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Exploring the conformations of polyflavanoids - An approach to understanding the significance of tannins

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INTRODUCTION

Reflection on my 25 years of research at our USDA Forest Service Laboratory in Pineville, Louisiana suggests that at least a third and possibly closer to half of the research we have done on polyflavanoids is in one or another way connected with attempts to understand the conformational properties of these compounds. Our concentration on definition of the preferred conformations and conformational flexibility of polyflavanoids is due to our belief that both the commercial and ecological significance of polyflavanoids rest, to large degree, on the relationship of conformation with the complexation of these compounds with other biopolymers (particularly proteins and carbohydrates).

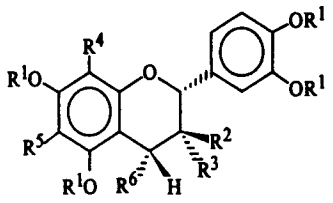
This review is not prompted by the notion that we have solved all the problems so it is now time for a synthesis of that work, but rather that I believe we have entered a new and exciting phase of tannin chemistry where both NMR and computational approaches have grown to considerable power at the same time when tannin research is increasingly being focused more on the biological significance of these compounds.

In this review, I have summarized both the research conducted at our laboratory in Pineville and the work done by our partners and colleagues who have collaborated with us at various laboratories around the world. We must limit this review to milestones that I consider to be the most important parts of that effort, what I think we now know, and some discussion of what I believe are priority issues that need attention. If we are to more fully understand the biological significance of condensed tannins, and particularly their complexation with other biopolymers, we must continue to advance our understanding of the conformational preferences

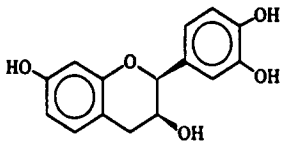
and flexibility of these compounds, particularly the free phenols in water, for which we now have only limited data.

CRYSTAL STRUCTURES OF CONSTITUENT FLAVAN-3-OLS

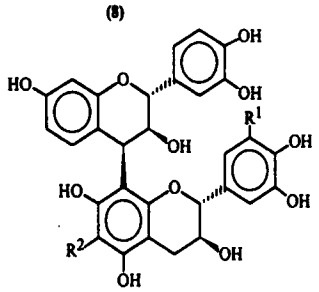
Before embarking on any attempt to define the conformations of polyflavanoids, by either physical means or molecular modeling, it is helpful, if not mandatory, to have solid data for bond lengths as well as both bond and torsional angles present in the monomeric units that are repeat units of a polymer. On meeting Professor Mattice, one of his first questions was " what crystal structures had been defined ?" He needed physical data to help guide molecular modeling experiments (in those days the MM2 force-field). Engel *et al.* (1978) had reported on the crystal structure of 8-bromo-tetra-*O*-methyl-(+)-catechin **1** but crystal structure data for the free phenols was lacking. It is important to note that, despite the fact that (+)-catechin **2** is easily crystallized from water, the solid state structure of this most common flavan-3-ol is still not defined because it has not yet been crystallized in a form amenable to a solid state structure determination. Therefore, focus of our initial work was to build a data base for the crystal structures of constituent flavan-3-ols and their derivatives and early molecular modeling work was centered on the conformations of these compounds centered on studies of tetra-*O*-methyl-(+)-catechin **3** and tetra-*O*-methyl-(-)-epicatechin **4** (Mattice *et al.*, 1982).



	R^1	R^2	R^3	R^4	R^5	R^6
(1)	OMe	OH	H	Br	H	H
(2)	H	OH	H	H	H	H
(3)	OMe	OH	H	H	H	H
(4)	OMe	H	OH	H	H	H
(5)	H	H	OH	H	H	H
(6)	OMe	H	OAc	H	Br	H
(7)	H	OH	H	H	H	OH
(9)	OAc	OAc	H	H	H	H
(10)	OMe	OH	H	H	H	Phloro-OMe$_3$



(8)



	R^1	R^2
(11)	H	H
(12)	OH	Fisetinidol

During our studies of the regioselectivity of bromination and hydroxybenzylation of catechin and epicatechin (McGraw and Hemingway, 1982), we noticed some beautiful crystals of (-)-epicatechin **5**. These crystals were particularly important because they were anhydrous so

they provided access not only to the solid state structure (fig. 1) but also their dipole moment (Fronczek *et al.*, 1984). These physical data provided information needed for interpretation of molecular modeling studies of the phenolic procyanidins using the MM2 force field (discussed more fully below). In those days, NMR data were not especially helpful because of poor resolution of the heterocyclic ring proton couplings, particularly for the 2,3-*cis* isomers. However, with newer high-field machines and lineshape analysis of C(3)-H of flavan-3-ols such as epicatechin (Hemingway *et al.*, 1996), or even dimeric procyanidins in the free phenolic form (see below), it is possible to obtain a close estimate of all coupling constants of the heterocyclic ring. For epicatechin (fig. 2), the resulting $J_{2,3}$ of 1.6 Hz is large in comparison with that predicted by crystal structure data. Analysis of the splitting pattern for C(3)-H also provides information on torsional angles between C(3)-H and C(4)-H_α and C(4)-H_β and for epicatechin these angles result in $J_{3,4}$ couplings of 3.3 and 4.5 Hz, respectively.

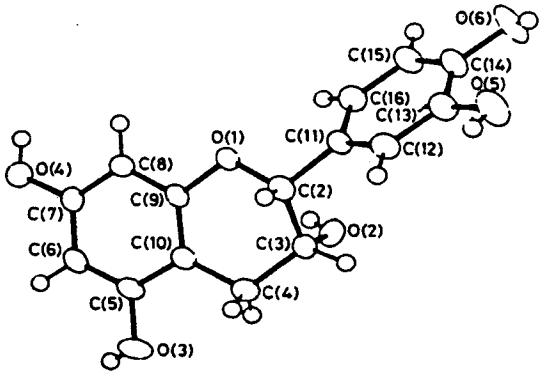


Figure 1. Crystal structure of (-)-epicatechin (Fronczek *et al.*, 1984)

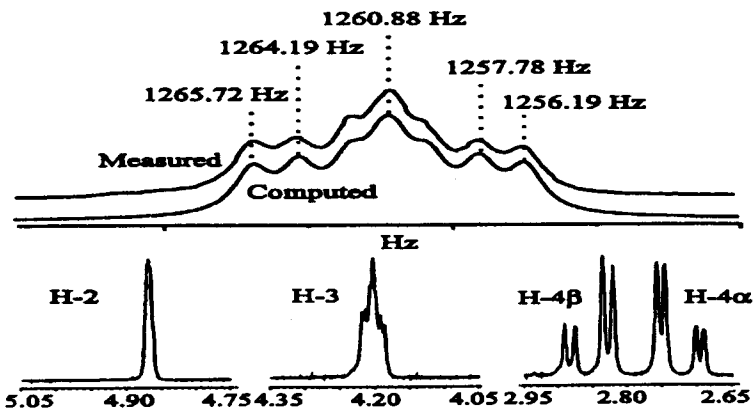


Figure 2. Heterocyclic ring coupling constants of (-)-epicatechin as established by lineshape analysis of C(3)-H (Hemingway *et al.*, 1996). These data together with results of a Multi-NOE experiment show $J_{2,3}$ = 1.6; $J_{3,4\alpha}$ = 3.3; $J_{3,4\beta}$ = 4.5; $J_{4\alpha,4\beta}$ = 16.8 Hz.*

* see p. 84

Boeyens *et al.* (1986) definition of the crystal structure of (-)-3-*O*-acetyl-6-bromo-3',4',5,7 tetra-*O*-methylepicatechin **6** was important in providing information on the effects of derivatization of these compounds through comparison with the solid state structure of epicatechin (Fronczek *et al.*, 1984). The conformation of the heterocycle in the brominated, methylated, and acetylated derivative is similar to that found for (-)-epicatechin or close to a half-chair but with some distortion to a C(2)-sofa. The catechol ring is approximately equatorial and the 3-acetoxyl is approximately axial (fig. 3).

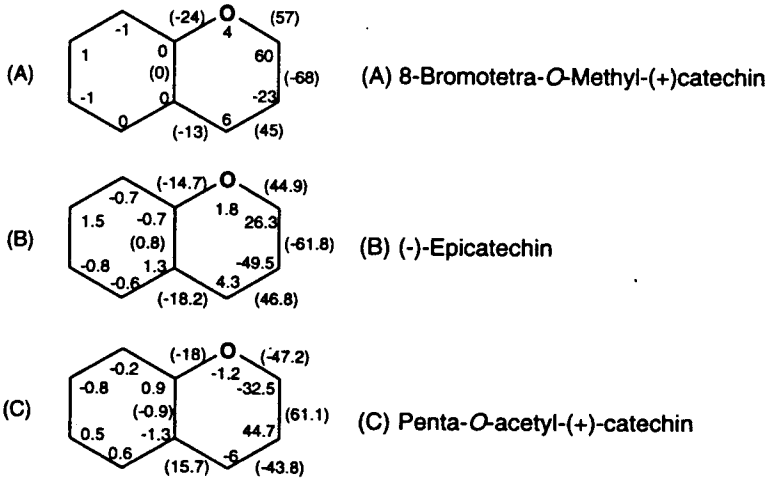


Figure 3. Comparison of A- and C-ring geometries in crystal structures of flavan-3-ols

Porter *et al.* (1985) added significantly to our store of information on the structure of the phenolic forms of flavan-3-ols and their derivatives through his work on the crystal structure of the flavan-3,4-diol, 2*R*,3*S*,4*R* 2,3-*trans*-3,4-*trans* leucocyanidin **7**, thereby largely overcoming the absence of solid state structure data for (+)-catechin. As was found in (-)-epicatechin, the heterocyclic ring of leucocyanidin also was basically a half-chair but with more C(3)-sofa character than was seen in the solid state structure of (-)-epicatechin.

