INTERACTION OF FLAVANOIDS WITH PEPTIDES AND PROTEINS AND CONFORMATIONS OF DIMERIC FLAVANOIDS IN SOLUTION

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1. INTRODUCTION

Although the physiological roles of tannins and related polyphenols in plants have not yet been clarified, their ability to form complexes with proteins or related biopolymers has been correlated with some protection of the plants from predators such as animals, insects, and microbes.[1] Similarly, commercial uses of tannins, especially in the leather and brewing industries, are also based on their binding with proteins.[2] Pharmacological properties of tannins have been investigated based on recent advances in the structural study of tannins in medicinal plants, and various actions of tannins including antitumor and antiviral effects have been revealed.[3] These effects are attributed to interactions with certain biomolecules in organisms, too.

Most of these effects are dependent on the chemical structures or molecular shapes of tannins.[3] Therefore, clarification of the molecular conformations of tannins and related polyphenols is requisite to understanding the process of molecular interaction of tannins with the biomolecules. Here we summarize our reports on the conformational analyses of dimeric flavanoids related to condensed tannins and interaction of these flavanoids with peptides. Because molecular

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conformations of oligomeric flavanoids[4] in the free phenolic form in solution with the biologically significant solvent (water) had not yet been characterized, we focused our efforts on definition of the conformations of the natural free phenols in aqueous solutions.

2. CONFORMATIONS IN ORGANIC SOLVENTS

Among various proanthocyanidins, we used catechin-(4α→8)-catechin and catechin-(4α→8)-epicatechin for the conformational analysis, since the presence of high rotational barriers around their interflavan linkages[5] resulted in sharp NMR spectra for the two conformers seen for each of the two compounds. However, complete assignment of these spectra had not been attempted. These dimeric procyanidins were synthesized as shown in figure 1.[6] The 2α,3β-dihydroquercetin, prepared by Karchesy at Oregon State University, was treated with sodium borohydride and the resulting hydroxyl group at C-4 of the reduction product was removed in acidic conditions to give a cation. Nucleophilic A-rings of either (+)-catechin or (−)-epicatechin then attacked the cation to form the dimeric procyanidins. These two dimers, which have (+)-catechin as the upper unit and either (+)-catechin or (−)-epicatechin as the lower unit, were then available without structural ambiguity.

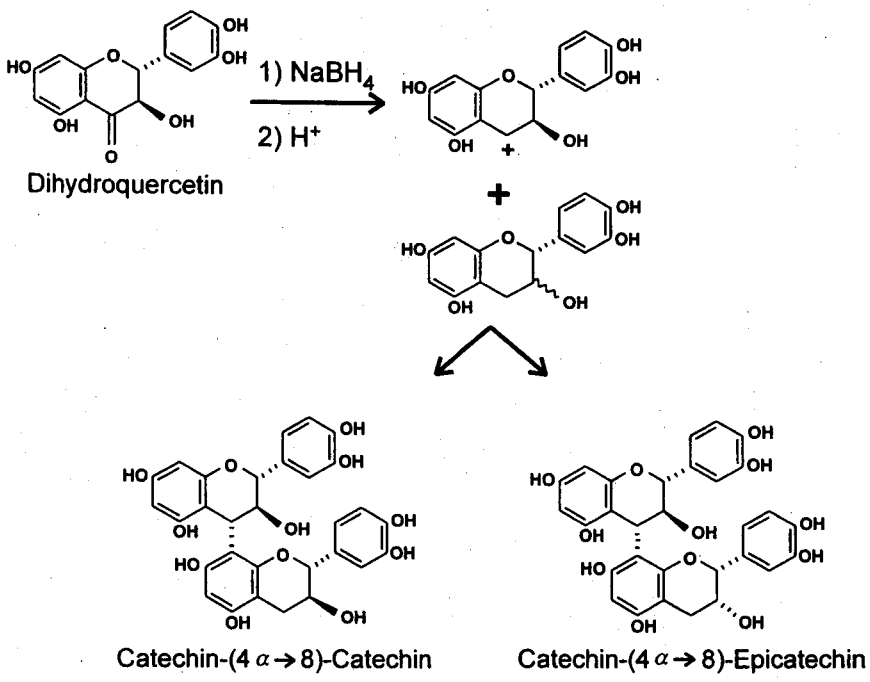


Figure 1. Syntheses of catechin-(4α→8)-catechin and catechin-(4α→8)-epicatechin.

The ^{1}H NMR spectrum of catechin-(4α→8)-epicatechin in acetone-d_6 is shown in figure 2. Steric hindrance minimizes rotation around the interflavan bond, and two relatively stable conformational forms were observed in the NMR spectra. An expanded spectrum shows the signals in the aromatic region at around 6 ppm are A-ring and D-ring protons. Signals at lower field of the aromatic region are composed of four sets of three protons each forming the ABC 3-spin system characteristic of catechol rings. These are attributable to protons of B-ring and E-ring of this dimeric procyanidin. Therefore, the aromatic protons indicate the presence of two conformers, one of which shows signals due to the minor conformer with significant upfield shifts relative to those of the major conformer. The upfield shift of these B- and E-ring protons indicates that the minor conformer adopts a conformation where the two aromatic rings are close to each other enabling these anisotropic effects.

To establish the spatial positions of the constituent flavan units around the interflavan bonds of the dimeric procyanidins, definition of the conformations of heterocyclic C- and F-rings that affect locations of B- and E-rings in each dimer is required. Coupling constants of heterocyclic C- and F-ring protons in the ^{1}H NMR spectra of the two dimers, catechin-(4α→8)-catechin and catechin-(4α→8)-catechin, were analyzed with the aid of spectral simulation. The coupling constants for the upper units of both dimers were similar to those for the half-chair form of the heterocyclic ring of the monomeric catechin in which the B-ring is equatorial. However, the coupling constants for the lower units are different from the constituent monomers.

