

Chapter 7

NMR Studies on the Conformation of Polyflavanoids and their Association With Proteins

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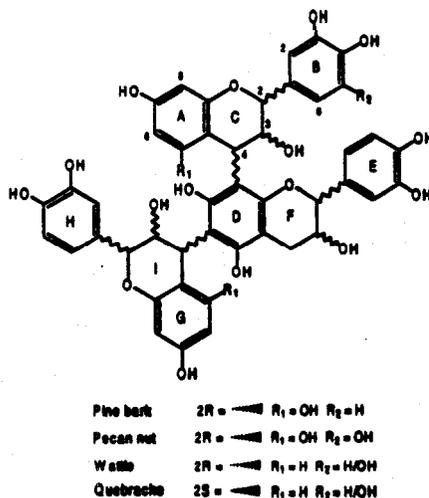
INTRODUCTION

Polyflavanoids (also named condensed tannins or proanthocyanidins) make up approximately half of the dry weight of most commercial tree barks, are often found **in even** higher concentrations in nut shells, and **are** important constituents of the **leaves of** plants. (18,27) The polyflavanoids rank second in abundance after lignin as a source of renewable phenolic materials. Most of their commercial and ecological **significance** centers on either their propensity to form complexes with proteins or on their potent anti-oxidant properties (21,42).

Because it is believed that the association of polyflavanoids with proteins is dictated by the shape and **flexibility** of these molecules, (15) we have undertaken an effort to try to define the conformational dynamics of polyflavanoids and to learn more about the interaction of **polyflavanoids with** proteins. (11,19,35,39) Our approach has been centered mainly on NMR experiments to obtain the necessary physical data to evaluate the results of computational chemistry. (22,41) Both **NMR** instrumentation and computational chemistry software **are** advancing at a fast pace and increasingly offer avenues to solution of questions surrounding tannin/protein interactions.

The conventional systems for naming and numbering the rings in a **trimeric** proanthocyanidin are shown in **Figure 1**.

Figure 1: General Structural Features of Plant **Polyflavonoids. (18,27)**



Note that each chain extender unit has three **chiral** centers so the absolute stereochemistry at each center must be defined. The structural **features** that relate to their shape **and** flexibility center on:

- 1) heterocyclic **ring stereochemistry**;
- 2) configurational isomerism due to heterogeneity (or **homogeneity**) in **the** location of **the** interflavanyl bond;
- 3) rotational isomerism due to restricted rotation about the **interflavanyl** bond, and
- 4) conformational isomerism due to flexing of the **pyran ring**.

NMR experiments provide access to resolution of the **questions** surrounding all of **these issues**. While an understanding of **the** conformational dynamics of these compounds is essential, it is also important to relate that information more directly to questions of how the polyflavonoids interact with proteins. **Here** too, NMR experiments provide critical physical data to better understand the interactions between **polyflavonoid** and polypeptides. NMR **experiments can** reveal intermolecular interactions **either** through **changes** in chemical shifts or **through** nuclear **Overhauser** effects. We have concentrated our work on the association of oligomeric **flavonoids** with oligomeric **peptides** in which both **NMR** data and **computational** chemistry approaches are **manageable** within the context of the instrumentation available to us. The results obtained from study of **oligomers** should provide a basis for understanding the behavior of the higher **polymers**.

Some of the NMR experiments we have used to address these problems are described here with the intent of demonstrating the wide range **experiments** that have been required to solve various problems. By necessity, this chapter concentrates on our own work and does not pretend to be a thorough review of the literature which is vast because most work on the chemistry of these compounds involves **NMR** experiments. The intent is to provide the reader with some examples how NMR experiments have been used to solve a variety of problems.

Heterocyclic Ring Stereochemistry

In **contrast** to **lignin**, the **polyflavonoids** are comparatively small molecules **and**, those within a plant species and tissue, are of a comparatively specific uniform structure (Figure 1). A typical condensed tannin isolate shows a broad distribution of molecular weight but with a number average molecular weight of only about **3,000-5,000** (between 10 and 17 flavan units) **(7,26)**.

The interflavanyl bond in the **5,7-dihydroxyflavans**, such as the procyanidins and prodelphinidins present in most commercially important tree barks, can be cleaved under mild acid conditions and the constituent units in the higher polymers can be established by analysis of the cleavage products. **(5,32)** The **interflavanyl** bond in the "5deoxy" **profisetinidins and** prorobinetinidins, such as in wattle tannins, resists cleavage under mild acidic or alkaline conditions but, fortunately, these extracts tend to be dominated by **trimers** and tetramers so most structural features can be defined by NMR experiments.

The absolute stereochemistry at **C-2c** is usually consistent within' the chain extender units. Compounds with **2R** absolute **stereochemistry** predominate being present in the vast majority of the hundreds of **plants** examined to date. **(18,27)** However, natural 'compounds with **2S** absolute stereochemistry do exist and are represented by the commercially important **Quebracho (44)** and **Rhus** spp. (4) tannins. The absolute stereochemistry at **C-2c** in the terminal unit is usually, but apparently not **always, (44)** similar to that in the chain extender units. Within the **5-deoxy profisetinidins** and prorobinetinidins in which the **interflavanyl** bond resists cleavage, complete proof of the absolute **stereochemistry** requires the synthesis of isomers of known stereochemistry. **(32,37)**

Once the absolute stereochemistry at the **C-2c** or **C-4c** positions is **established**, the relative stereochemistry at the pyran ring **chiral** centers can usually be deter **ined** by **¹H NMR** experiments **from** the heterocyclic ring proton coupling (Table 1). **(24)**