As a final example of the solid state structure of a flavan-3-ol in the phenolic form, we (Tobiason *et al.*, 1993) have examined the structure of the 5-deoxy 2*S*,3*S* *ent*-epifisetinidol **8**. In this compound (fig. 4) the catechol B-ring is in an approximate equatorial position forcing a "reverse" half chair heterocyclic ring conformation. As was found in previous examples, there is a tendency to distortion of the ring to a C(3)-sofa. Except for differences expected from the opposite absolute stereochemistry, bond lengths and angles are fairly similar in *ent*-epifisetinidol and (-)-epicatechin and are predicted reasonably by a variety of forcefields.

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* Dr. Adrienne Davis recently pointed to a discrepancy in earlier assignments for (-)-epicatechin and Dr. Petrus J. Steynberg, using a NOEMULT experiment, verified that assignments of the H-4 protons in (-)-epicatechin should be inverted from those commonly quoted in the literature (Hemingway *et al.*, 1996). These results agree with assignments in the Ph.D. Thesis of Dr. L. Balas and sent to me recently by Professor Vercauteren.

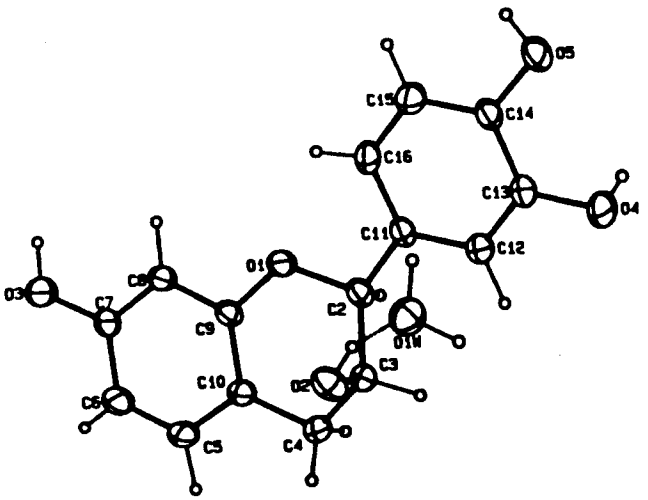


Figure 4. Crystal structure of *ent*-epifisetinidol (Tobiason *et al.*, 1993)

Perhaps the most significant result of our crystal structure work was the observation of Fronczeck *et al.* (1985) that penta-*O*-acetyl-(+)-catechin **9** assumed a diaxial orientation of the catechol B-ring and the 3-acetoxyl moiety, thus forcing the pyran ring into an approximate "reverse" half chair conformation in the crystal state (fig. 5). However, NMR data (showing a much larger $J_{2,3}$ coupling constant, $J_{2,3}$=6.5 Hz, than would be expected for this compound in a diaxial conformation and likewise significantly smaller than the $J_{2,3}$ coupling constant predicted from the conformer in which the catechol B-ring was approximately equatorial) suggested a rapid flipping in the conformation of the heterocyclic C-ring on an NMR time-scale when this compound is in solution.

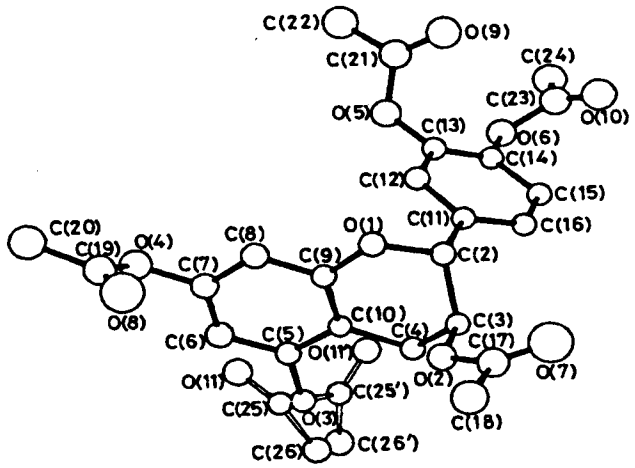


Figure 5. Crystal structure of penta-*O*-acetyl-(+)-catechin (Fronczeck *et al.*, 1985)

This work was closely followed by an important contribution by Porter *et al.* (1986), a part of which defined the crystal structure of the methylether derivative of the phloroglucinol adduct of (+)-catechin **10**. Here the heterocyclic ring tends toward a C(2)-sofa. As was observed in other studies, in the solid state the plane of the B-ring adopts a conformation where it eclipses the C(2)-H bond and the appending phloroglucinol ring at C(4) eclipses the C(4)-H bond.

The crystal structure of tetra-*O*-methyl-(+)-catechin **3** is interesting because two conformations are found in the unit cell (fig. 6), apparently the result of hydrogen bonding between the C(3) hydroxyl proton and the oxygen of methoxyl groups in both the A- and B-rings (Fronczek *et al.*, 1993). This compound also gave us important physical data on C(2)-H to C(3)-H torsional angles in the solid state as compared to those measured through $J_{2,3}$ coupling constants in solution. The discrepancy between solid and solution conformations was once more addressed in terms of a rapid interchange between A- and E-conformations in the NMR time-scale.

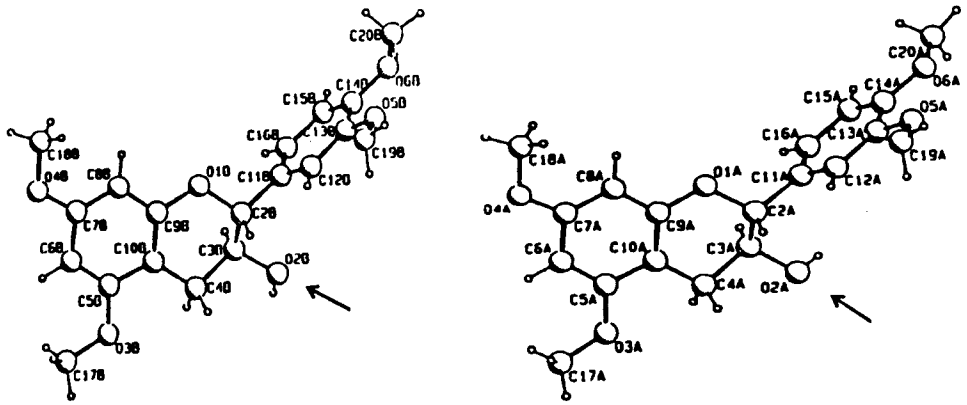


Figure 6. Crystal structure of tetra-*O*-methyl-(+)-catechin (Fronczek *et al.*, 1993)