For the lower residue of catechin-(4α→8)-epicatechin, estimation of the $J_{2,3}$ coupling constant is difficult, since H-2 appears as a broad singlet and H-3 proton

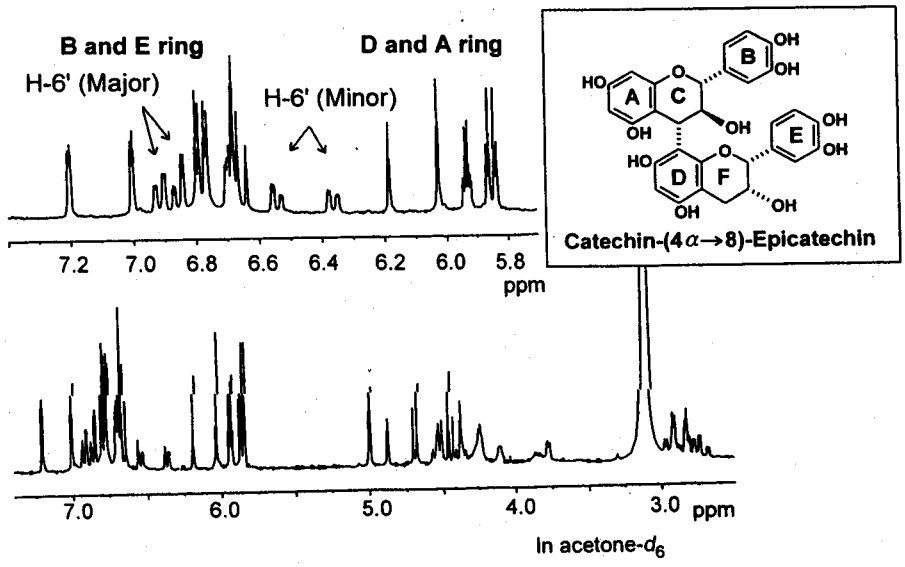


Figure 2. ^{1}H NMR spectrum of catechin-(4α→8)-epicatechin.

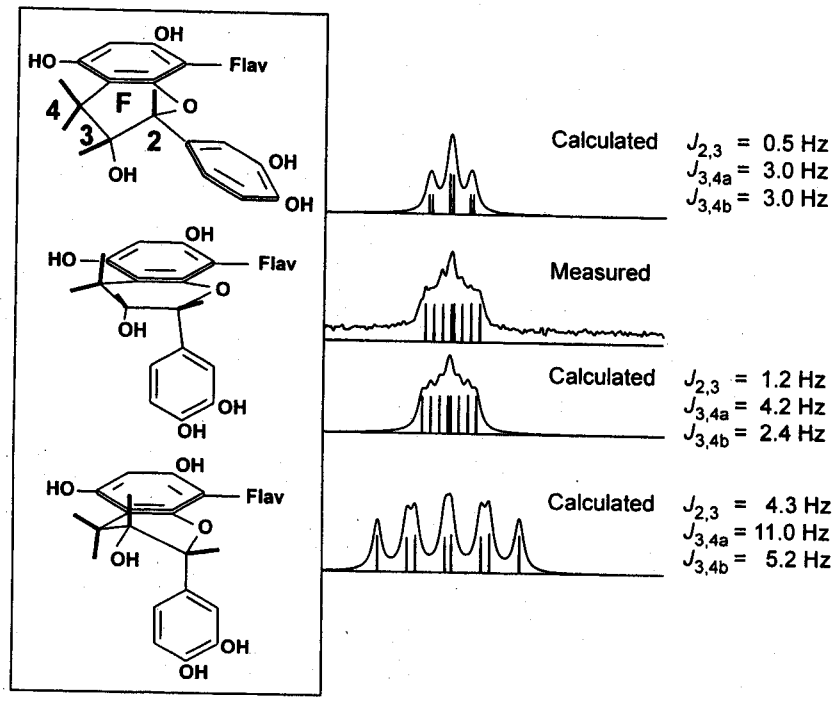


Figure 3. Conformations and coupling patterns for F-ring H-3 of catechin-(4α→8)-epicatechin in acetone-d_6 containing D_2O.

shows a multiplet signal due to coupling with the two H-4 protons as shown in figure 3. However, spectral simulation using PCPMR[7] led to the assignment that the coupling constant between H-2 and H-3 is 1.2 Hz, and $J_{3,4a}$ and $J_{3,4b}$ are 4.2 and 2.4, respectively, when recorded in acetone-d_6. These F-ring coupling constants are consistent with neither the half-chair conformation with an equatorial phenyl ring nor the reverse half-chair conformation with an axial phenyl ring. An energetically unfavorable skewed-boat conformation was thus suggested for the F-ring, even though the observed coupling may be the result of an "averaged" coupling over the time frame of the NMR experiment[8] for reasons described below.

In D_2O, catechin-(4α→8)-epicatechin showed a coupling pattern (fig. 4) somewhat different from that in acetone-d_6 as shown in figure 3. Obviously, the observed signal pattern of F-ring H-3 is far from that for the energetically preferable half-chair or reverse half-chair conformation, and the simulated signal patterns indicated that the F-ring adopts the C3 sofa conformation.

On the other hand, the dimer catechin-(4α→8)-catechin showed a signal pattern of F-ring H-3 as shown in figure 5. The observed signal pattern is closer to the calculated signal pattern for the half-chair heterocyclic ring with an equatorial phenyl group. The calculated signal pattern for the reverse chair form with an axial phenyl group is far from the measured one, indicating that this form does not participate strongly in the conformation of this compound.

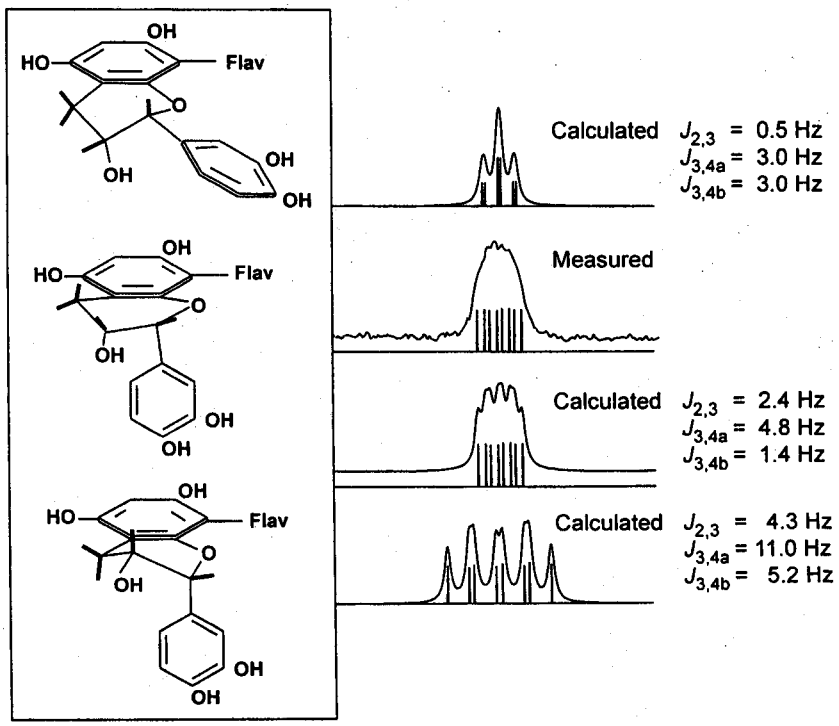


Figure 4. Conformations and coupling patterns for F-ring H-3 of catechin-(4α→8)-epicatechin in D_2O.

