 depicts the covalent structure of two bridged dimers, procyanidin A1 and A2. Their longer names are epicatechin-(4β→8;2β→0→7)-catechin and epicatechin-(4β→8;2β→0→7)-epicatechin, respectively. The bridges from C(2) to C(7) in procyanidin A1 and A2 prevent internal rotation about the interflavan bond.

Since it was first reported a few years ago,^{10,11} the fluorescence of the oligomeric procyanidins has played an important role in the development of an understanding of the conformations of the

higher polymers. Upon excitation near 280 nm in dilute solution in 1,4-dioxane, both monomers show a single emission band at 320–324 nm.^{11,12} The decay of the intensity of the fluorescence, $I(t)$, can be described by a single exponential.

$$I(t) = \alpha \exp(-t/\tau) \quad (1)$$

The fluorescence lifetimes, τ , for the two monomers are indistinguishable.¹¹ Higher oligomers exhibit an emission maximum in the same spectral region, but with a lower fluorescence quantum yield, Q , than that obtained with the monomers.^{11,12} Although a constrained dimer, procyanidin A1, exhibits a fluorescence decay that can be described by eq 1, two unconstrained dimers, procyanidins B1 and B7, exhibit a heterogeneous decay that requires the sum of two exponentials for an adequate description.¹¹

$$I(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \quad (2)$$

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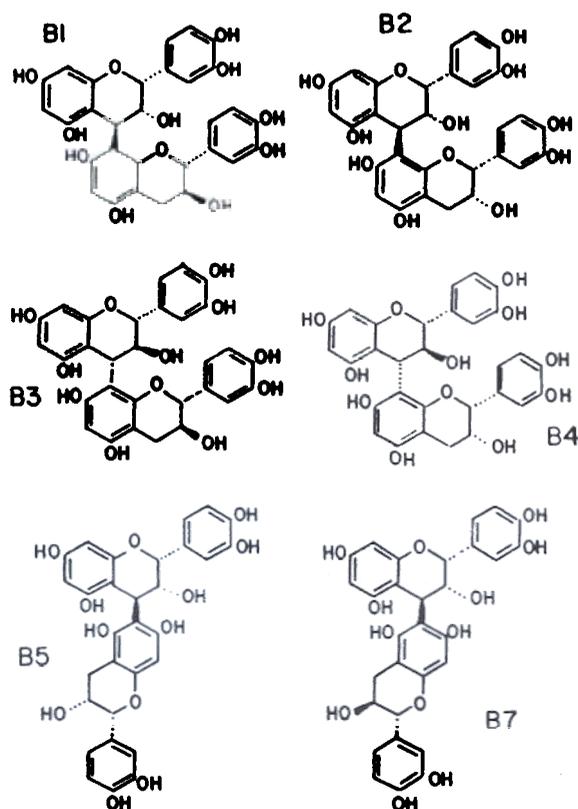


Figure 1. Covalent structures of six unbridged dimers. Procyanidins: B1, epicatechin-(4 β →8)-catechin; B2, epicatechin-(4 β →8)-epicatechin; B3, catechin-(4 α →8)-catechin; B4, catechin-(4 α →8)-epicatechin; B5, epicatechin-(4 β →6)-epicatechin; B7, epicatechin-(4 β →6)-catechin.

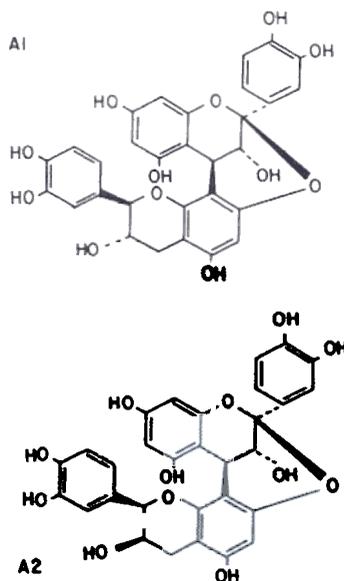


Figure 2. Covalent structures of two bridged dimers. Procyanidins: A1, epicatechin-(4 β →8;2 β →0→7)-catechin; A2, epicatechin-(4 β →8;2 β →0→7)-epicatechin.