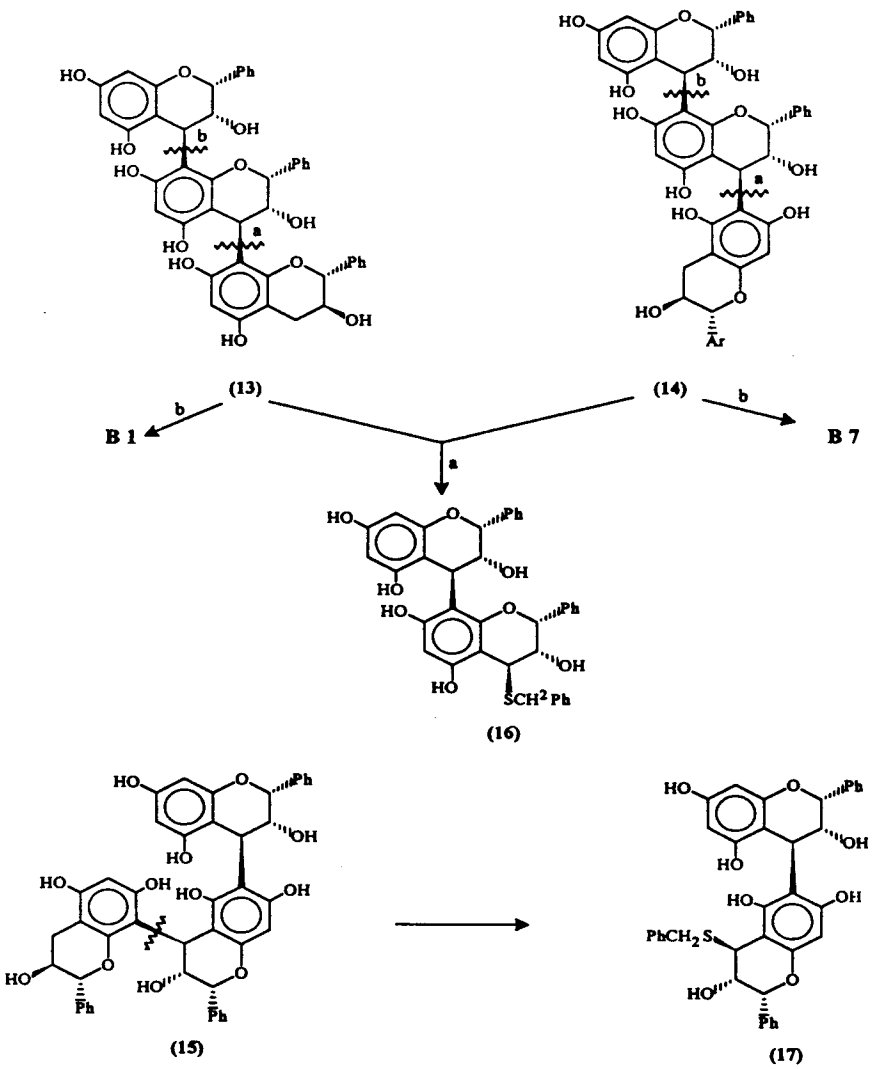
Problems associated with a diversity of conformations (evidence of considerable flexibility even in the solid state) in the same unit cell were amplified by Drs. Karin and Jannie Steynberg who were able to make visually beautiful crystals of the dimeric flavan-3-ol fisetinidol-(4→8)-catechin **11**. However, hydration was so critical to the solid state structure of these crystals that they decomposed when dried. X-ray diffraction indicated that many conformations were present. The Steynbergs and Fronczek (unpublished results) made one of the most important contributions to our understanding of these compounds even though this effort did not result in a publication. Fronczek's finding of considerable disorder in these crystals provided important evidence for the conformational flexibility of these compounds. We are still working to find a way to obtain some crystal structure data for dimeric proanthocyanidins.

CONFORMATIONAL VARIATION ASSOCIATED WITH INTERFLAVANOID BOND LOCATION

Roux and Ferreira (1982) at Bloemfontein have shown that condensation of the 5-deoxy profisetinidols or prorobinetinidols (5-deoxy electrophiles) with 5,7-dihydroxyflavan-3-ols as chain terminating nucleophiles result in "angular" polymers in which two chains of profisetinidin or prorobinetinidin units, predominantly linked carbon-4 to carbon-6, are linked to both the carbon-8 and carbon-6 positions of a 5,7 dihydroxy-flavan-3-ol terminating unit, typically catechin or analogous 5,7-dihydroxyflavans such as epicatechin, or gallocatechin *c.f.* **12**. Preference for substitution at both the carbon-8 and carbon-6 of the 5,7-dihydroxyflavan chain terminating unit and for substitution at carbon-6 of a chain extender in the profisetinidins or prorobinetinidins is understandable in terms of the relative electrophilicy or nucleophilicity of the partners of this polymerization process.

By contrast, the procyanidins and prodelphinidins tend to be linear. Haslam (1977) published a paper in 1977 that captured considerable attention because of the beauty of possible helically wound polymers with the "handedness" of the helix dependent on the stereochemistry at C(4). We set out a series of experiments directed to determining if the procyanidins really existed as linear C(4)-D(8) linked polymers in natural extracts.

At our Pineville laboratory, we were able to isolate a series of trimers in which both C(4)-D(8) and C(4)-D(6) interflavanoid bonds were present. I then had the great pleasure to spend 8 months working with Lawrence Porter and Yeap Foo at DSIR's laboratory in Petone, New Zealand to prove the structures of the three trimers (Hemingway *et al.*, 1982) epicatechin-(4→8)-epicatechin-(4→8)-catechin **13**, epicatechin-(4→8)-epicatechin-(4→6)-catechin **14**, and epicatechin-(4→6)-epicatechin-(4→8)-catechin **15**. Synthesis and characterization of the benzylthioether derivatives of both epicatechin-(4→8)-epicatechin **16** and epicatechin-(4→6)-epicatechin **17** provided probes of interflavanoid bond heterogeneity in polymers by thiolytic cleavage (scheme 1). We argued that it was unlikely that the C(4)-D(6) bonds were formed during mild acid-catalyzed cleavage in the presence of such a large excess of thiol as a capture nucleophile in the reaction.



Scheme 1

Soon after publication of that work, Yeap Foo came to our Pineville laboratory and we examined the potential for branching in procyanidins. He was able to synthesize the first representative of trimer epicatechin-(4→8)-catechin-(6→4)-epicatechin that represents a potential branch point in the procyanidins (Foo and Hemingway, 1984). Mattice and Porter (1984) pursued that work further, searching for possible evidence of branching in polymers using carbon NMR experiments, and concluded that branching was likely to exist, but infrequently, in natural polymers.

Possibly because so much attention has been diverted to rotational isomerism and heterocyclic ring conformations, questions surrounding angularity or branching in natural proanthocyanidins have not received the attention they deserve in the past 10 years. Definitive proof of whether or not some natural procyanidins are linear C(4)-D(8) linked polymers (Gupta and Haslam, 1978) and, if so, how they might be produced in plants (Stafford, 1988) has not been shown.

ROTATIONAL ISOMERISM

Resistance to rotation about the interflavanoid bond is often so large as to result in two sharp NMR spectra for each compound. This is particularly true for the 4 -linked proanthocyanidins whether representatives of the 5-deoxy profisetinidins or procyanidins. Where rotation is significant but slow on an NMR time-scale, the more difficult problem of extreme broadening of proton and carbon signals is encountered. Bergmann *et al.* (1987) (later extended by Cho *et al.* (1990; 1991)) demonstrated that time-resolved fluorescence offered a powerful probe of rotational isomerism in compounds that showed broadened but first order NMR spectra at ambient temperatures because of the much faster time scale of the fluorescence experiment. For example, the fluorescence decay of epicatechin-(4→8)-catechin **18** in dioxane is closely fitted to an equation with two exponential terms (fig. 7) that indicate the presence of two rotamers in approximate relative proportions of 3:1 even though the two rotamers could not be resolved in the ^{1}H-NMR spectrum.

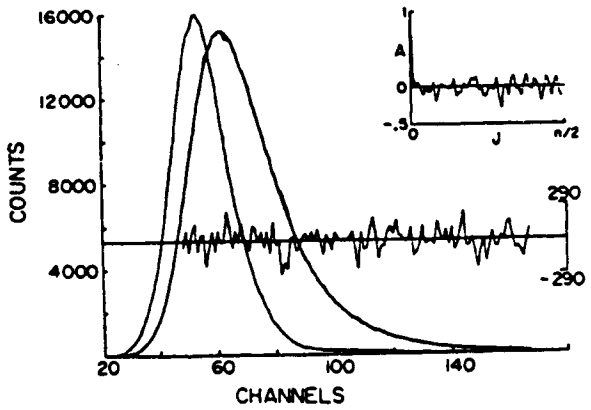
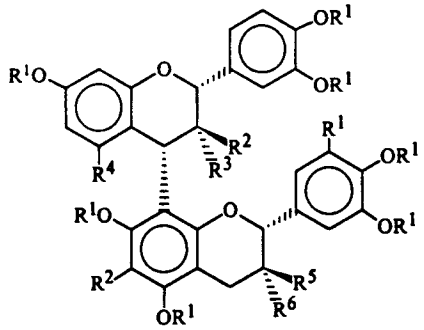


Figure 7. Fluorescence decay curves for epicatechin-(4β→8)-catechin in dioxane. Excitation wavelength is 280 nm. Emission wavelength is 315 nm. Temperature = 25° C. Data fit to biexponential function, x^2 = 1.1 (Bergmann *et al.*, 1987)

Applications of NMR methods to accurately permit measures of the relative proportions of two rotational isomers in dimeric proanthocyanidins are limited to special cases of high

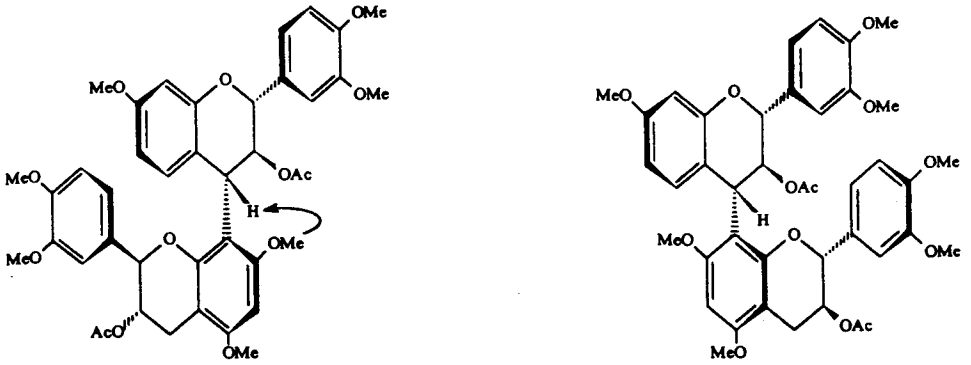
resistance to rotation about the interflavanoid bond. Therefore, studies have concentrated on derivatives such as peracetate (Foo and Porter, 1983) or methylether acetates (Steynberg *et al.*, 1995).