Table 1: ^1H NMR Coupling Constants of Methyl Ether Acetate and Peracetate Derivatives of Procyanidin Dimers. (24)

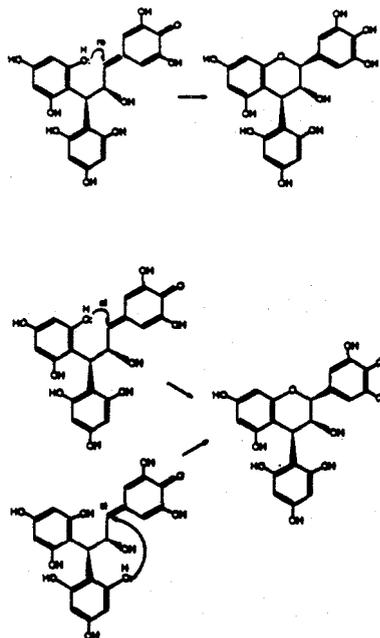
Stereochemistry	Methyl Ether Acetate		Acetate	
	$J_{2,3}$	$J_{3,4}$	$J_{2,3}$	$J_{3,4}$
2,3-trans-2,3-trans I	10	8.7-9.5	9.6-10.0	9.0-9.6
2,3-trans-3,4-cis	9.5	5.5	9.6	6.5
3,4-cis-3,4-trans	0-1.7	2.0-2.2	1	1.5
2,3-cis-3,4-cis	1	4.7-4.9	1	5.5-6.0

Evidence for either **2,3-cis** or **2,3-trans** relative stereochemistry can also be obtained from the **C-2c** chemical shift in a ^{13}C NMR experiment. (28) However, care must be exercised in the interpretation of proton coupling constants as described more fully in discussion of the **2,3-cis-3,4-trans** and **2,3-cis-3,4-cis** isomers as well as difficulties associated with conformational flexing of the pyran ring reviewed below.

In the **5-deoxy profisetinidins** and **prorobinetinidins** such as those in wattle tannins, the chain extender units are either **2,3-trans-3,4-trans** or **2,3-trans-3,4-cis** relative stereochemistry. (30) Profisetinidins that are of **2,3-cis-3,4-trans** stereochemistry are extremely rare, the tannins from *Pithecellobium dulce* (Madras thorn) bark being the best source known of that stereochemistry to date. (36) Within the **5,7-dihydroxyflavans** typical of most tree bark tannins, the chain extender units are predominantly of **2,3-cis-3,4-trans** relative stereochemistry. (18,27) The terminal unit is typically **2,3-trans** as in (+)-catechin. This monomeric flavan-3-ol is also normally found in plant extracts. (27)

The isolation of the **2S all-cis ent-epicatechin-(4 β →2)-phloroglucinol (37)** from acid-catalyzed cleavage of 2R prodelfinidins from pecan nut pith tannins in the presence of phloroglucinol as a capture nucleophile is an example of the care that must be taken in assigning the stereochemistry of these compounds (Figure 2).

Figure 2: Origins of **Epigallocatechin-(4 β →2)-Phloroglucinol** and **ent-Epigallocatechin-(4 β →2)-Phloroglucinol** from Cleavage of Pecan Pith Tannins. (37)



It has long been assumed that **cleavage** of the **interflavanoid** bond of procyanidins and **prodelphinidins** can be achieved with retention of configuration at **C-2_c** when reacting these compounds with capture **nucleophiles** such as thiols or phenols such as phloroglucinol. (5) However, two **epigallocatechin-(4→2)-phloroglucinol** adducts with similar proton coupling constants of $J_{2,3} = < 1$, $J_{3,4} = 1.4$ and $J_{2,3} = 1.5$, $J_{3,4} = 2.0$ Hz (as measured from their methyl ether acetate derivatives) were obtained from reactions that were prolonged to 24 and 48 hours at 100 °C in an attempt to increase the yield of **flavan-(4→2)-phloroglucinol** cleavage products from pecan pith tannins.

Circular dichroism (CD) measurements showed that the absolute stereochemistry at **C-4_c** was **4S** (a 4 β →2 linkage) in both products. (37) While evident in the study of other compounds in which **H-2_c** and **H-4_c** are both axial, no NOE correlation was observed between **H-2_c** and **H-4_c** in either **NOESY** or NOE difference experiments on either compound. However, the **NOESY** experiment **did** show a correlation between the **methoxy** protons of the pyrogallol B-ring and the phloroglucinol D-ring in one of **these products**. When coupled with the CD results, the compound showing a strong NOE cross peak must be of a **2S, 3S, 4S (all-cis)** stereochemistry. As would be **expected**, no NOE was seen between **H-2_c** and **H-4_c** or between the B- and D-rings in

the *2R*, *3R*, *4S* isomer that would be expected from a 4β substitution of the carbocation derived from *2,3-cis* constituent units in the polymer.

In the proof of the structures of the novel *2,3-cis* proflisetinidins isolated from Guamuchil,(36) the relative stereochemistry of the chain extender and terminal units was obvious, from ^1H and ^{13}C NMR spectra. The configuration at C-4 of the chain extender units was apparent from the CD spectra and consistent with *4S* absolute stereochemistry. The most difficult challenge came when the absolute stereochemistry of the *2,3-cis* and *2,3-trans* terminal units had to be proven. Because it was not possible to cleave the interflavanyl bond under mild conditions, synthesis of dimers and trimers with *2R* and *2S* absolute stereochemistry in the chain extender and more importantly also the terminal units from precursors of defined absolute stereochemistry was required. (36) The ^1H NMR spectra of many of these compounds exhibited severe broadening due to slow rotation at ambient temperature (see discussion below) but first order spectra were obtained when spectra were recorded at 343 °K.