In addition, the molecular shapes of proanthocyanidins are strongly affected by steric hindrance around their interflavan bonds, so it is necessary to define the dihedral angles between the two flavan units of dimeric procyanidins. Spatial positions of the two halves in each of the two dimers were analyzed based on ^1H-^1H long-rang COSY[9] and NOESY measurements.

The ^1H-^1H long-range COSY spectrum of catechin-(4α→8)-epicatechin shown in figure 6 enabled the assignments of the B- and E-ring protons to each conformer by cross peaks due to the coupling between C-ring H-2 and B-ring H-2′ and H-6′, and between F-ring H-2 and E ring H-2′ and H-6′. In addition, this measurement permitted assignment of the orientation of D-ring relative to the upper half of the dimer in the following way. The ^1H-^1H long-range COSY spectrum showed strong cross peaks between C-ring H-4 and H-6 and H-8 of A-ring where the A-ring plane is at 90 degrees to the C4-H4 bond. The cross peak between D-ring H-6 and C-ring H-4 of the major conformer therefore indicates that the bond C4-H4 and the D-ring plane form an angle of about 90 degrees. On the other hand, the minor conformer showed a much weaker cross peak for the coupling between the C-ring H-4 and D-ring H-6. Therefore, the dihedral angle between C4-H4 and the D-ring plane in the minor conformer is larger or smaller than 90 degrees tending to either 180 or 0 degrees when the cross peak is absent.

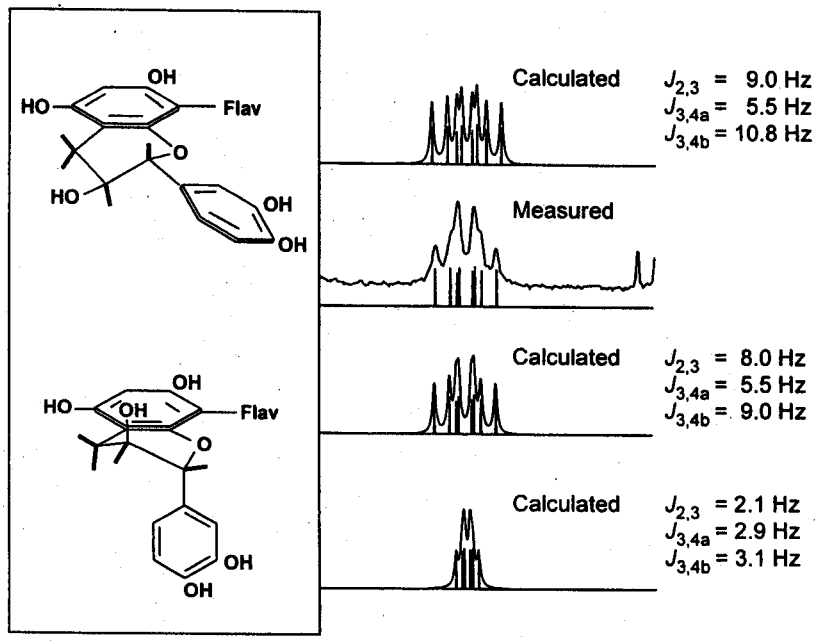


Figure 5. Conformations and coupling patterns for F-ring H-3 of catechin-(4α→8)-catechin in D_2O.

In support of the above conclusions, the NOESY spectrum of catechin-(4α→8)-epicatechin shown in figure 7 indicates that the C- and E-rings of this molecule are spatially close to each other. A cross peak due to NOE correlation between C-ring H-4 and E-ring H-2′ of the major conformer indicated that the major conformer of this dimeric procyanidin adopts a conformation in which the α-oriented E-ring of the lower unit locates near the β-oriented H-4 of the upper unit. Although the spectrum also showed many correlations between the protons of one conformer and the corresponding protons of the other conformer, these cross peaks are not due to NOE correlations but to the conformational exchange. For example, the H-2′ of the lower half of one conformer shows a correlation with H-2′ of the lower half of another conformer.

The presence of the NOE between C-ring H-4 and E-ring H-2′ in the molecule of catechin-(4α→8)-epicatechin was confirmed by measurement of a NOE difference spectrum (fig. 8). Irradiation of H-2′ of the lower residue of the major conformer caused increase of the peak area of C-ring H-4 along with that of F-ring H-2. Therefore, the orientation of the lower residue is that shown in figure 7, where the E-ring of the lower residue locates on the same side as that of H-4 of the upper residue. Irradiation of H-2′ of the lower unit of the major conformer also caused a negative peak for the corresponding proton of the minor conformer. The appearance of the negative peak of the corresponding proton of another conformer is explained by conformational exchange accompanied by saturation transfer.