	R^1	R^2	R^3	R^4	R^5	R^6
(18)	H	H	OH	OH	OH	H
(19)	Me	OAc	H	H	OAc	H
(20)	H	OH	H	H	OH	H
(21)	H	OH	H	OH	H	OH
(22)	H	OH	H	OH	OH	H

Foo and Porter (1983) studied a series of 2*R* and 2*S* isomers of procyanidins and showed that for the "normal" 2,3-*trans* (either 2*R*,3*S* or 2*S*,3*R* for both the upper and lower units) dimers, two rotamers in a relative population of about 3.5:1 were present whereas for the "crossed" 2,3-*trans* (2*R*,3*S* in the upper unit and 2*S*,3*R* stereochemistry in the lower unit) isomer, the ratio of two rotamers decreased to 1.8:1. Peracetate derivatives were used to define the influence of stereochemistry on the relative proportions of rotamers in the 2,3-*cis* isomers because first order spectra are seen in the free phenols. To assign the conformations of the two rotamers they assigned the set of signals that were similar to the peracetate derivatives of the flavan-3-ol or its phloroglucinol adduct to the more extended conformation, whereas the set of signals that were shifted upfield to the more crowed rotamer in which the E-ring is back behind the plane of the A- and C-ring.

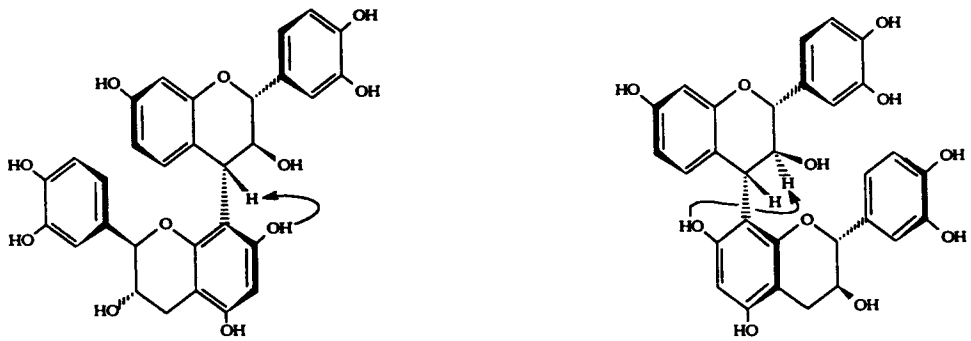
The methylether acetates offer a distinct advantage because of the power of n.O.e. experiments using methoxyl protons in defining the conformations of the two rotamers. In the methylether acetate derivative of fisetinidol-(4→8)-catechin **19** for example, n.O.e. is typically seen between the C(4)-H and the D(7)-methoxyl in the rotamers in which the E-ring is back behind the plane of the A- and C-ring of the upper unit (fig. 8) (Steynberg *et al.*, 1995). The more extended rotamer, in which the E-ring extends out from the A and C-ring plane, is usually seen as the minor rotamer when these derivatives are analyzed in $CDCl_3$. These results suggest that these compounds favor conformations which minimize their surface area, an observation first made by Foo and Porter (1983) studying peracetate derivatives of procyanidins.



A(10)-C(4)-D(8)-D(7) = (-) **A(10)-C(4)-D(8)-D(7) = (+)**

Figure 8. NOE definition of the two rotational isomers of fisetinidol-(4α→8)-catechin (Steynberg *et al.*, 1995)

Comparatively few attempts have been made to define the conformations of the more biologically significant free phenolic forms of these compounds. Steynberg *et al.* (1992) studied the conformations of fisetinidol-(4→8)-catechin **20** in specially dried d_6-acetone where exchange of the hydroxyl protons was slowed sufficiently to permit n.O.e. experiments using the hydroxyl protons. The observation of n.O.e. from the D(7)-hydroxyl to C(4)-H in one rotamer and to C(3)-H of the other rotamer defined their conformations as the more compact isomer in which the E-ring was back behind the plane of the A- and C-ring for the "major" and the more extended conformer in which the E-ring projects out from this plane for the "minor" rotamer (fig. 9).



A(10)-C(4)-D(8)-D(7) = (-) **A(10)-C(4)-D(8)-D(7) = (+)**

Figure 9. NOE definition of the two rotational isomers of fisetinidol-(4α→8)-catechin as the free phenol (Steynberg *et al.*, 1992)

More recently, Hatano and Hemingway (1996) studied the conformations of the two procyanidins catechin-(4→8)-epicatechin **21** and catechin-(4→8)-catechin **22**. In d_6-acetone or d_8-dioxane, the spectrum of **21** show the B and E ring protons of one of the rotamers are seen at the "normal" chemical shifts whereas the B- and E-ring resonances are shifted upfield in the other rotamer, similar to the observations of Foo and Porter (1983). Long-Range COSY spectra show strong cross peaks between C(4)-H and both A(6)-H and A(8)-H in both rotamers. By contrast, strong cross peaks between C(4)-H and D(6)-H are seen for only the rotamer in which the B- and E-ring protons are observed at "normal" chemical shifts (fig. 10).

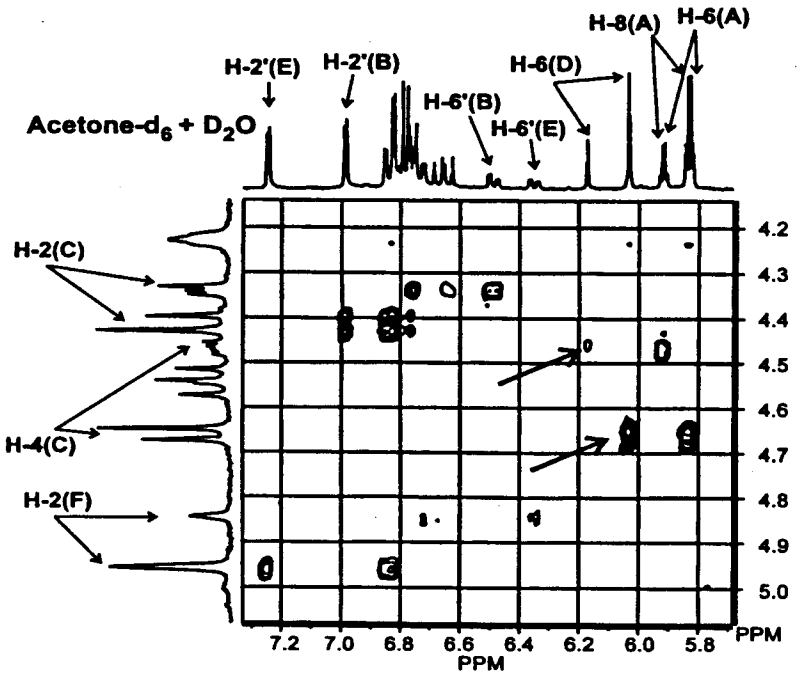


Figure 10. Long-range COSY of catechin-(4α→8)-epicatechin (Hatano and Hemingway, 1992)

On the premise that this correlation is due to an approximate 90° angle between C(4)-H and the A(6)-H and A(8)-H in both rotamers and D(6)-H in only one of the rotamers, we proposed that the rotamer in which a strong cross peak is observed is that which has greater mobility (*i.e.* the more extended rotamer in which the E-ring extends out away from the plane of the A- and C-ring plane). In addition, both NOESY (fig. 11) and NOE Difference experiments show correlation between E(2)-H and C(4)-H for this rotamer. These results, together with the skewed-boat conformation of the F-ring discussed more fully below, are consistent with a C(4)-H;C(4);D(8);D(7) torsion of about (-) 90° where the E-ring extends out form the plane of the A- and C-rings.