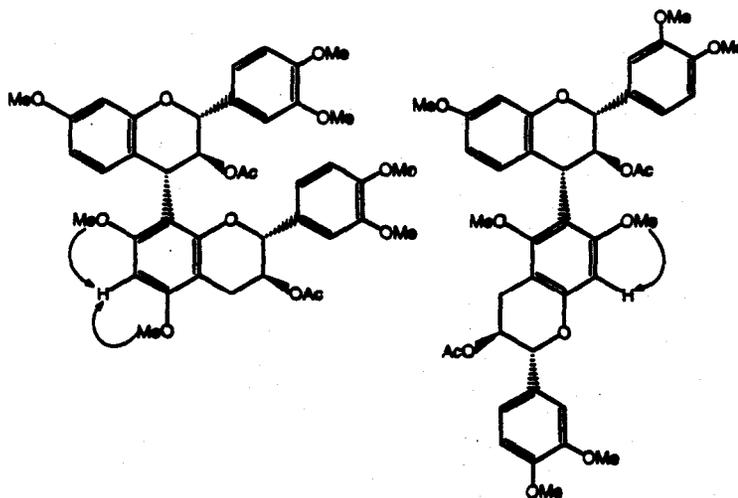
Configurational Isomerism at the Interflavanyl Bond

Regio-isomerism involved with the location of the interflavanyl bond in dimeric procyanidins was first described by Weinges (46) following from the strong effort made by Freudenberg, Mayer, and Weinges and their coworkers at Heidelberg through the 1960s. That work is summarized in a review (45) that still is most valuable. Since the late 1960's, there has been explosive growth in our understanding of oligomeric proanthocyanidins driven by the works of Roux, (31-33) Haslam, (14,15) and many other laboratories around the world.

In studies of the procyanidins in southern pine bark, three trimers that differed in the location of the interflavanyl bond a) epicatechin-($4\beta\rightarrow 8$)-epicatechin-($4\beta\rightarrow 8$)-catechin. b) epicatechin-($4\beta\rightarrow 8$)-epicatechin-($4\beta\rightarrow 6$)-catechin and c) epicatechin-($4\beta\rightarrow 6$)-epicatechin-($4\beta\rightarrow 8$)-catechin were isolated and partial thiolytic cleavage of the polymer gave dimeric cleavage products representing both regio-isomers.(20) It is interesting to reflect on the considerable work with unpleasant thiols that was required to synthesize the different dimer thioether derivatives and how simple the proof of structure would have been if we had access to HMQC and HMBC experiments then. Despite the progress on the chemistry of procyanidins, the question of whether plants produce linear all-($4\rightarrow 8$) linked procyanidins and heterogeneity in the location of the interflavanyl bond in procyanidins is due to rearrangement in the isolation of these compounds or if these compounds are produced by plants with a mixture of ($4\rightarrow 6$) and ($4\rightarrow 8$) interflavanyl bonds is still not resolved.

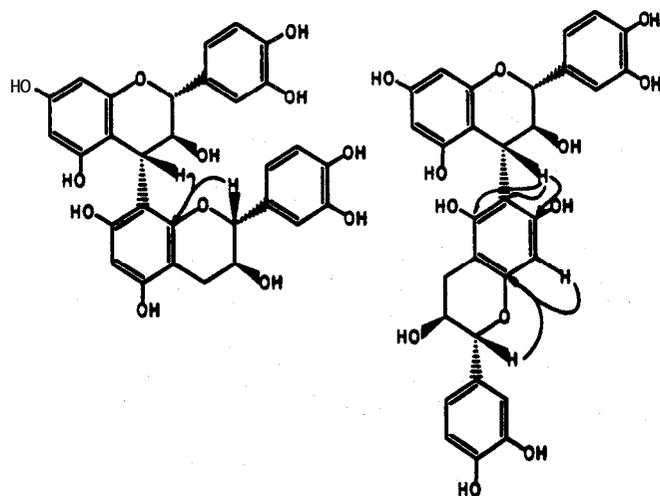
When examined carefully, the differences between these structures are slight and near-symmetry presents a challenge in distinguishing between the procyanidins and prodelphinidins with either (4→6) or (4→8) interflavanoid bonds using simple ^1H - or ^{13}C -NMR experiments. One approach to resolution of questions about interflavanoid bond location is to make the methyl ether or methyl ether acetate derivatives and to use methoxy NOE experiments. (34) Both the MeO-5_A and MeO-7_A methoxy groups are correlated with H-6_A whereas only the MeO-7_A will see H-8_A permitting differentiation of the A- and D-ring methoxyls in both structures. In compounds with a (4→8)-linkage, both the MeO-5_D and MeO-7_D methoxy groups will show correlation to H-6_D . By contrast, in compounds with a (4→6)-linkage, only a correlation between MeO-7_D and H-8_D is observed (Figure 3).

Figure 3: Methoxy NOE Experiments Define Interflavanoid Bond Location In Methylated Derivatives. (34)



We have also used both HMBC and COLOC experiments to resolve the question of interflavanoid bond location in the mixture of rotational isomers seen in 2,3-*trans* procyanidins in their free phenolic form. (17) In the (4→8)-linked structure, long-range correlations can be seen from H-4_C and H-2_F to C-10. However, in the (4→6)-linked structure, there are long range correlations between H-4_C and C-5_D as well as C-7_D and these carbon signals can be distinguished from C-10_D by its correlation with H-8_D and H-2_F (Figure 4).