The heterogeneous decay in the unconstrained dimers arises from the presence of two rotational isomers at the interflavan bond, interconversion of which is slow on the time scale for fluorescence. The relative populations of the two rotational isomers can be extracted from the contribution to the fluorescence decay curves of the two terms in eq 2.¹¹ Rotational isomeric state analysis of the higher polymers,^{13,14} utilizing the relative populations of the

two rotational isomers deduced from the analysis of the fluorescence decay curves for procyanidins B1 and B7, leads to the conclusion that the polymers are disordered and more compact than polystyrene chains of the same molecular weight.

A more extensive examination of the decay of the fluorescence in this class of compounds is reported here. Reported for the first time are the decays of the fluorescences of procyanidins B2, B3, B4, B5, and A2. While confirming the earlier conclusions about the origin of the heterogeneous fluorescence decay from rotational isomerism, the data for the more extensive set of dimers show that there is a much broader range for the relative contributions of the two terms in eq 2 than was apparent from the study of procyanidins B1 and B7. When combined with rotational isomeric state theory, this result has profound implications for the conformational versatility of the polymeric procyanidins. It implies that plants can manipulate the stiffness and the extension of their polymeric procyanidins over a broad range by manipulation of the location and stereochemistry of the interflavan bond.

Experimental Methods and Details

Catechin and epicatechin were purchased from Sigma Chemical Co. The details of the preparation and purification of the dimers and characterization via ¹³C nuclear magnetic resonance, specific rotation, and FAB mass spectrometry,¹² have been described elsewhere.^{12,15-17} 1,4-Dioxane (spectrophotometric grade) purchased from Aldrich Chemical Co. was used without further purification. Freshly prepared solutions were used through this study due to the sensitivity of solutions of the samples to exposure to light for long periods of time.

Ultraviolet absorption measurements were performed at ambient temperature with a Perkin-Elmer Lambda Array 3840 UV/vis spectrophotometer equipped with a photodiode array and a deuterium lamp and interfaced with Perkin-Elmer Computerized Spectroscopic Software. Absorbances at 295 nm for the monomers and 290 nm for the dimers, which were the wavelengths used for excitation in the fluorescence lifetime measurements, were in the range of 0.07–0.11.

Time-resolved fluorescence measurements achieved by time-correlated single-photon counting^{18,19} were used to determine the decay curves for the fluorescence of the monomeric and dimeric procyanidins in solution. The excitation source was a Model 702 ultrafast dye laser (Coherence Co.), synchronously pumped by a frequency doubled, mode-locked Model Antares 76-s Nd:YAG laser (Coherence Co.). A 7220 cavity dumper (Coherence Co.) was used in the dye laser systems to provide selectable output pulse repetition rates. A Model 5-14A autocorrelator (Inred Co.) was used to measure the temporal width of the pulse emitted from a high repetition rate mode-locked laser. The fwhm (full width, half maximum) of the dye laser pulse was about 4 ps. The fwhm of the instrumental response function was about 580 ps. A repetition rate of 5.4 MHz was used with the time-to-amplitude converter. An R928 photomultiplier, manufactured by Hamamatsu Corp., was used. The filter used was a 305-nm cutoff filter (WG-305, Rolyon Arcadia, CA). A counting rate of about 18K counts/s was used for detecting the decay of the fluorescence. The collection of data was continued until the decay curves had above 20K counts in the peak. The decay curves were stored in the range of 0–441 channels. Time calibration was 0.03623 ns/channel. The data acquisition time required was usually in the range of 20–40 s. Electronics manufactured by EG & G Ortec Co. were used for photon timing. Magic-angle conditions were employed. The solvent blank for 1,4-dioxane was subtracted. Measurements were performed at ambient temperature.