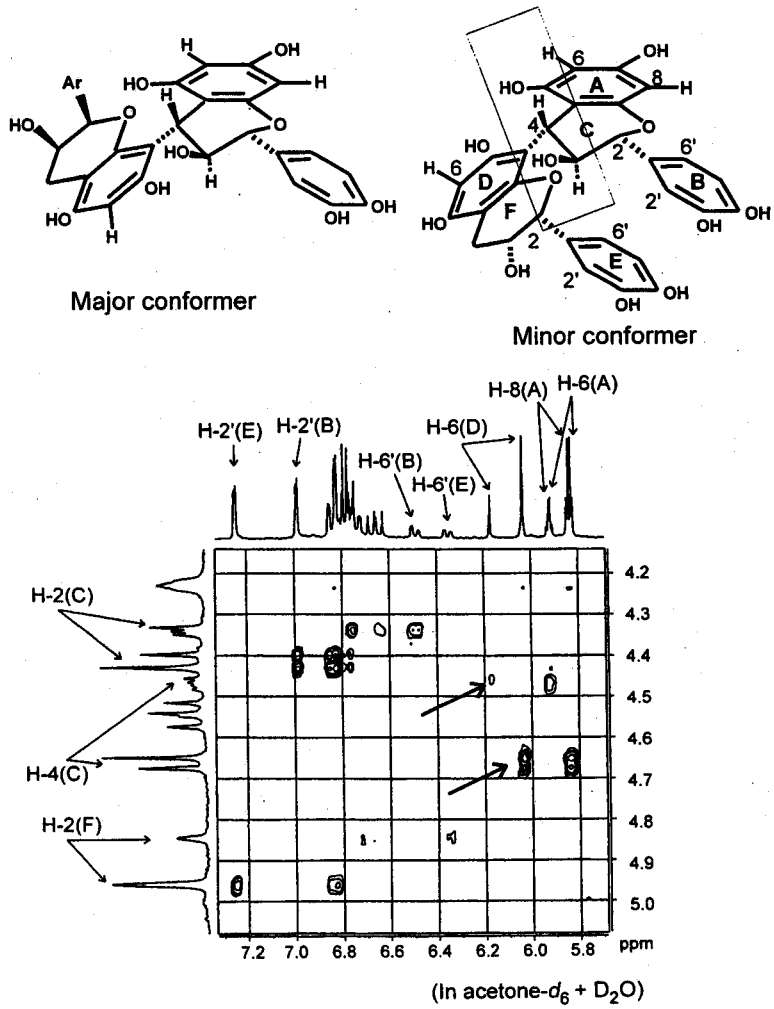


Figure 6. ^1H-^1H Long-range COSY spectrum of catechin-(4α→8)-epicatechin in acetone-d_6 containing D_2O.

Analogous spatial correlations between the protons of C- and E-rings in one conformer of the dimeric procyanidin, catechin-(4α→8)-catechin were also observed in the NOESY and NOE difference spectra.

3. CONFORMATIONS IN WATER

The ^1H NMR spectra of catechin-(4α→8)-epicatechin in three different solvents, acetone-d_6, dioxane-d_8 and D_2O, differed as shown in figure 9. The chemical shifts of several aromatic protons in the spectrum in dioxane-d_8 varied from those in the spectrum in acetone-d_6, whereas relative abundances of the two conformers in

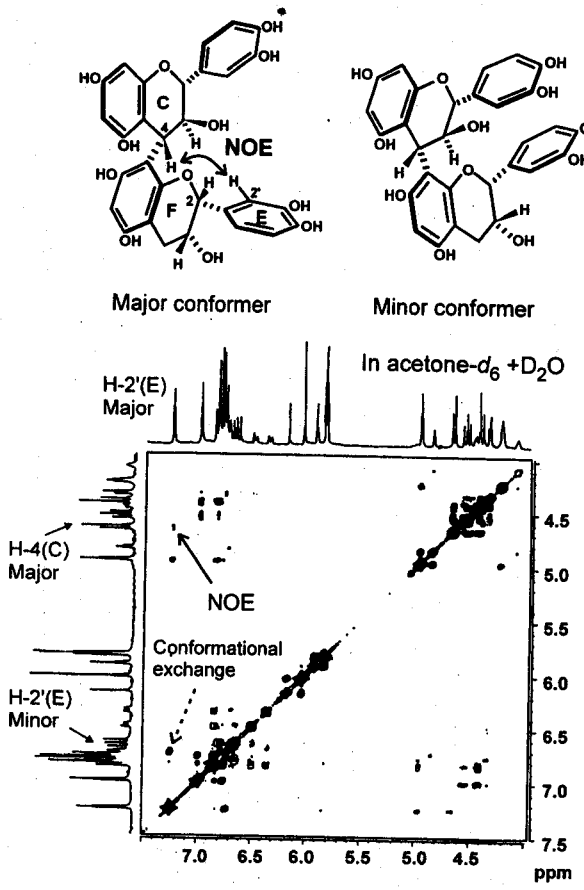


Figure 7. NOESY Spectrum of catechin-(4α→8)-epicatechin in acetone-d_6 containing D_2O.

these two solvents were almost the same. In D_2O, the signals of the major conformer in the organic solvents almost disappeared, and only the signals of another conformer were clearly observed. The aromatic protons of the major conformer in D_2O (i.e., the minor conformer in the organic solvents) shifted upfield noticeably relative to those of the other conformer. These changes are attributable to the anisotropic effects between B- and E-rings where the two aromatic rings overlap each other.

The composition of the two conformers of catechin-(4α→8)-epicatechin was dependent on the water content in the organic solvent. As shown in figure 10, the abundance of the major conformer in the organic solvents increases in the presence of a small amount of D_2O and then rapidly decreases upon further addition of D_2O. The relative proportion of the two conformers was estimated from the peak area in the ^1H NMR spectra. This change in rotamer population can be explained in the following way. The more "extended" conformer (i.e., the major conformer in

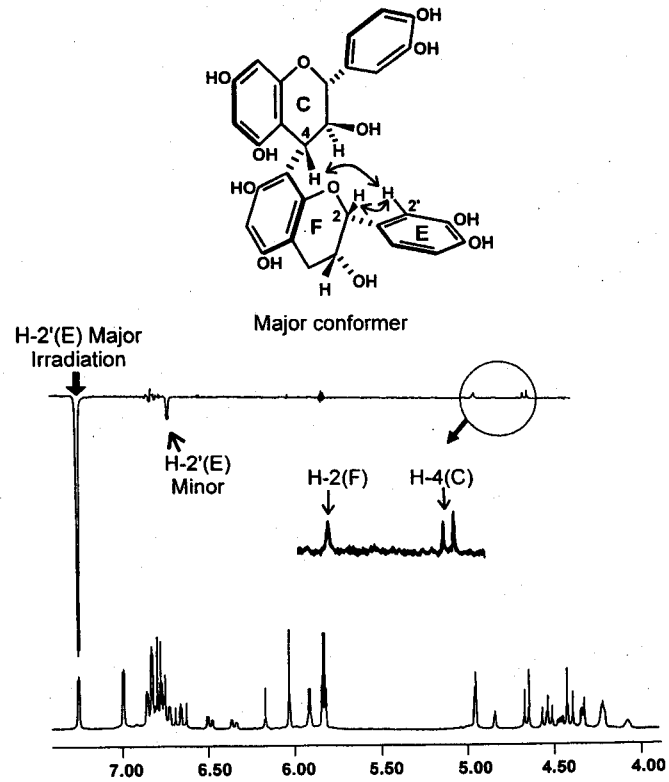


Figure 8. NOE difference spectrum of catechin-(4α→8)-epicatechin in acetone-d_6 containing D_2O.

acetone-d_6) is considered to be energetically preferable in the organic solvents. On the other hand, in D_2O, the dimer prefers the more "compact" conformation that is stabilized by hydrophobic or π-π interactions. These two conformations, which have been discussed for catechin-(4α→8)-epicatechin, are represented by those shown in figure 11.[10]

Interestingly, for catechin-(4α→8)-catechin, the relative abundance of the more "extended" conformer where the C- and E-rings are close to each other is smaller than that of the more "compact" conformer even in organic solvents such as acetone-d_6 and dioxane-d_8. In D_2O, this dimer exclusively adopts the more "compact" conformation.