Figure 4: COLOC and HMBC Experiments Define Interflavanyl Bond in Phenolic Forms of Proanthocyanidins. (17)

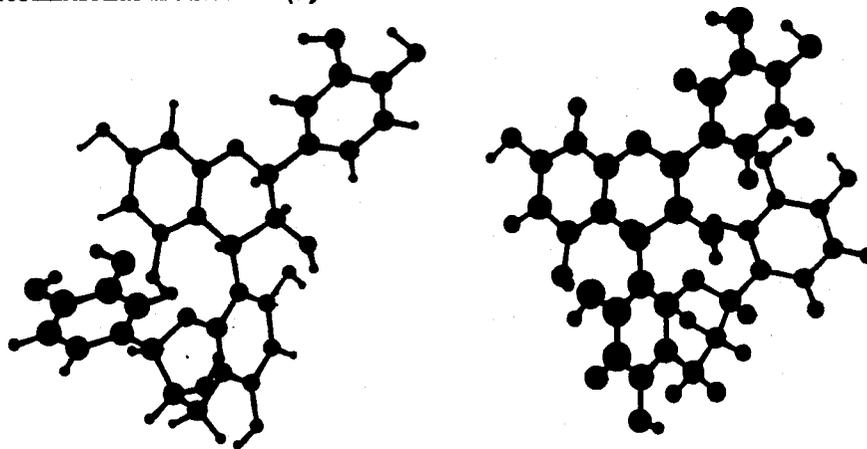


Balas and Vercauteren (1,2) have used a similar approach to **define the** location of **the** interflavanyl bonds in **peracetate derivatives** of dimeric and trimeric procyanidins.

Rotational Isomerism

Hindered rotation about **the** interflavanoid bond (8) results in doubling of **the** signals in the NMR spectra where **rotation** is restricted (Figure 5).

Figure 5: Restricted Rotation about the Interflavanyl Bond Leads to Conformational Isomerism. (9)

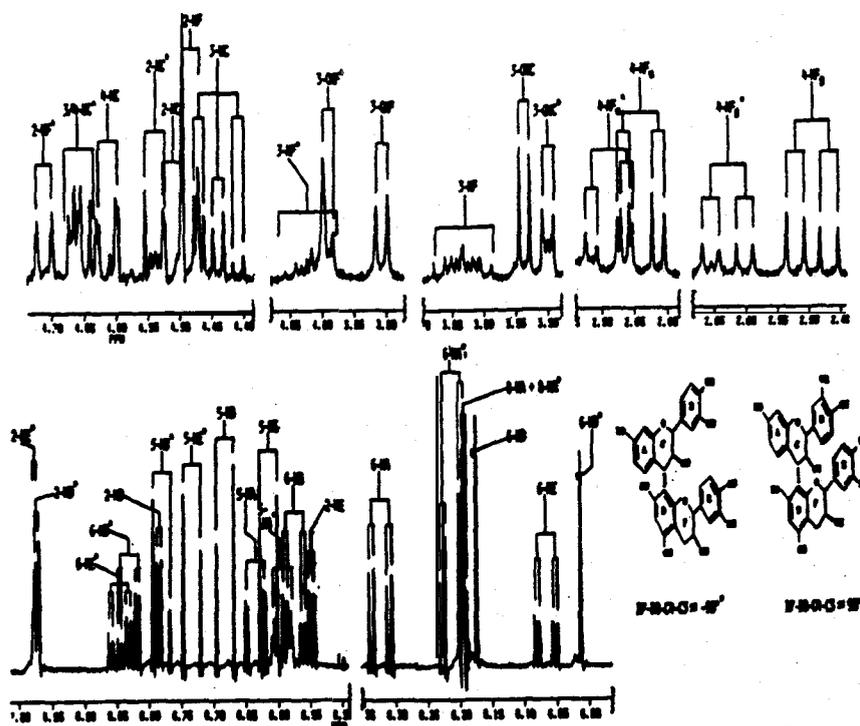


Severe broadening of the proton signals is seen where slow rotation occurs during the time frame of the NMR experiment. Fletcher (3) examined this phenomenon for the procyaaidias that are most commonly found in commercially important tree barks. At ambient temperature, two sharp sets of signals due to each rotamer are seen in the proton spectra of the **2,3-trans-3,4-trans** procyaaidias such as **catechin-(4 α →8)-catechin** whereas only broadened signals are seen for the **2, 3-cis-3, 4-trans** isomers such as **epicatechin-(4 α →8)-catechin** in which the lower unit is quasi-axial to the plane of the A and C ring of the upper unit. Even where rotation is fast enough to show a first order NMR spectrum, the presence of two preferred rotational isomers can be seen using time-resolved fluorescence decay analyses because of the much shorter time frame for those experiments (3).

A combination of COSY and NOE difference experiments was used in order to fully assign the ¹H NMR spectra for each of two rotamers observed in the methyl ether acetate derivatives of a series of dimeric profisetinidins. (34) A COSY experiment permits one to assign the heterocyclic ring protons of the upper and lower units in each of the rotamers and long range correlations allow assignments of the methoxy protons as well as the B and E rings for each rotamer. Once the Ar-H and methoxy protons for each rotamer are assigned, the orientation of the two rotamers can be established. In the more compact rotamer in which the E- and F-rings are back behind the plane of the A- and C-ring, NOE between the MeO-7_D and H-4_C is evident. In the more extended rotamer in which the E- and F-rings extend out from the plane of the A- and C-ring, no correlation between these protons is observed.