The standard fluorophore for calibration was anthracene in cyclohexane. The lifetime of the anthracene in cyclohexane was about 4.29 ns, with a decay described by a single exponential. This value agreed with the literature value¹⁹ of 4.10 ns. Coffee creamer was used as a scatterer. All samples were maintained at ambient temperature. Data acquisition was controlled by both multichannel analyzer (MCA) and deconvolution software produced by Edinburgh Instruments, Ltd. (Scotland). A non-linear least-squares method was used to analyze the time-resolved decay

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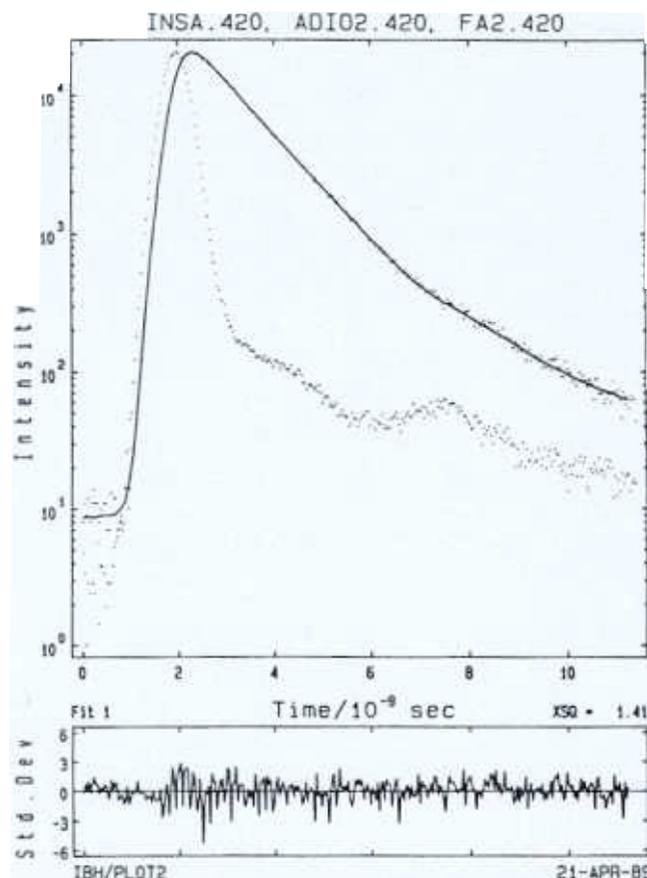


Figure 3. Fluorescence decay curve fitted to a single-exponential function for procyanidin A2 in 1,4-dioxane. In the upper panel, the sharper peak with dots is the measured excitation pulse profile; the broader peak with dots is the measured decay of the sample used; the solid line is the curve fitted to exponential functions. The bottom panel represents the residuals. XSQ means the value of χ^2 .

of the fluorescence. The goodness of fit is determined by the value of χ^2 , which is calculated by the following equation.²¹

$$\chi^2 = \sum w_i [R(t) - R_c(t)]^2 \quad (3)$$

Here w_i is a statistical weighting factor ($1/R(t)$) and $[R(t) - R_c(t)]$ is the residual. The residuals must be randomly distributed around zero for a good fit. The fraction of the fluorescence intensity contributed by an individual component is determined by

$$f_i = \alpha_i \tau_i / \sum (\alpha_i \tau_i) \quad (4)$$

The α_i and τ_i are varied during calculations until the best fit is achieved, $0 < f_i < 1$.

Results

The ultraviolet absorption spectra show the characteristics reported earlier.^{12,20} In 1,4-dioxane, dilute solutions of all samples except the bridged dimer had a maximum absorption at 280–282 nm. The maximum for the bridged dimer was at 277 nm.

Fluorescence Lifetime Measurements. A. Monomers. The decays of the fluorescence of the monomers were measured in 1,4-dioxane. The decay curves were adequately fitted to a single-exponential decay function, with this decay function being independent of emission wavelength in the range of 310–350 nm. The values of the χ^2 were 1.55–1.67, the residuals fluctuated randomly around zero, and the quality of the fit did not improve when the decay function was assumed to be a sum of two exponentials. The values of τ are 2.06 ± 0.01 ns for catechin and 2.05 ± 0.02 ns for epicatechin. Previously Bergmann et al.¹¹ reported τ 's of 2.00 ± 0.05 and 2.04 ± 0.05 ns for catechin and epicatechin, respectively, in 1,4-dioxane.