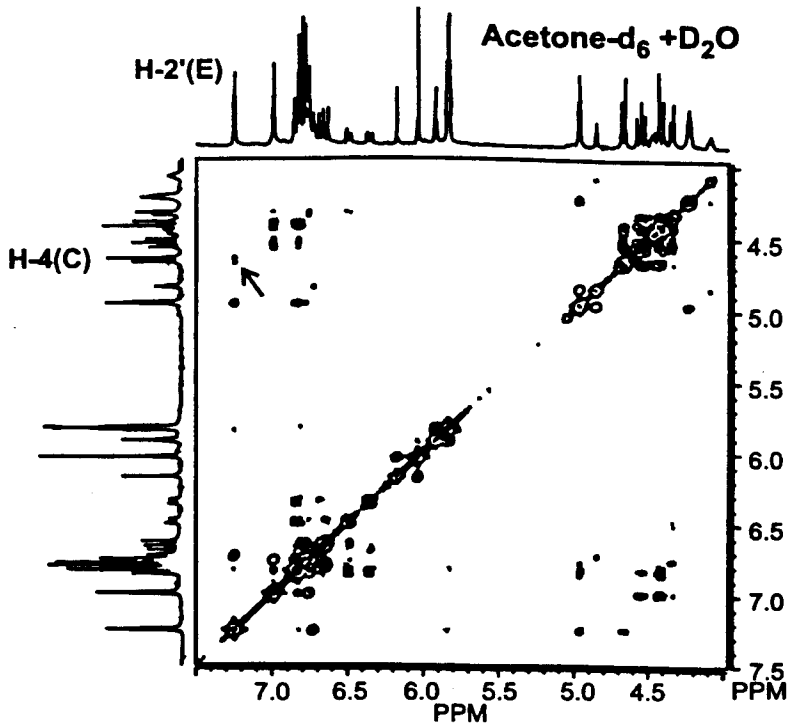


Figure 11. NOESY of catechin-(4α→8)-epicatechin (Hatano and Hemingway, 1996)

Hatano and Hemingway (1996)'s observations of only weak if detectable correlations between C(4)-H and D(6)-H, the requirement for a skewed-boat conformation of the F-ring (see below), and the observation of a characteristic up-field shift of the B- and/or E-ring protons all are consistent with the other rotamer being the more crowded. Because of the skewed-boat conformation shown by coupling constants for the F-ring, resulting in the E-ring being in a more axial orientation, the most favorable orientation to account for the required interaction between the B- and E-rings is that in which the C(4)-H;C(4);D(8);D(7) torsion is about (+) 120°. The conformation suggested for the minor rotamer could be stabilized by a tendency for *Pi-Pi* stacking of the B- and E-rings as observed by Steynberg *et al.* (1992), for catechin-(4→2)-phloroglucinol and Brandt *et al.* (1992), for 2,3-*trans*-3,4-*trans* or 2,3-*cis*-3,4-*cis* (both 2,4-*cis*) isomers of flavan-4-resorcinol adducts. However, n.O.e. between the Ar-H protons of the B- and E-rings was not seen as evidence to support this hypothesis.

Both NOESY and NOE Difference spectra showed strong cross peaks that are due to conformational exchange in which the proton of one rotamer is correlated with the same proton in the other rotamer (fig. 12). This "conformational exchange" within the time-scale of the NMR experiment indicates that rotational interchange occurs even though two rotamers are seen

as distinct sets of sharp signals in the time scale of the n.O.e experiment (Hatano and Hemingway, 1996).

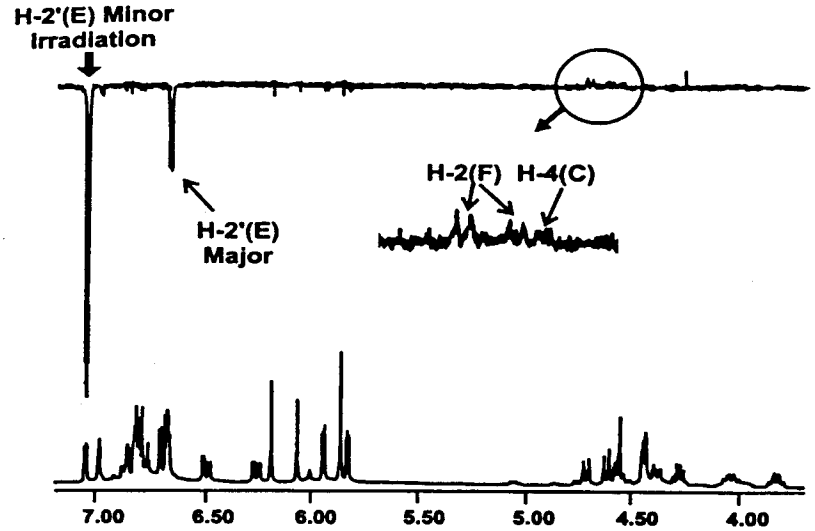


Figure 12. NOE Difference spectrum of catechin-(4α→8)-catechin, showing conformational exchange with n.O.e. to both rotamers, from E(2)-H to F(2)-H as well as C(4)-H (Hatano and Hemingway, 1992)

This conformational exchange is not observed in NOE-Difference spectra of the methylether acetate derivatives of proanthocyanidins. When these compounds are methylated and acetylated, rotation is slowed to the extent that irradiation of a signal is not translated to the analogous signal in the other rotamer in the NMR time-scale (Steynberg *et al.*, 1995). The appearance of two sharp sets of signals due to two rotamers in the NMR spectra of the 4α→8 linked procyanidins in the free phenolic form must be interpreted as being caused by the exceptionally high probability of the 4α→8 procyanidins existing as one or another of two rotational conformations together with a comparatively small time that intermediate conformations exist in the time frame of the NMR experiment.

Both Haslam (1977) studying catechin-(4α→8)-catechin **22** and Steynberg *et al.* (1992) studying fisetinidol-(4α→8)-catechin **20** noted that one rotamer dominated when these compounds were dissolved in D_2O. Therefore, Hatano and Heminway (1996) examined the effect of D_2O concentration in d_6-acetone on the populations of rotational isomers seen for **21**. Results (fig. 13) show the importance of studying the natural product in biologically significant solvents. Unfortunately, the majority of the NMR data available for oligomeric proanthocyanidins is of either the methylether acetates or peracetates rather than the free phenolic form of biological significance, and even when studies have been made on the free phenol, most of these were made in organic solvents.

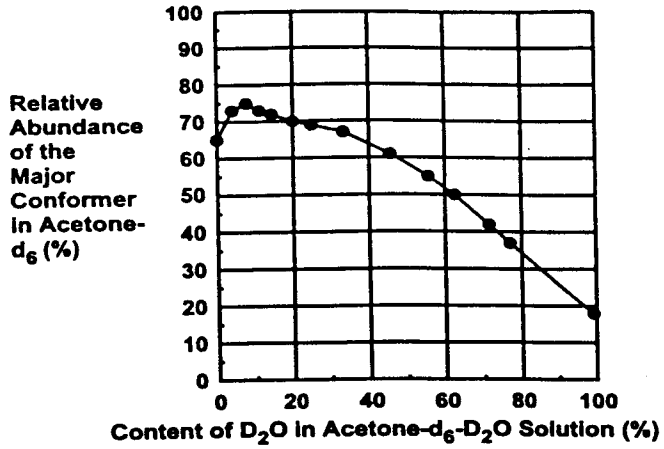


Figure 13. Effect of solvent on the relative proportions of the two rotamers in catechin-(4α→8)-epicatechin (Hatano and Hemingway, 1996)

When either **21** or **22** is dissolved in D_2O, the proton spectra show predominantly the rotamer in which both B- and E-ring protons are shifted upfield. The Long-Range COSY experiment did not show strong cross peaks between C(4)-H and D(6)-H, and coupling constants observed for the F-ring were consistent with a skewed-boat conformation. Therefore, the more compact rotamer is strongly, although not exclusively, preferred in solutions of these dimers in D_2O.

HETEROCYCLIC RING CONFORMATIONS

Viswandhan *et al.* (1987), and Viswandhan and Mattice (1987) established the basis for our work on molecular mechanics approaches to the conformations of flavan-3-ols and dimeric proanthocyanidins. Their work using the MM2 forcefield led to descriptions of the heterocyclic ring conformations, B-ring orientations, and orientation of the upper and lower flavan units through the interflavanoid bonds in dimeric procyanidins. These calculations also supported the view that rapid exchange between A- and E-conformers was probable and that, although the A-conformers generally exhibited higher total steric energy, differences were typically in the range of 3 to 5 kcal/mol so A-conformations should be considered. As mentioned previously, this work led to the "classic" paper by Porter *et al.* (1986), in which A- and E-conformational interchange was formally proposed. This theme was explored in many experiments that followed.

A variety of different approaches to modeling of the flavan-3-ols have now been examined including the MNDO, AM1, and PM3 semi-empirical molecular orbital computations (Steynberg *et al.*, 1992; Tobiason, 1992). Although there are differences in the conformations

of the heterocyclic rings of the low energy states as predicted by these different methods, these differences are usually not large. Steynberg *et al.* (1992) explored the energy barriers to E-/A-conformational interconversion going through either an -boat or a -boat and showed that with the MMX forcefield these interconversions of (+)-catechin involved energy barriers of only between 5.4 and 7.9 kcal/mole, respectively (fig. 14).