Steynberg (35) used a similar series of NMR experiments in a conformational analysis of the profisetinidin dimer (-)-fisetinidol-(4 α →8)-catechin in the free phenolic form. Here the ¹H NMR spectrum shows two rotational isomers in approximately equal proportion (Figure 6).

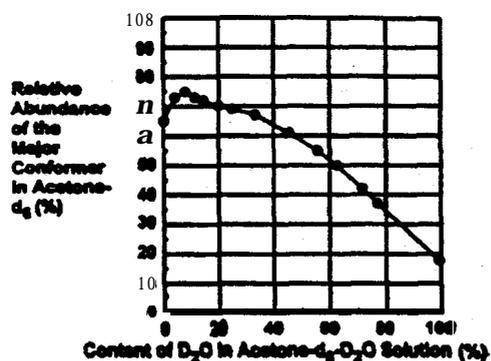
Figure 6: The Proton NMR Spectrum of **Fisetinidol-(4 α →8)-Catechin** Displays Two Rotational Isomers in Nearly Equal Proportion (35)



A combination of **homonuclear J-Resolved**, **COSY**, and **HETCOR** experiments **provided most** of the **information** needed to unequivocally assign the protons of the two **rotamers**. By working in an especially dry **environment** with extensively dried **d₆-acetone**, exchange of **the hydroxy** protons could be slowed sufficiently to allow **hydroxy NOE** experiments. **NOE from OH-7_D to H-4_C** in one **rotamer** and from **OH-7_D to H-3_C** in the other defined the conformations of the two rotamers. As was seen in the methyl **ether acetates** described above, the slightly **preferred rotamer** was the more **compact** one in which the **E- and F-rings** are back behind the plane of the **A- and C-rings**. **Steynberg** suggested on the basis of chemical **shifts** of the E-ring protons that the **compact rotamer** is heavily favored when this **compound** is dissolved in the biologically **significant** solvent, water (35).

Hatano (17) studied the effect of solvent on the rotational isomer preferences of the procyanidin dimers catechin-(4 α - \rightarrow 8)-catechin and catechin-(4 α - \rightarrow 8)-epicatechin and found a similar result with one predominant rotamer evident when dissolved in D₂O. When the proton spectrum of catechin-(4 α - \rightarrow 8)-epicatechin is recorded in δ -acetone, the more extended rotamer is present at about 65% of the population. Addition of increasing amounts of D₂O resulted in a gradual decline in the population of the extended rotamer until it represented only 20% when dissolved in D₂O (Figure 7).

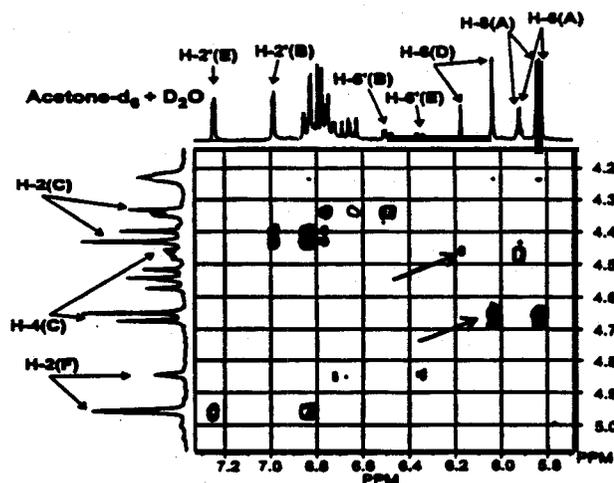
Figure 7: Conformational Preferences of Dimers are Influenced Strongly by Solvent Composition (17)



Similarly, the extended form of the dimer catechin-(4 α - \rightarrow 8)-catechin represents about 40% of the population when dissolved in δ -acetone and only signals representing the more compact rotamer are seen when dissolved in D₂O.

A long-range COSY experiment provided evidence for the orientation of the two flavan units in the two rotamers. (17) Strong cross peaks were seen between H-4_C and both H-6_A and H-8_A, analogous to allylic coupling where the overlapping of the C-H σ -bond and the π orbitals is maximized at a 0° dihedral angle. The H-6_A and H-8_A form an approximate 90° angle relative to H-4_C in both rotamers and strong cross peaks to both are observed (Figure 8).

Figure 8: **A Long-Range COSY Experiment Provides Information on the Torsion Between Flavan Units of Dimers.(17)**