4. INTERACTIONS BETWEEN POLYPHENOLS AND PEPTIDES CONTAINING PROLINE RESIDUES

Utilization of tannins for leather-tanning is based on the affinity of tannins for collagen, which is rich in proline and hydroxyproline residues.[2] The abundant prolyl residues in salivary proteins also are considered to contribute substantially

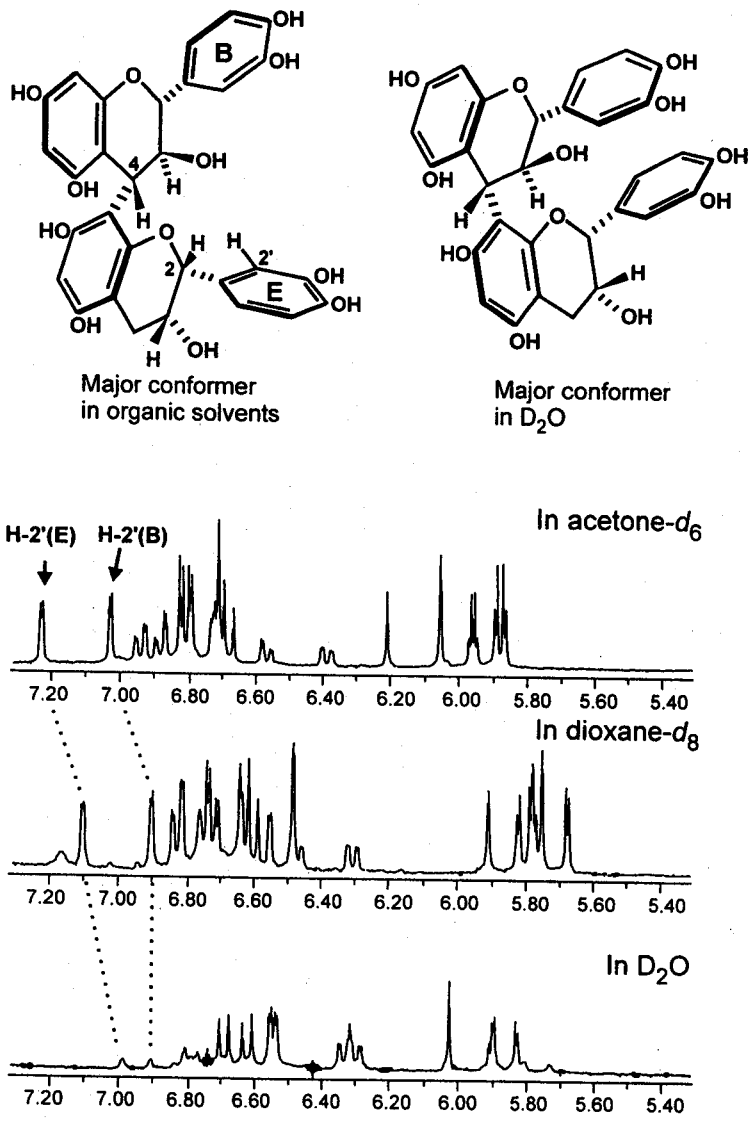


Figure 9. Solvent dependent changes of the ^1H NMR spectrum of catechin-(4α→8)-epicatechin.

to preferential complexation with tannins resulting in the astringency so important to the flavor of fine red wines.[2,11] The benefits of traditional uses of medicinal plants containing high levels of tannins on such problems as skin diseases or digestive disorders may also be attributable to the interaction of tannins with proline-rich proteins. Participation of proline residues in the complexation between pentagalloylglucose and proline-rich peptides has been shown.[12]

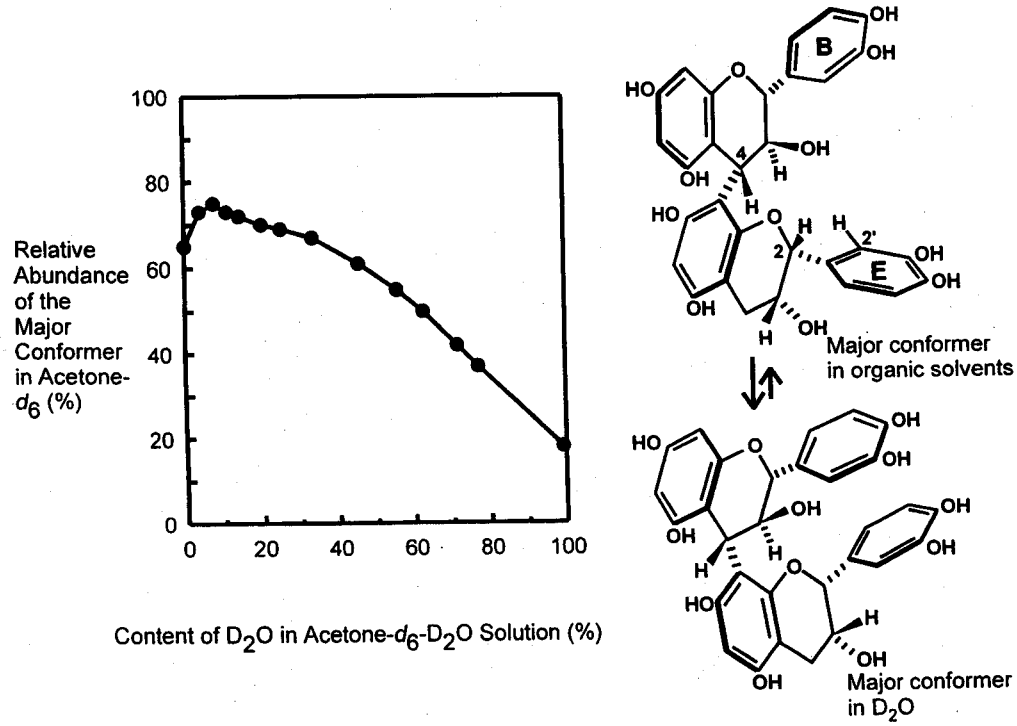


Figure 10. Effects of D_2O content on relative abundances of two conformers of catechin-(4α→8)-epicatechin.