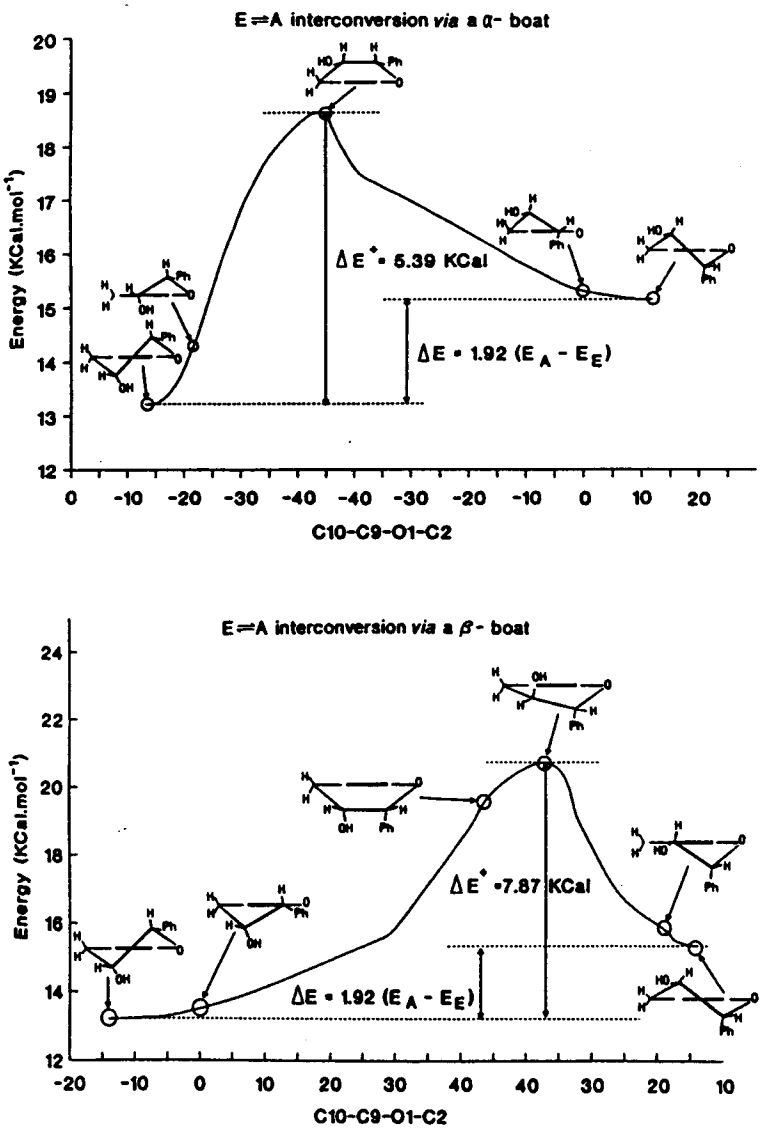


Figure 14. E↔A interconversion for (+)-catechin as predicted by the MMX forcefield (Steynberg *et al.*, 1992)

The A conformer was only 1.9 kcal/mol higher energy than the E-conformer for (+)-catechin. By calculating the heterocyclic ring $^3J_{HH}$ coupling constants from the low energy

conformations of both the E- and A-conformers and comparing these couplings with observed values, it is possible to obtain an estimate of the average distribution of the E- and A-conformers observed on an NMR time-scale. Importantly, the distribution one predicts through such an "averaging" was not related to the relative conformational energies of the two conformers! That discrepancy casts considerable doubt on the validity of the force field used for these computations. Tobiason (1992) found that MNDO computations gave the best fitting relative energies of the E and A-conformers of catechin and epicatechin when compared with observed $^3J_{HH}$ heterocyclic ring coupling constants. On the other hand, AM1 gave the most reasonable heterocyclic ring torsional angles overall. However, the problem of matching the proportions of the E- and A-conformers predicted from relative steric energy and averaging based on observed $^3J_{HH}$ coupling constants remains.

To explore the concept of E/A interconversion further, we examined the conformations of tetra-*O*-methyl-(+)-catechin **3** by molecular dynamics methods using Sybyl 4.1c (Fronczek *et al.*, 1993). This method showed a very fast interconversion of the heterocyclic ring between E and A conformations in comparison with an NMR time-scale. From these data it was possible to plot the probability of the torsional angle about the C(2) to C(3) bond (fig. 15) as well as the interdependence of the orientation of the B-ring in either the E- or A-conformers (fig. 16).

Averaging these conformations weighted by the sum of the probabilities of E (0.6) and A (0.4) conformers resulted in a predicted $J_{2,3}$ of coupling of 7.3 Hz as compared with the experimental result of 8.1 Hz. Therefore, while not an exact prediction of the observed $J_{2,3}$ coupling, these results certainly provided substantial additional support for E- and A-conformational interchange during the NMR experiment.

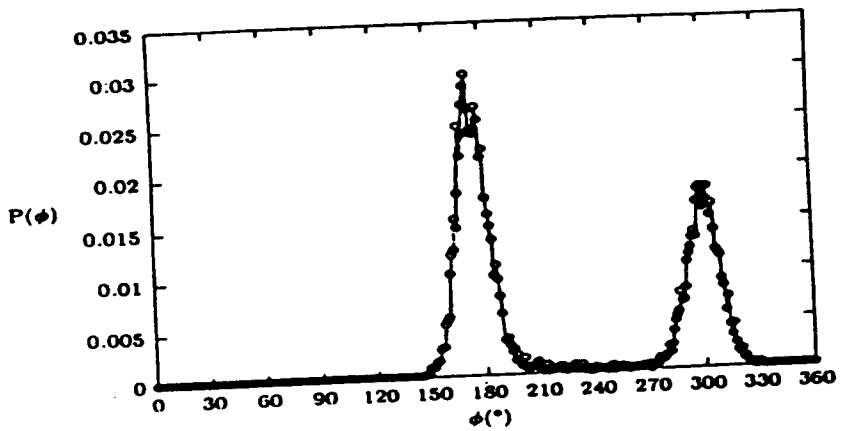


Figure 15. Average populations for the dihedral angle at C(2) - C(3) in tetra-*O*-methyl-(+)-catechin as predicted by molecular dynamics (Fronczek *et al.*, 1993)

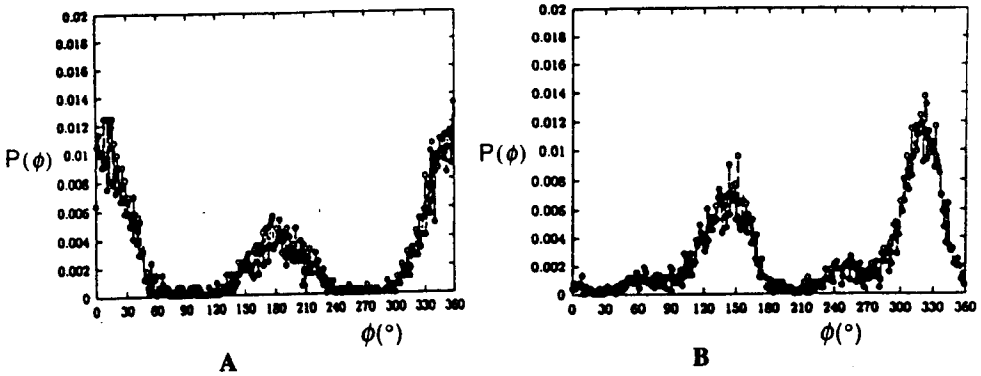


Figure 16. Average populations for the dihedral angle at C(2) - B(1) when the heterocyclic ring is in axial conformation (A) or equatorial conformation (B) as predicted by molecular dynamics for tetra-*O*-methyl-(+)-catechin (Fronczek *et al.*, 1993)

Tobiason and Hemingway (1994) took a similar approach by examining the conformational space in a global search of tetra-*O*-methyl-(+)-catechin (**3**) using the GMMX 1.0 program. Here too, we found that both the E- and A-conformers were represented in the ensemble of conformers that one would expect within a 3 kcal window (fig. 17). A Boltzmann averaging of these data weighted by the probability in the conformational space gave a predicted $J_{2,3}$ of between 7.68 and 8.15 Hz, as compared with 8.1 Hz observed; a $J_{3,4\alpha}$ of between 5.05 and 5.25 as compared with 5.5 Hz observed, and a $J_{3,4\beta}$ of between 9.33 and 9.88 as compared with 9.0 Hz observed. Application of this molecular search approach to other flavan-3-ols and their derivatives, while not as accurate, gave reasonable predictions of observed coupling constants except for those compounds with an acetoxyl at C(3) (Hemingway *et al.*, 1996).

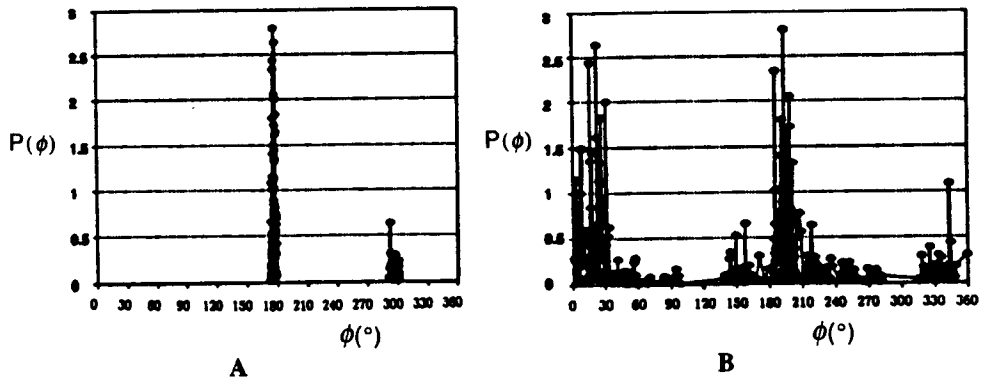


Figure 17. GMMX predicted populations (%) of C2 - C3 torsional angles A and of C2 - C11 torsional angles B for tetra-*O*-methyl-(+)-catechin (Tobiason and Hemingway, 1994)

Steynberg *et al.* (1995) studied the conformations of methylether acetate derivatives of a series of profisetinidins in which the effects of absolute stereochemistry of the upper unit

(2*R*,3*S*) and (2*S*,3*R*) absolute configuration, and interflavanoid bond location (4,8) and (4,6) as well as stereochemistry (4α) and (4β) were considered. For all these compounds, $J_{2,3}$ and $J_{3,4}$ couplings observed for the heterocyclic ring of the upper unit were close to 9 to 10 Hz, indicating an E-conformer with a half-chair conformation for the 2*R*, 3*S* isomers and a "reverse" half-chair conformation for the 2*S*, 3*R* isomers. The conformation of the terminal catechin unit was much more variable with many examples of $J_{2,3}$ coupling in the terminal unit as small as 5.0 to 7.0 Hz implying a strong preference for an A-conformation, especially in the more extended rotamer where the E-ring extends out from the plane of the A- and C-rings.