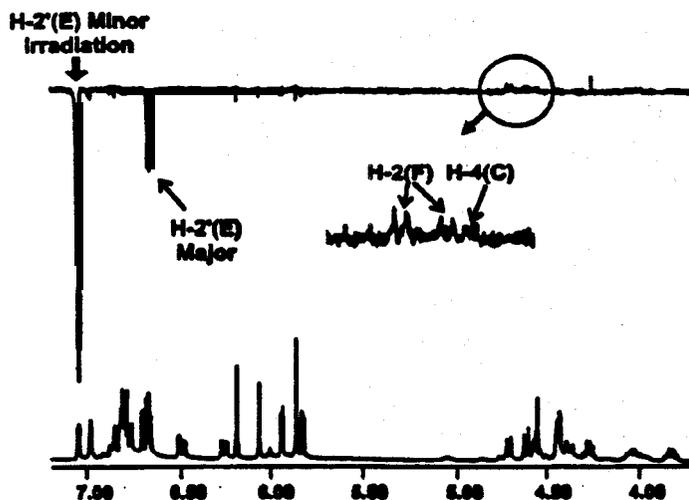
The important aspect of this experiment is that it provides a probe of the orientation of the terminal unit. For the **dimer catechin-(4 α →8)-epicatechin** in δ -acetone strong cross peaks are **seen** in the major rotamer between **H-4_C** and **H-6_D** indicating an angle between these protons of approximately **90°**. By contrast, the minor rotamer showed only a very weak cross peak between **H-4_C** and **H-6_D** suggesting an angle deviating to **0°** or **180°**. Just the opposite situation was observed in study of **catechin-(4 α →8)-catechin** in δ -acetone. Here **there** was a strong cross peak between **H-4_C** and **H-6_D** in the minor rotamer and no correlation between those protons in the major rotamer. These results indicate that the angle between **H-4_C** and **H-6_D** is about **90°** in the minor rotamer **but** that it is **distorted** more toward either **0°** or **180°** in the major rotamer.

The conclusions **reached** from the long range COSY experiments were supported by measurements of NOE difference and **NOESY** experiments. (17) Irradiation of **H-2_B** resulted in a strong positive NOE with **H-4_C** as well as a negative (**conformational** exchange) signal for **H-2_B** of the minor rotamer in **catechin-(4 α →8)-epicatechin**. By contrast, irradiation of **H-2_B** of **the** minor rotamer of **catechin-(4 α →8)-catechin** resulted in comparatively weak positive NOE with both **H-2_F** and **H-4_C** for the minor rotamer **and** a stronger negative (**conformation** exchange) signal for **H-2_B** of the major rotamer.

It is important to **recognize** the dynamics involved in the **conformations** of these **molecules**. Even though two distinct rotational isomers are seen as sharp **¹H NMR**

signals in dimers such as the *2,3-trans-3,4-trans* procyanidins, conformational exchange (17) is an important feature of the NOESY and NOE difference spectra obtained from these compounds (Figure 9).

Figure 9: **Conformational** Exchange is a Prominent Feature of NOESY and NOE Difference Spectra Despite High Rotational Energy Barrier. (17)



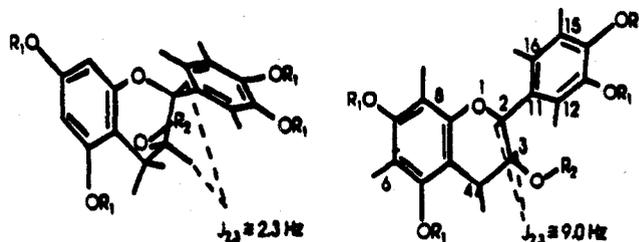
When examined using the MM2 forcefield, two rotational isomers with only small (ca. 3.7 kcal/mol) energy differences are seen in which the C-3_C-C-4_C-C-8_D-C-9_D torsion angles center on $\pm 90^\circ$. The flexibility in rotation is limited to a narrow range of $\pm 75^\circ$ to $\pm 104^\circ$ for acceptable energy because of severe steric interactions. (43) The high energy barrier to rotation through 360° begs the question of whether the two "rotamers" correspond to conformations in which the lower unit is really either out from or back behind the plane of the A- and C-rings with conformational exchange through such high energy barriers. The same question arose from study of the conformation of the lower unit pyran ring through line shape analysis of the H-3_F coupling as discussed below.

Conformational Isomerism of the Pyran Ring

Considerable effort has been put to understanding of the conformational dynamics of the the pyran ring because observed couplings between H-2_C, H-3_C and H-4_C do not agree with values calculated for a low energy 'half-chair' conformation in which the B-ring is quasi-equatorial to the plane of the A-C rings. For example, measured $J_{2,3}$ coupling for the *2,3-trans* flavan-3-ol derivatives generally fall in the range of 6-7 Hz

in contrast to expected values of about 10 Hz. The observation that the pyran ring in the crystal form of the **penta-acetate** of **(+)-catechin** is in a "reverse **half-chair**" with the B-ring *quasi-axial* (10) prompted Porter (29) to suggest that the small coupling constants observed in NMR experiments result from averaging of the E-(B-ring equatorial) and A- (B-ring axial) conformers due to rapid conformational exchange in the time frame of the NMR experiment (Figure 10).

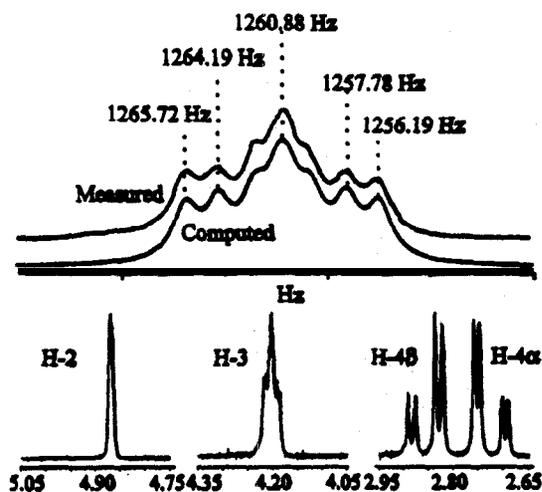
Figure 10: Computations Show A- and E-Conformers of Similar Energy and Observed $J_{2,3}$ Coupling is Typically Intermediate Suggesting A- to E-Conformational Averaging in Monomeric Flavan-3-ol Derivatives (29,40)