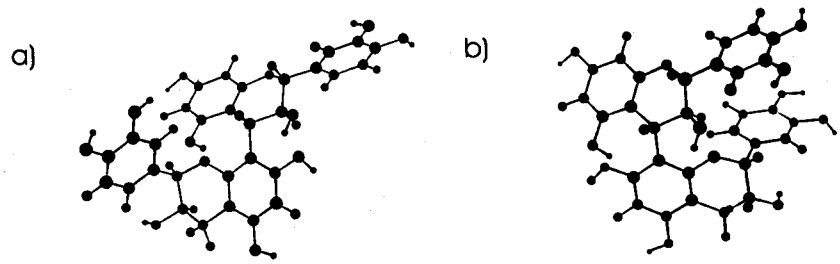


Figure 11. Conformations of catechin-(4α→8)-epicatechin in solutions a) More "extended", major conformation in acetone-d_6 and dioxane-d_8. b) More "compact", minor conformation in D_2O.

Our studies started with the analysis of the simplest combination, catechin and proline (or hydroxyproline), using NOESY experiments as a tool for clarifying the positions in each of the two molecules involved in molecular interaction. Catechin has been regarded as too small to interact to form precipitates with proteins.[13] However, when even a small amount of catechin was added to an aqueous solution (containing 10 percent of CD_3OD) of poly-L-proline (m.w. 10,000–30,000)

(9 mM for catechin and 120 mM for a monomeric proline residue), a white precipitate was formed. The interaction of catechin with a local portion of the polypeptide chain was proposed as the mechanism of this interaction.[14] NMR experiments on mixtures of catechin and proline in H_2O (containing 10 percent CD_3OD) with the NOESYHG pulse sequence[9] showed cross peaks of intermolecular NOEs between catechin B-ring protons and the proline C_γ protons (fig. 12). The C_β and C_δ protons of proline showed weaker interactions with aromatic protons of both the A- and B-rings of catechin. On the other hand, the NOESY spectrum of a mixture of catechin and *cis*-4-hydroxy-L-proline showed significant cross peaks between the C_δ protons of hydroxyproline and B-ring protons of catechin. The spectrum also showed correlations between a hydroxyproline C_δ proton (α-oriented

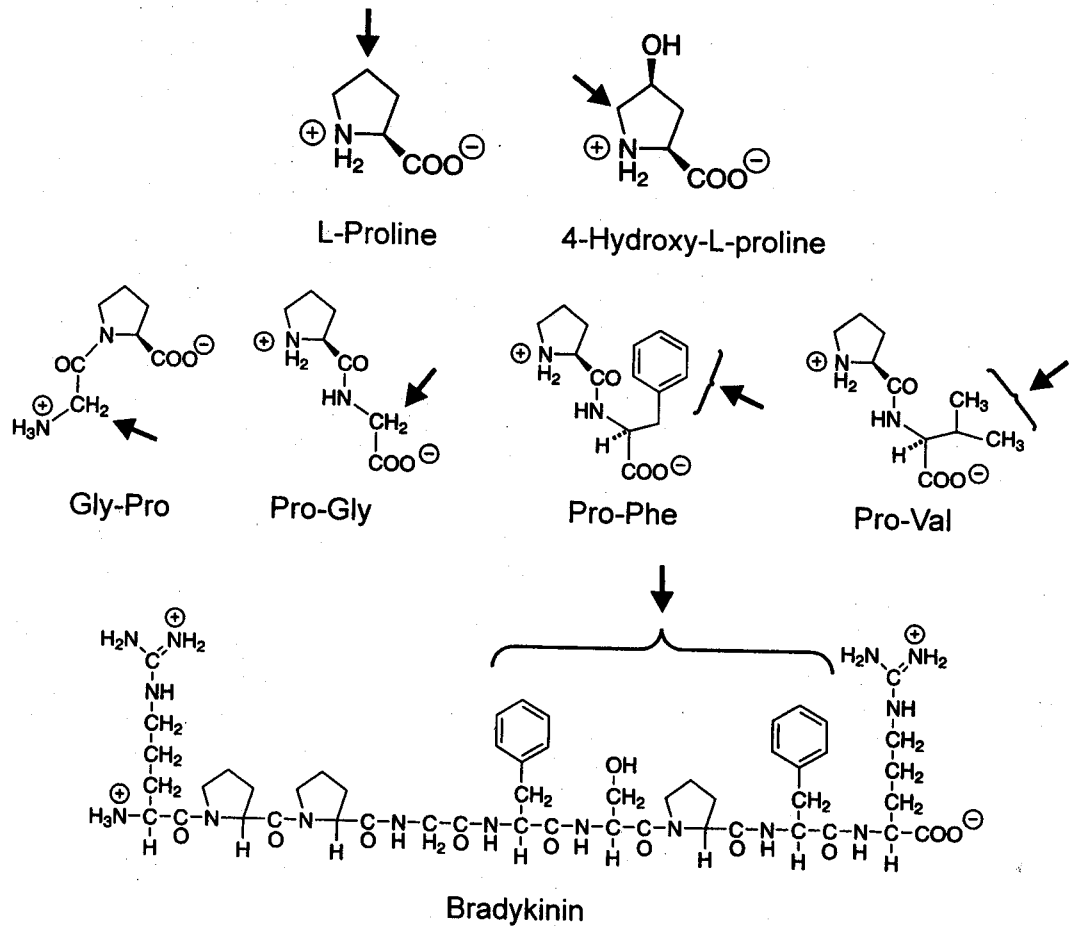


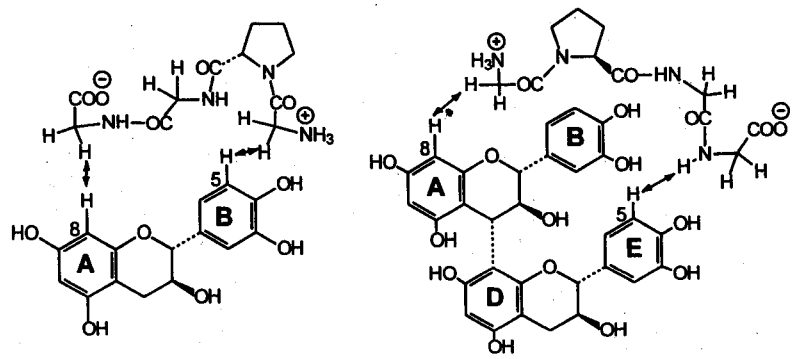
Figure 12. Association of (+)-catechin with proline, hydroxyproline, and dipeptides containing proline. The arrows indicate the sites that showed NOE interaction with catechin protons.

proton) to catechin H-6 and H-8. Importantly, noticeable changes in the ^{1}H or ^{13}C chemical shifts, which would suggest the presence of strong hydrogen bonding effects, were not observed. Therefore, hydrophobic interaction seems to be the dominant feature of these intermolecular associations. The aromatic rings of catechin and the aliphatic regions of proline or hydroxyproline come in close contact, but the complex is not so tight that we see significant changes in chemical shifts.