In studies of the free phenolic forms of the procyanidins **21** and **22** in D_2O, Hatano and Hemingway (1996) found that the upper units in both rotamers of each of these compounds showed $J_{2,3}$ and $J_{3,4}$ coupling constants close to 10 and 8 Hz, respectively, consistent with an approximate half-chair conformations for the upper unit. However, the coupling constants found for the heterocyclic F-rings in the terminal units were vastly different from those expected of a half -chair E-conformation for either **21** with epicatechin (fig. 18) as the terminal unit or **22** with a catechin terminal unit.

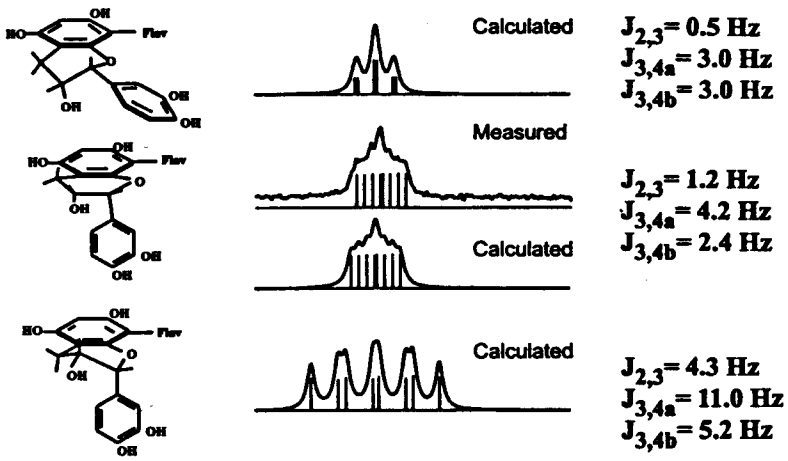


Figure 18. Conformations and coupling patterns for F(3)-H of catechin-(4α→8)-epicatechin in acetone/D_2O

Analysis of the splitting pattern of F(3)-H of the major rotamer of **21** in d_6-acetone using PCPMR suggested $J_{2,3}$ of 1.2; $J_{3,4\alpha}$ of 4.2; and $J_{3,4\beta}$ of 2.4 Hz. The splitting pattern of F(3)-H as observed could be closely matched and we found this approach to be very sensitive, providing estimates of coupling constants close to +/- 0.1 Hz. We then used the MMX forcefield in PCModel to optimize the heterocyclic ring conformations of epicatechin in both the E- and A-conformations and entered those coupling constants into PCPMR to estimate the line

shape of F(3)-H that would result from those coupling constants. To our surprise, this comparison showed conclusively that it is not possible to average the F(3)-H signals of the E- and A-conformers and come to a line shape as observed in the NMR spectrum. The heterocyclic ring of the terminal unit had to be close to a skewed boat in order to account for the observed coupling.

We used a similar protocol to study the conformation of the terminal unit of **21** when dissolved in D_2O (fig. 19). Here, the $J_{2,3}$ was higher (2.4 Hz) and $J_{3,4\beta}$ substantially lower (1.4 Hz) than was found for the same compound in d_6-acetone with a small amount of water. As was observed previously, it is not possible to account for the splitting pattern of F(3)-H through any kind of average of the E- and A-conformers. Rather, the heterocyclic ring of the terminal unit must be in a conformation between a F(3)-sofa and a boat.

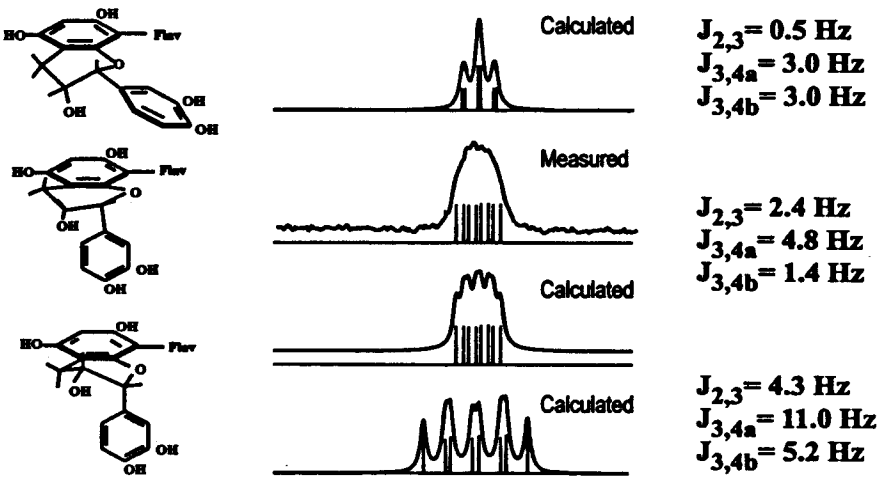


Figure 19. Conformations and coupling patterns for F(3)-H of catechin-(4α→8)-epicatechin in D_2O

A similar result was obtained in analysis of **22** in D_2O where catechin is the terminal unit (fig. 20). Here the E-conformer would result in wider splitting with much stronger signals on the wings of the multiplet and substantial proportions of the A-conformer would result in a much more close spacing of the central two peaks than observed.

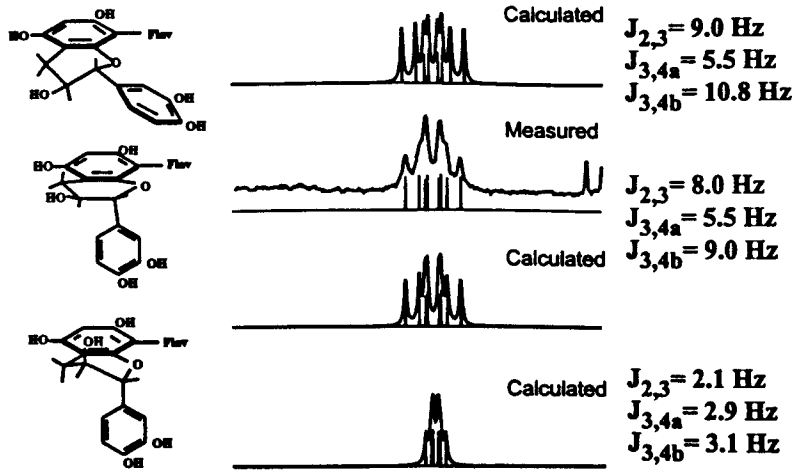


Figure 20. Conformations and coupling patterns for F(3)-H of catechin-(4α→8)-catechin in D_2O

Hatano and Hemingway (1996)'s results demonstrate the considerable power in use of PCPMR line shape analyses particularly on C(3)-H or F(3)-H to define the heterocyclic ring conformation in these compounds. Most importantly, these results suggest that one should use considerable caution in explaining the $^3J_{HH}$ coupling patterns of the heterocyclic ring protons in oligomeric proanthocyanidins through assumptions of a distribution of E- and A-conformers. The observations made here of skewed boat conformations of these compounds were supported by NOESY and NOE-Difference experiments that showed n.O.e. between C(4)-H and E(2)-H in the more extended rotamer that can only be accounted for by the E-ring being close to axial. It seems clear from Hatano's results that we should examine the proton spectra of the free phenolic forms of a wider range of proanthocyanidins, especially in D_2O, if we are to gain information useful in the interpretation of the biological properties of these compounds.

CONCLUSIONS

The most significant revelation of all this work is that the polyflavanoids are extremely flexible compounds and readily adapt different rotational and heterocyclic ring conformations in different solvents. Although computational approaches to conformational analyses of these compounds have been extremely useful, considerable caution must be exercised in interpretation of those results. The force fields in molecular mechanics need modification, particularly to loosen up the constraints on the heterocyclic ring if we are to obtain results from modeling that are consistent with NMR observations. We obviously need to devote more energy to NMR studies of the free phenolic forms of these compounds in water. Our results suggest that the

conformations of derivatives such as peracetates or methylether acetates have little relevance to the conformations of the natural phenolic compounds. The influence of solvent on the conformational properties of the phenols is so strong that, if we are interested in the conformations of these compounds in biological systems, we really should be studying their spectral properties in D_2O and not in organic solvents.