Evidence supporting this A- to **E-conformational** exchange in the **2,3-trans flavan-3-ol** derivatives has been obtained in molecular dynamics computations (6,12) and, as well as conformational search methods (40) including reasonably close approximations of the **changes** in heterocyclic ring coupling constants associated with variation in temperature. (41)

Similar studies on the **2,3-cis flavan-3-ol ent-epifisetinidol**, (39) while consistent with the hypothesis of A- to **E-conformational** exchange, were not totally conclusive because **H-2c** appears as a broad signal with **about 2Hz** coupling. However, good estimates of the coupling constants for the heterocyclic ring could be obtained by line shape analysis of **H-3c** (Figure 11). (22)

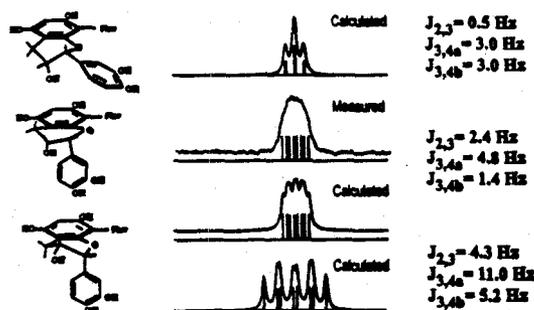
Figure 11: Lineshape Analysis of H-3_C Provides Access to all the Coupling Constants in the Pyran Ring of 2,3-cis Flavan-3-ols.(19,22)



The assignments given to H-4, and H-4_β in (-)-epicatechin (22) were later shown to be incorrect by a multi-NOE experiment (9) and should be reversed. The correct coupling constants are: $J_{2,3} = 1.6$; $J_{3,4\alpha} = 3.3$; $J_{3,4\beta} = 4.5$ Hz. These coupling constants are also consistent with the results of a GMMX global search computation which indicated that a Boltzmann average of the A- and E-conformer populations could suitably predict the observed coupling constants.

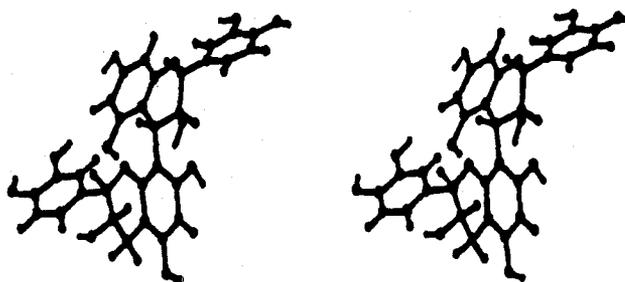
The coupling constants observed for the upper unit heterocyclic ring (C-ring) in both rotational isomers of either catechin-(4 α →8)-epicatechin or catechin-(4 α →8)-catechin were consistent with a half-chair conformation in which the B-ring was approximately equatorial suggesting that substitution at C-4 α with a bulky flavan unit stabilizes the conformation of the C-ring. (17) However, line shape analysis of H-3_F in either rotamer of catechin-(4 α →8)-epicatechin suggested that this pyran ring is distorted toward a sofa conformation (Figure 12).

Figure 12: Lineshape Analysis of H-3_F in the Lower Unit of Procyanidin Dimers Suggests a Sofa Conformation that is Supported by NOESY and Long-Range COSY Experiments. (17)



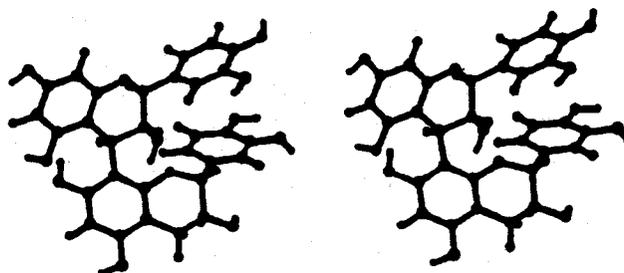
There would not seem to be any way to make a Boltzmann sum of the A- and E-conformer couplings and arrive at the observed splitting for H-3_F. NOESY and NOE difference experiments also supported the conclusion that the lower unit heterocyclic ring is distorted toward a sofa conformation. In the extended conformer, NOE is seen between H-2_E and H-4_C while a long range COSY spectrum shows an approximate 90° angle between H-4_C and H-6_D. A preferred conformation similar to that shown in Figure 13 is consistent with all these observations.

Figure 13: Stereoview of the Extended Conformer of Catechin-(4 α - \rightarrow)-Epicatechin that is Preferred in Acetone. (17)



By contrast, the evidence suggests that π - π interaction between the B- and E-rings stabilizes the conformation shown in Figure 14 for the more compact rotamer that predominates in water.

Figure 14: Stereoview of the Compact Conformer of Catechin-(4 α →8)-Epicatechin that is Preferred in Water. (17)



Interaction Between Flavanoids and Oligopeptides

Haslam (15) made an outstanding review of our understanding of the complexation of plant polyphenols with proteins and carbohydrates up to 1987. Murray and Haslam (25) studied changes in chemical shifts to show that, in the interactions of pentagalloyl glucose with proline rich peptides, the interaction was selectively with proline and its neighboring residues. In a more recent review, Haslam (14) stressed the importance of hydrophobic interactions between plant polyphenols and other biopolymers including proteins or plant polypeptides.