As mentioned above, polyphenols are believed to preferentially interact with proline residues in peptides. However, catechin showed intermolecular NOEs with the other amino acid residues rather than proline when dipeptides containing a proline residue, shown in figure 12, were used as targets for intermolecular recognition. An aqueous solution (containing 10 percent CD_3OD) of a mixture of Pro-Gly and (+)-catechin showed intermolecular NOE between the glycine C_α protons and the (+)-catechin H-8 proton. Pro-Val showed intermolecular NOE with catechin between methyl signals of the valine residue and the catechin H-8 as well as the H-2′ proton. Pro-Phe showed intermolecular NOE with catechin between the phenyl ring of the peptide and the catechin B-ring. Gly-Pro showed two sets of ^{1}H and ^{13}C signals, which are attributable to the isomers concerning the amino (imino) nitrogen of proline. The major isomer, which showed a 2H singlet-like signal for glycine C_α protons, was assigned the *trans*-configuration. The minor isomer, which showed two doublets of the corresponding protons whose chemical shifts are separated due to the effect of the neighboring proline carbonyl carbon, was assigned the *cis*-configuration. The NOESY spectrum of a mixture of catechin and Gly-Pro showed significant cross peaks between the glycine protons of the *cis*-isomer and the catechin H-6 and H-8. On the other hand, the *trans*-isomer showed only very weak cross peaks with the B-ring protons of catechin. These results indicate that two aromatic rings of the catechin molecule preferentially associate with hydrophobic moieties in peptides other than the proline residues. Self-association of (+)-catechin in water was also shown by an NOE correlation of H-2′ with H-8 of another molecule of catechin, even in competition with these peptides.

NOESY experiments on the interactions of polyphenols with two oligopeptides containing proline residues suggested the importance of the molecular conformations of both the polyphenol and peptide in addition to the hydrophobicity, to the specificity of their complexation. Bradykinin is a nonapeptide, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, containing three prolyl residues. This peptide exhibits various physiological effects including the regulation of blood pressure, and conformational studies on it in various solvents have been conducted.[15–17] Complexation of this peptide with polyphenols has also been investigated.[18] Our NOESY study on the interaction of bradykinin and (+)-catechin showed NOEs of the proline C_δ protons, the Ser C_β methylene protons, and the phenyl protons of the phenylalanyl residues with the catechin A-ring protons. The phenylalanyl residue also showed correlations with catechin B-ring protons. The amino acid residues in bradykinin showing these NOEs are contained in the sequence. Phe_5-Ser_6-Pro_7-Phe_8. The interaction of this same sequence in bradykinin with micelles has also been reported.[15]

Gly-Pro-Gly-Gly is a tetrapeptide with inhibitory effects on the activity of dipeptidyl peptidase IV. This compound has been reported to inhibit the entry of



Gly-Pro-Gly-Gly
+
(+)-Catechin

Gly-Pro-Gly-Gly
+
Catechin-(4 $\alpha \rightarrow$ 8)-catechin

Figure 13. Observed NOE interaction of flavanoids with Gly-Pro-Gly-Gly in water.

HIV into cells.[19] Although the *cis*-isomer of the proline nitrogen is present, its content is low, so the mixture can be treated practically as *trans*-isomer. Differentiation of the three sets of methylene protons of the glycine residues in the peptide was substantiated by the ^1H-^1H and ^1H-^{13}C long-range COSY analyses.

The NOESY spectrum of the mixture of (+)-catechin and Gly-Pro-Gly-Gly in water (containing 10 percent CD_3OD) showed correlations of catechin H-6 and H-8 with the C_α methylene protons of the *C*-terminal glycine residue, and of the catechin H-5 proton with methylene protons of the *N*-terminal glycine residue, suggesting that the catechin A-ring preferentially associates with the *C*-terminal and the B-ring with the *N*-terminal glycine residues.[20] Intermolecular NOEs, which suggest participation of the proline residue in the complexation, were not observed. Proline probably plays a role in the restriction of the relative spatial positions of the two glycine residues.

Intermolecular NOEs were also observed for the combination of Gly-Pro-Gly-Gly and catechin-(4$\alpha \rightarrow$8)-catechin. The association sites in the molecules of the polyphenol and peptide are, however, different from those observed in catechin. The NOESY spectrum showed intermolecular NOE between H-8_A (the upper unit of the procyanidin dimer) and the methylene protons of the *N*-terminal glycine residue and between the E-ring protons, H-5_E and H-2_E of the dimer with the amide proton of the *C*-terminal glycine residue. These cross peaks suggest an association as exemplified by figure 13.

5. CD SPECTRAL ANALYSIS OF POLYPEPTIDE CONFORMATION CHANGES

The NMR results described above indicated that complexation is directed to conformationally accessible hydrophobic regions; therefore, the molecular shapes

of both the polyphenol and polypeptide are important to selectivity. The NMR measurements of mixtures of flavans and bradykinin did not show significant changes in the chemical shifts of proton signals. This fact suggested interaction without conformational change of either the polyphenol or the peptide. Because circular dichroism (CD) is known to be sensitive to the conformational changes of peptides,[21] we used CD to verify whether the conformational changes in the peptide molecule occur with addition of polyphenols or not. When bradykinin was used as the peptide for the complexation, CD spectral measurements did not show any significant spectral changes upon the addition of either catechin or catechin-(4α→8)-catechin.