Table I. Double-Exponential Fluorescence Decays for Five Dimers in 1,4-Dioxane^a

compnd	lifetime (ns)		$f_1 = I - f_2$	χ^2 range
	τ_1	τ_2		
B1	1.523 ± 0.004	0.855 ± 0.083	0.940 ± 0.006	1.46–1.66
B2	1.570 ± 0.016	0.823 ± 0.015	0.974 ± 0.003	1.55–1.72
B3	1.472 ± 0.010	0.955 ± 0.012	0.586 ± 0.022	1.37–1.58
B4	1.341 ± 0.021	0.486 ± 0.028	0.791 ± 0.015	1.46–1.68
B5	1.420 ± 0.007	0.880 ± 0.050	0.950 ± 0.012	1.53–1.67

^a Mean values and standard deviations obtained from three to five experiments. The excitation wavelength is 290 nm. The decay of the fluorescence for procyanidin B2 can be fit equally well by a single exponential with $\tau = 1.54$ ns.

B. Bridged Dimer. The fluorescence decay curve of procyanidin A2 is described by a single exponential, eq 1, with a τ of 1.07 ± 0.01 ns. This value of τ is virtually identical with the result (1.06 ± 0.10 ns) obtained previously for another bridged dimer, procyanidin A1.¹¹ The only difference in the covalent structure between procyanidin A1 and A2 is in the configuration at C(3) of the bottom monomer unit. A single-exponential fit for the procyanidin A2 decay curve is depicted in Figure 3, representing the random fluctuation of the residuals around zero with the value of χ^2 being 1.41. When the double-exponential function in eq 2 was assumed to fit the decay curve, neither the χ^2 value nor the residuals were improved at all. Furthermore, the contribution by the longer lifetime component was negligible (less than 1%). These results for procyanidin A2 confirm the prior conclusion¹¹ that prevention of rotation about the interflavan bond in a bridged dimer results in a single-exponential decay for the fluorescence. In contrast, a heterogeneous decay is seen in most dimers that are not bridged, as described in the next section.

C. Dimers without a Bridging Ring. The fluorescence decay curves for five unbridged dimers, procyanidin B1, B2, B3, B4, and B5, were measured in 1,4-dioxane. The results are summarized in Table I. The decay curve for epicatechin-(4 β →8)-catechin (procyanidin B1) was adequately fitted by a double-exponential function, with random fluctuation of the residuals. The major component of the decay has the longer lifetime (1.52 ns), with the fraction of the intensity from this component being about 94%. The minor component is the one with the shorter lifetime (0.86 ns). When the present decay data were fitted to a single-exponential function, a slightly poorer fit was obtained, as indicated by the χ^2 value of 1.77. The small contribution to the overall decay by the process with the shorter lifetime has a detectable influence. Fitting this decay curve to the sum of three exponentials was impossible because it led to a negative preexponential factor for one of the components. An earlier experiment also found that the decay of the fluorescence of procyanidin B1 is described by a sum of two exponentials, but the τ_i and f_i were somewhat different.¹¹ The significance of the difference will be addressed in the next section.

The decay curve for epicatechin-(4 β →8)-epicatechin (procyanidin B2) was fitted almost equally well to eq 1 and 2. This decay was well approximated as a single exponential due to a very small (2.6%) fractional contribution by the component with the shorter lifetime and to no significant improvement in the χ^2 (from 1.55 to 1.58) upon changing from a single- to a double-exponential fit. Fitting to three exponentials was impossible for the same reason as described above in the case of epicatechin-(4 β →8)-catechin.

The decay curves for catechin-(4 α →8)-catechin (procyanidin B3) and catechin-(4 α →8)-epicatechin (procyanidin B4) in 1,4-dioxane were best described by eq 2. When these data were fitted to single-exponential functions, the values of the χ^2 were very large (3.06 for catechin-(4 α →8)-catechin and 12.17 for catechin-(4 α →8)-epicatechin). Fitting to the sum of two exponentials resulted in a large improvement of the χ^2 values, to 1.37 and 1.46, respectively, and random fluctuation of the residuals around zero. There was no further improvement in the χ^2 when the decays were