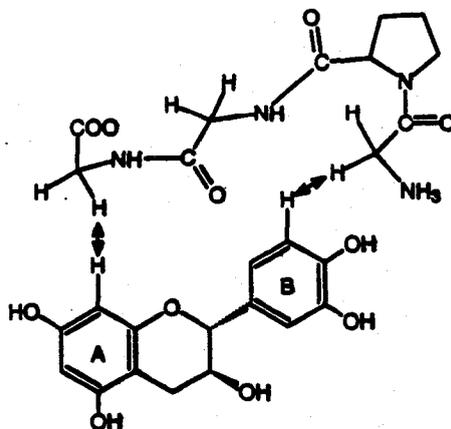
Hatano (16) used NOESYHG experiments in an attempt to define the interactions of flavanoids with oligomeric peptides containing prolyl residues where changes in chemical shift were usually too small to measure. Here a challenge was to work with very dilute water or water/D₂O solutions to prevent precipitation of the complex while also suppressing the large water signal. Because of rapid exchange of protons of interest, it was not always possible to work with D₂O as the solvent. We have found the P1-3-3-1 experiment particularly useful in minimizing the large water signals in the proton spectra.

When (+)-catechin and L-proline are combined, the NOESYHG experiment showed correlation between the catechin B-ring protons and the L-proline C γ protons. (16) However, in combinations of (+)-catechin with the dipeptide Gly-Pro in water, significant cross peaks were seen between the (+)-catechin A-ring protons and the glycine protons. Turning the sequence the other way to combinations with Pro-Gly,

once more a significant cross peak was seen **between** the catechin **H-8_A** and glycine protons. The importance of hydrophobic interactions with residues other than **proline** was further emphasized in NOESYHG experiments with the dipeptides Pro-Val and Pro-PhA. (16)

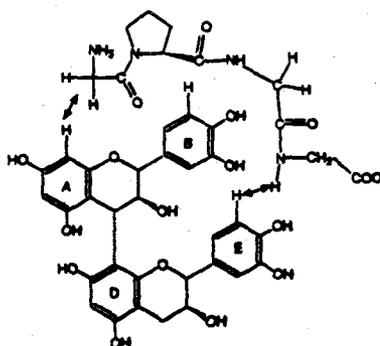
Experiments using the oligopeptides **Gly-Pro-Gly-Gly** and bradykinin (**Arg-Pro-Pro-Gly-PhA-Ser-Pro-PhA-Arg**) showed that it is possible to obtain information from NOESYHG experiments at 300 MHz for fairly complex oligopeptides and that conformational properties of the flavan and the oligopeptide are indeed important (16) For example, interactions of **(+)-catechin** or **catechin-(4 α →8)-catechin** with the tetrapeptide **Gly-Pro-Gly-Gly** differ. In the interaction with **(+)-catechin**, **H-8_A** shows correlation with the **C α** methylene protons of the C-terminal Gly unit and a cross peak is also observed between the **H-5_B** proton and the methylene protons of the N-terminal Gly residue (Figure 15).

Figure 15: Intermolecular NOE Correlations Between (+)-Catechin And Gly-Pro-Gly-Gly in Water.(16)



By contrast, the NOESYHG experiment on the interaction between the **dimer catechin-(4 α →8)-catechin** with **the this** same **tetra-peptide** shows correlation of **the** A-ring **H-8_A** with the **methylene** protons of the **N-terminal** Gly residue while a cross peak is also evident between **H-2_B** and **H-5_B** and the amide proton of the C-terminal Gly unit (Figure 16).

Figure 16: Intermolecular NOE Correlations Between Catechin-(4 α - \rightarrow 8)-Catechin and Gly-Pro-Gly-Gly in Water. (16)



In all the work done to date, we have not seen **significant** changes in chemical shifts that suggest strong polar interaction. This is to be expected because the presence of water should disrupt hydrogen bonding. However, we have found that NOESYHG experiments can reveal intermolecular associations in water that emphasize the importance of conformations for both the flavan and **peptide** as well as their potential for hydrophobic association.

It is important to note that cross peaks representing intermolecular associations between the flavanoids themselves are also prominent in these NOESY experiments. (16) Self association of the **polyflavanoids** is particularly evident when dissolved in water and other flavanoid **molecules** compete strongly with the polypeptides in the systems we have examined thus far. It is generally considered important that the tannin have a molecular weight *equivalent* to about 3 to 4 flavanoid units in order to complex strongly to proteins. However, we have found that even addition of the **monomeric flavan-3-ol (+)-catechin** and in low molar ratios to the number of prolyl units in polyproline results in precipitation. **Solubility** constraints are at least **as** important as the problem of working with large HOD **resonance** in **these** experiments.

CONCLUSIONS

Progress in our understanding of the chemistry of condensed tannins has been intimately tied to the **development** of NMR technology **and**, fortunately, there seems no end in sight to **progress** in new **NMR** instrumentation and experiments. It does *not* seem long ago that the first ^{13}C -NMR experiments on polymeric **procyanidins** were **presented** simultaneously by Lawrence Porter (7) and **Karchesy** (23) at a 1979 **ACS** symposium **organized** by J.W. Rowe in Hawaii. The great progress that has been

made since then is largely thanks to the development of NMR instrumentation. This review, by necessity, is focused on providing some examples from our own work and is not meant to be a thorough review of all the NMR experiments that have been so important to advancing plant polyphenol chemistry. We hope the examples and literature cited here will be of some help in providing an understanding where we have been and where we are headed in applying NMR experiments to understanding the chemistry and significance of an important class of plant polyphenols.

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