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REFERENCES

BERGMANN W.R., BARKLEY M.D., HEMINGWAY R.W., MATTICE W.L., 1987. Heterogeneous fluorescence decay of 4-6 and 4-8 linked dimers of (+)-catechin and (-)-epicatechin as a result of rotational isomerism. *J. Am. Chem. Soc.*, 109, 6614-6619.

BOEYENS J.C.A., DANNER L., KOLODZIEJ H., FERREIRA D., ROUX D.G., 1986. Crystal structure and absolute configuration of (-)-3-*O*-Acetyl-6-bromo-3',4',5,7-tetra-*O*-methylepicatechin. *J. Chem. Soc. Perkin Trans. 2*, 301-303.

BRANDT E.V., STEYNBERG J.P., FERREIRA D., 1992. Conformational analysis of arylflavan-3-ols as models for C-ring isomerized condensed tannins. *In* : Hemingway R.W., Laks P.E., Branham S.J. (Eds.): *Plant Polyphenols: Synthesis, Properties Significance*. Plenum Press: New York, p.487-499.

CHO D., TIAN R., PORTER L.J., HEMINGWAY R.W., MATTICE W.L., 1990. Variations in the heterogeneity of the decay of the fluorescence in six procyanidin dimers. *J. Am. Chem. Soc.*, 112, 4273-4277.

CHO D., PORTER L.J., MATTICE W.L., 1991. Conformations and time-resolved fluorescence of oligomers of (-)-epicatechin with (4β→8)-interflavan bonds. *Biopolymers* , 31, 537-545.

ENGEL D.W., HATTINGH M., HUNDT H.K.L., ROUX D.G., 1978. X-ray structure, conformation, and absolute configuration of 8-bromotetra-*O*-methyl-(+)-catechin. *J. Chem. Soc., Chem. Communications*, 695-696.

FOO L.Y., PORTER L.J., 1983. Synthesis and conformation of procyanidin diastereoisomers. *J. Chem. Soc. Perkin 1*, 1535-1543.

FOO L.Y., HEMINGWAY R.W., 1984. Condensed Tannins: Synthesis of the first "branched" procyanidin trimer. *J. Chem. Soc. Chem. Communications*, 85-86.

FRONCZEK F.R., GANNUCH G., MATTICE W.L., TOBIASON F.L., BROEKER J.L., HEMINGWAY R.W., 1984. Dipole moment, solution, and solid state structure of (-)-epicatechin, a monomer unit of procyanidin polymers. *J. Chem. Soc., Perkin Trans. 2*, 1611- 1616.

FRONCZEK F.R., GANNUCH G., MATTICE W.L., HEMINGWAY R.W., CHIARI G., TOBIASON F.L., HOUGLUM K, SHANAFELT A., 1985. Preference for occupancy of axial positions by substituents bonded to the heterocyclic ring in penta-*O*-acetyl-(+)-catechin in the crystalline state. *J. Chem. Soc., Perkin Trans. 2*, 1383-1386.

FRONCZEK F.R., HEMINGWAY R.W., MCGRAW G.W., STEYNBERG J.P., HELFER C.A., MATTICE W.L., 1993. Crystal Structure, conformational analysis, and molecular dynamics of tetra-*O*-methyl-(+)-catechin. *Biopolymers*, 33, 275-282.

FRONCZEK F.R., STEYNBERG E.C., STEYNBERG J.P., (unpublished results).

GUPTA R.K., HASLAM E., 1978. Plant proanthocyanidins. Part 5. Sorghum polyphenols. *J. Chem. Soc. Perkin Trans. 1*, 892-896.

HASLAM E., 1977. Symmetry and promiscuity in procyanidins biochemistry. *Phytochemistry*, 16, 1625-1640.

HATANO T., HEMINGWAY R.W., 1996. Conformational isomerism of phenolic procyanidins: preferred conformations in organic solvents and water. *J. Chem. Soc. Perkin 2.* (in press, paper no. 6/05592c) and presented to the Anselme Payen Award Symposium, ACS, New Orleans, L.A., 1996.

HEMINGWAY R.W., FOO L.Y., PORTER L.J., 1982. Linkage isomerism in trimeric and polymeric 2,3-*cis* procyanidins. *J. Chem. Soc. Perkin Trans. 1*, 1209.

HEMINGWAY R.W., TOBIASON F.L., MCGRAW G.W. and STEYNBERG J.P., 1996. Conformation and complexation of tannins: NMR spectra and molecular search modeling of flavan-3-ols. *Magnetic Resonance in Chemistry*, 34, 424-433.

MATTICE W.L., TOBIASON F.L., HOUGLUM K., SHANAFELT A., 1982. Conformational analysis and dipole moments of tetra-*O*-methyl-(+)-catechin and tetra-*O*-methyl-(-)-epicatechin. *J. Am. Chem. Soc.*, 104, 3359- 3362.

MATTICE W.L., PORTER L.J., 1984. Molecular weight averages and ^{13}C-NMR intensities provide evidence for branching in proanthocyanidin polymers. *Phytochemistry*, 23, 1309-1311.

MCGRAW G.W., HEMINGWAY R.W., 1982. Electrophilic aromatic substitution of catechins: bromination and benzylation. *J. Chem. Soc., Perkin Trans. 1*, 973-978.

PORTER L.J., WONG R.Y., CHAN B.G., 1985. The molecular and crystal structure of (+)-2,3-*trans*-3,4-*trans*-leucocyanidin (2*R*,3*S*,4*R*)-(+)-3,3',4,4',5,7-hexahydroxyflavan)-dihydrate and comparison of its heterocyclic conformation in solution and the solid state. *J. Chem. Soc. Perkin Trans. 1*, 1413-1418.

PORTER L.J., WONG R.Y., BENSON M., CHANG B.G. VISWANADHAN V.N., GANDOUR R.E., MATTICE W.L., 1986. Conformational analysis of flavans: ^{1}H-NMR and molecular mechanical (MM2) studies of the benzopyran ring of 3',4',5,7-tetrahydroxyflavan-3-ols: the crystal and molecular structure of the procyanidin (2*R*,3*S*,4*R*)-3',4',5,7-tetramethoxy-4-(2,4,6-trimethoxyphenyl)-flavan-3-ol.; *J. Chem. Res.* S86, M830.

ROUX D.G., FERREIRA D., 1982. The direct biomimetic synthesis, structure and absolute configuration of angular and linear condensed tannins. *Progr. Chem. Org. Nat. Prod.*, 41, 47-76.

STAFFORD H.A., 1988. The enzymology of proanthocyanidin biosynthesis. *In*: Hemingway, R.W., Karchesy, J.J., Branham, S.J. (Eds.): *Chemistry and Significance of Condensed Tannins.* Plenum Press, New York, p.47-70.

STEYNBERG J.P., BRANDT E.V., HOFFMANN M.J.H., HEMINGWAY R.W., FERREIRA D., 1992. Conformations of Proanthocyanidins. *In*: Hemingway R.W., Laks P.E., Branham S.J. (Eds.): *Plant Polyphenols: Synthesis, Properties, Significance*. Plenum Press, New York, p.501-520.

STEYNBERG J.P., BRANDT E.V., FERREIRA D., HELFER C.A., MATTICE W.L., GORNIK D., HEMINGWAY R.W., 1995. Conformational analysis of oligomeric flavans. 2 - Methylether acetate derivatives of profisetinidins. *Magnetic Resonance in Chemistry*; 33, 611-620.

TOBIASON F.L., 1992. MNDO and AM1 molecular orbital and molecular mechanics analyses of (+)-catechin, (-)-epicatechin, and their 3-*O*-acetyl derivatives. *In*: Hemingway R.W., Laks P.E., Branham S.J. (Eds.): *Plant Polyphenols: Synthesis, Properties Significance*. Plenum Press, New York, p. 459-478.

TOBIASON F.L., FRONCZEK F.R., STEYNBERG J.P., STEYNBERG E.C., HEMINGWAY R.W., 1993. Crystal structure, conformational analyses, and charge density distributions for *ent*-epifisetinidol: an explanation for regioselective aromatic substitution of 5-deoxyflavans. *Tetrahedron.*, 49, 5927-5940.

TOBIASON F.L., HEMINGWAY R.W., 1994. Predicting heterocyclic ring coupling constants through a conformational search of tetra-*O*-methyl-(+)-catechin. *Tetrahedron Lett.*, 35, 2137-2140.

VISWANADHAN V.N., MATTICE W.L., 1987. Preferred conformations of the sixteen (4-6) and (4-8) linked dimers of (+)-catechin and (-)-epicatechin with axial or equatorial dihydroxyphenol rings at C(2). *J. Chem. Soc. Perkin Trans. 2*, 739-743.

VISWANADHAN V.N., BERGMANN W.R., MATTICE W.L., 1987. Configurational statistics of C(4)-C(8) linked homopolymers of (+)-catechin and (-)-epicatechin. *Macromolecules*, 20, 1539-1543.