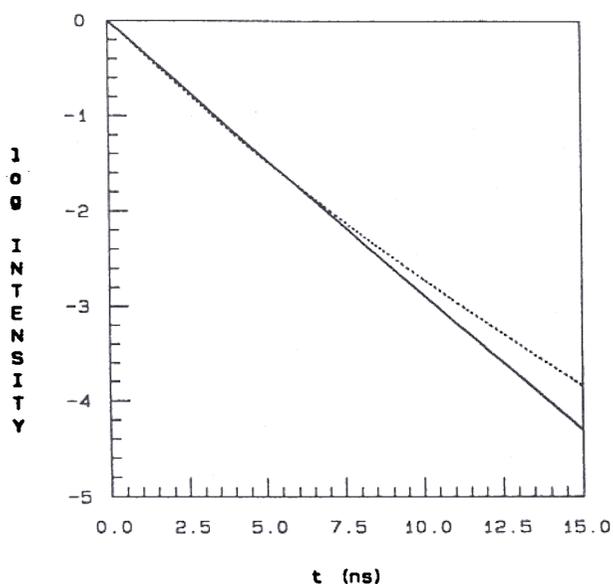


Figure 4. Comparison of the fluorescence decays, as $\ln I$ vs t , described by the parameters deduced for procyanidin B1 in the present work (solid line) and in Bergmann et al.¹¹ (dashed line).

In catechin-(4 α →8)-catechin the contribution by the shorter component to the fluorescence decay is about two-thirds of the contribution by the longer component, and in catechin-(4 α →8)-epicatechin the contribution by the shorter component is about one-fourth of that by the longer component.

The fluorescence decay data of epicatechin-(4 β →6)-epicatechin (procyanidin B5) were similar to those of epicatechin-(4 β →8)-epicatechin (procyanidin B2), but τ of the major component in procyanidin B5 was $\approx 10\%$ shorter than that of procyanidin B2, as shown in Table I. The small contribution (about 5%) to the decay by the shorter component is responsible for the heterogeneity of the fluorescence decay. Fitting this curve to the sum of three exponential functions was impossible due to a negative relative amplitude of one of the components.

Discussion

Three of the compounds (epicatechin, catechin, procyanidin B1) studied here with a laser as the source of excitation were also studied earlier¹¹ with a system that employed a deuterium flash lamp. Comparison can also be made between procyanidins A1 and A2, because these two constrained dimers differ only in the stereochemistry of the attachment of the hydroxyl group at C(3) of the lower monomeric unit, as depicted in Figure 2.

The two studies yield experimentally indistinguishable decay curves for epicatechin and catechin in 1,4-dioxane, and the decay curve for procyanidin A2 in this solvent is indistinguishable from the one measured earlier for procyanidin A1. All of these decay curves can be described by eq 1 with a value of τ that is about 2.05 ns (for epicatechin and catechin) or 1.07 ns (for procyanidins A1 and A2). Hence, both systems yield the same results for those samples that have a fluorescence decay describable by a single exponential with $\tau > 1$ ns.

The decays obtained for procyanidin B1 in 1,4-dioxane, with either system, cannot be successfully described by eq 1, but they can be described by eq 2. Synthetic decay curves calculated from the two sets of decay parameters are compared in Figure 4. The differences between the two curves are very small over the first decade. The value of τ_1 in the present work, 1.523 ± 0.004 ns, is very close to the weighted average of τ_1 (1.25 ± 0.08 ns) and τ_2 (2.0 ± 0.4 ns) from the previous work. The present results are likely to be the more accurate because they were obtained with instrumentation with a faster instrument response function and under conditions where data collection required a much shorter time.

The minor alteration in the description of the heterogeneity of the decay of the fluorescence of procyanidin B1 does not require

any alteration in the interpretation of the molecular origin of the decay parameters. The interpretation is that each of the two rotational isomers at the interflavan bond is responsible for one of the two terms in eq 2. Of course, restriction of the populations to a single rotational isomer, by the introduction of a bridging ring, must produce the simpler decay described by eq 1, as measured initially for procyanidin A1¹¹ and as confirmed here for procyanidin A2. The population of the two rotational isomers in procyanidin B1 was extracted from the two preexponential factors in eq 2. The value of $\alpha_1(\alpha_1 + \alpha_2)^{-1}$, 0.75 ± 0.15 , reported earlier¹¹ is not convincingly different from the value, 0.90 ± 0.01 , found in the present work.