However, the CD spectrum of cytochrome *c* showed a noticeable change in the visible region when either catechin or catechin-(4α→8)-catechin was added to the solution of cytochrome *c* (fig. 14). This CD spectral change can, however, be attributed to the reduction of Fe^{3+} to Fe^{2+} of the heme prosthetic group based on the change in the visible spectrum (fig. 14). Most proteins in aqueous media, including cytochrome *c*, adopt conformations where the hydrophobic and hydrophilic side chains of the constituent amino acid residues are inside and outside, respectively. The reduction of the heme group by the polyphenols must be explained in terms of the interaction at the hydrophobic "inside" region in the cytochrome *c* molecule. Further study on the interaction of flavans with cytochrome *c* is now in progress.

6. CONCLUSIONS

Complete assignment of the ^1H and ^{13}C NMR spectra of the two conformational isomers for each of the dimeric proanthocyanidins catechin-(4α→8)-catechin and catechin-(4α→8)-epicatechin, in the natural free phenolic form and in the biologically significant solvent water, has been accomplished.[10] Knowledge of the assignments of the proton spectra, together with the application of various two-dimensional NMR experiments, has permitted the definition of the shapes of the heterocyclic C and F rings in the upper and lower units, the dihedral angles between the upper and lower flavan units, and the effect of changes in solvent composition on the relative proportions and shapes of the two conformers of these dimeric proanthocyanidins in organic and water solvents.[10] In organic solvents, the more extended conformation is favored for catechin-(4α→8)-epicatechin; and a similar extended conformation, although not the major isomer, is present in nearly equal proportion to a more compact conformer for catechin-(4α→8)-catechin. However, in water the more compact conformers dominate. These "unfavorable" conformations seem to be stabilized by hydrophobic (or π-π) interactions between the upper and lower unit catechol rings.

The importance of hydrophobic interactions was further highlighted in studies of the interaction of proanthocyanidins with oligopeptides. Even though trimeric and higher molecular weight proanthocyanidins are commonly assumed to be responsible for the precipitation of proteins, when even low mole ratios of the monomeric flavan-3-ol (+)-catechin was combined with polyproline, a white precipitate was obtained.[20] Evidence for hydrophobic interaction was seen in NOESYHG experiments, but no noticeable changes in ^1H or ^{13}C chemical shifts

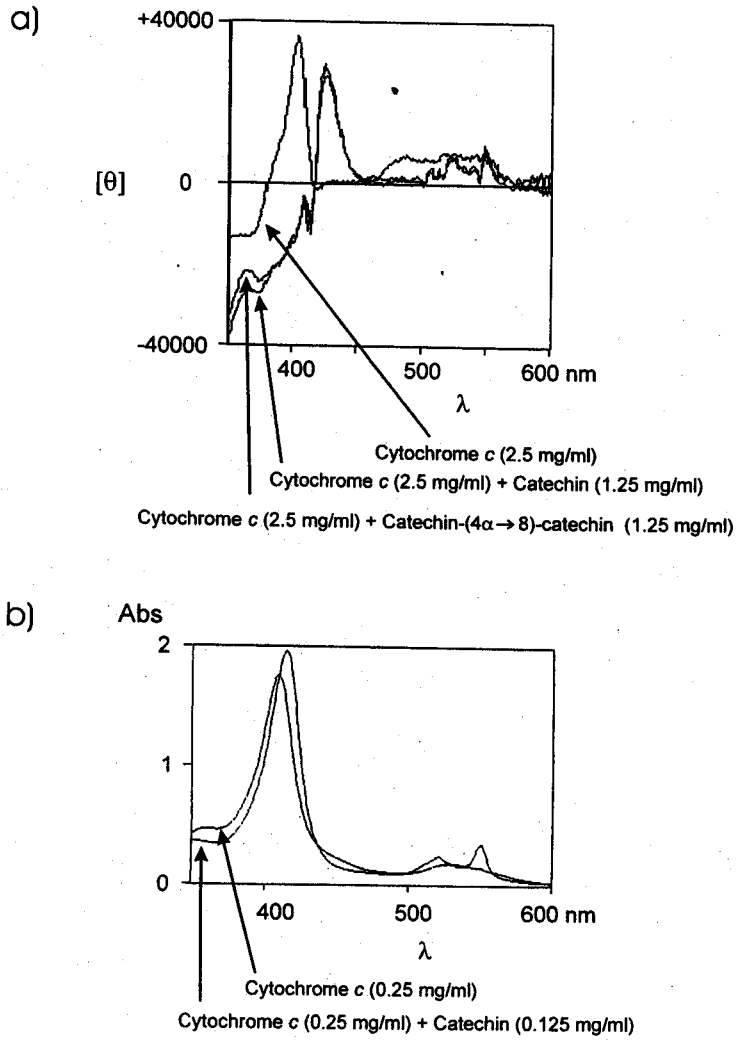


Figure 14. Spectral changes of cytochrome *c* upon the addition of flavans a) CD spectra (in H_2O-CH_3OH, 9:1, v/v). b) Visible spectra (in H_2O-CH_3OH, 9:1, v/v).

were seen that would suggest strong hydrogen bonding in mixtures of (+)-catechin with L-proline or 4-hydroxy-L-proline. Studies of a series of dimeric peptides with various amino acid residues coupled with proline once more highlighted the significance of hydrophobic interaction. In association of catehin with dimeric peptides, stronger cross peaks were seen between either the A- or B-ring protons with the glycine, valine, or phenylalanine protons than to the prolyl unit protons. Once more, no measurable changes in chemical shifts suggesting strong hydrogen

bonding were observed. It is important to note that intermolecular self-association of catechin also resulted in strong cross peaks in these experiments.

Addition of catechin or its dimer to Gly-Pro-Gly-Gly did not show association with the prolyl residue but rather to the conformationally accessible methylenes of glycine.[20] No significant changes in NMR chemical shifts or CD spectra that would suggest a "tight" binding or strong hydrogen bonding effects were noted. Studies of the interaction of catechin and dimeric procyanidins with bradykinin and cytochrome *c* also showed no significant change in the conformation of the peptides. Rather, association of the polyphenol with the presumed biologically active center of bradykinin and reduction of the heme group that resides within the interior of the cytochrome *c* polymer suggested that hydrophobic interaction dominates in the association of polyphenols with proteins in water. No significant changes in NMR chemical shifts that might suggest strong hydrogen bonding were observed. The evidence of all these experiments suggests strong interaction of polyphenols with hydrophobic centers where both self-association and preference for accessible hydrophobic regions of oligomeric peptides dominate.

ACKNOWLEDGMENTS

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