$$\alpha_1(\alpha_1 + \alpha_2)^{-1} = f_1\tau_2[\tau_1 - f_1(\tau_1 - \tau_2)]^{-1} \quad (5)$$

The results reported here for procyanidins B1, B2, B3, B4, and B5 do modify in a significant way the qualitative picture of the conformational characteristics of the family of procyanidin polymers. The values of $\alpha_1(\alpha_1 + \alpha_2)^{-1}$ obtained for procyanidins B1 and B7 by Bergmann et al.¹¹ were 0.75 ± 0.15 and 0.85 ± 0.08 , respectively. Qualitatively, these results show that the less abundant rotational isomer is present in sufficient amount to cause the polymeric procyanidins of high molecular weight to be relative compact, disordered polymers. The basis for this conclusion is a rotational isomeric state analysis of the unperturbed mean square end-to-end distance, $\langle r^2 \rangle_0$, of the high polymers.^{13,14} The values of $\langle r^2 \rangle_0$ obtained from this analysis depend strongly on the relative population of the two rotational isomers at the interflavan bond. If the relative population is that deduced from the values of $\alpha_1(\alpha_1 + \alpha_2)^{-1}$ for procyanidins B1 and B7 by Bergmann et al.,¹¹ the high polymers will be disordered, with values of $\langle r^2 \rangle_0$ that are somewhat smaller than those for polystyrene chains of the same molecular weight.

The present results modify the picture of the conformational versatility of the polymeric procyanidins because the range for $\alpha_1(\alpha_1 + \alpha_2)^{-1}$ is much broader, extending from a low of 0.48 ± 0.02 (for procyanidin B3) to a high of 0.95 ± 0.01 (for procyanidin B2). The location and stereochemistry of the interflavan bond can produce dimers in which the two rotational isomers are populated on an equal basis, as illustrated by procyanidin B3, or dimers in which one rotational isomer is populated very nearly to the exclusion of the other, as illustrated by procyanidin B2. This range has profound implications for the values of $\langle r^2 \rangle_0$ obtained for polymers of high molecular weight because the rotational isomeric state analysis shows that the mean square unperturbed dimensions increase dramatically when one rotational isomer has a strong dominance over the other. In the limit where only a single rotational isomer is populated, the chains become rodlike with a local helical character. When the experimental values for $\alpha_1(\alpha_1 + \alpha_2)^{-1}$ reported here are interpreted in light of the rotational isomeric state analysis,^{13,14} it is apparent that the conformations of the high polymers can range all the way from molecules that are disordered and quite compact to molecules that are highly extended with a local helical structure. Hence, plants can control both the overall extension and the helical nature of the polymeric procyanidins that they produce by selection of the location and stereochemistry of the interflavan bond.

The values of f_1 for the five dimers in Table I separate naturally into two groups. Procyanidins B1, B2, and B5 have $\alpha_1(\alpha_1 + \alpha_2)^{-1} > 0.9$, and procyanidins B3 and B4 have $\alpha_1(\alpha_1 + \alpha_2)^{-1} < 0.6$. The three procyanidins in the first group have β stereochemistry for the attachment of the interflavan bond at C(4), and the two procyanidins in the latter group have α stereochemistry for this attachment. The results suggest that high polymers with interflavan bonds with predominantly α stereochemistry may be more disordered than those with predominantly β stereochemistry. It is difficult to draw any conclusions about the influence of the location of the interflavan bond, i.e., C(4)-C(6) vs C(4)-C(8), because only one dimer, procyanidin B5, has the former type of interflavan bond.

Control of the extension and local helical structure may be related to the biophysical role of the polymeric procyanidins as a plant defense mechanism if it provides a basis for specificity

in the formation of complexes between the polymeric procyanidins and other types of biopolymers. It may also become important in potential commercial applications of these polymers.

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Registry No. Procyanidin B1, 20315-25-7; procyanidin B2, 29106-49-8; procyanidin B3, 23567-23-9; procyanidin B4, 29106-51-2; procyanidin B5, 12798-57-1; procyanidin A1, 12798-56-0.