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THE OXIDATIVE TRANSFORMATIONS OF (+)CATECHIN AND (–)EPICATECHIN AS STUDIED BY ESR

FORMATION OF HYDROXYCATECHINIC ACIDS

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Abstract—The alkaline air oxidation of (+)catechin and (–)epicatechin has been studied by ESR spectroscopy. Both compounds give rise to the anion radicals of 2'-hydroxycatechinic acid and 6'-hydroxycatechinic acid by way of a hydroxylation and a rearrangement reaction. The primary spectra of (+)catechin, catechinic acid and the intermediate radicals of 2'-hydroxy-(+)catechin and 6'-hydroxy-(+)catechin are observed and characterized by ESR.

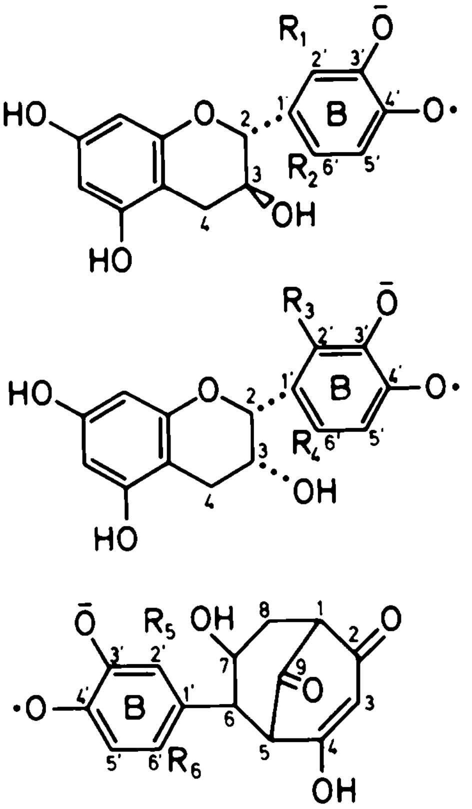
The flavan-3-ol compounds (+)catechin and its diastereomer (–)epicatechin are widely distributed throughout the plant kingdom.[1] They are found unconjugated, not as glycosides, in the vegetative tissues (leaves and fruits) of herbaceous plants, and as structural units in phenolic polymers (e.g. condensed tannins) in plants with woody habit.[2] The phenolic component of condensed tannins are usually extracted with hot dilute aqueous base or alkaline bisulfite solutions.[3] The extracts contain numerous chemical components for which the structural information is limited. In a previous paper[4] we showed how compounds with a quinone or quinol nucleous could be detected as the corresponding anion radicals in crude extracts by the ESR technique. Since we have observed in a number of plant extracts a radical, seemingly derived from a flavan-3-ol, we became interested in how the tannin precursors (+)catechin and (–)epicatechin react in alkaline solutions. Here we report the ESR data of the anion radical of (+)catechin generated in alkaline *oxygen saturated* solutions, and of a number of radical intermediates derived from it. The (+)catechin radical has been characterized previously by ESR, but some spectra from secondary radicals representing derivatives of (+)catechin could not be interpreted.[5] Sears *et al.*[6] have reported the rearrangement reaction of (+)catechin in *oxygen free* alkaline solutions to give catechinic acid, (6-(3,4-dihydroxyphenyl)-7-hydroxy-2,4,9-bicyclo [3.3.1] nonatrione). Accordingly, we found it worthwhile to include this acid in the present investigation in order to elucidate the reactions taking place. From the reactions of (–)epicatechin we have observed the same end product as from (+)catechin. Our failure to observe the radical of (–)-epicatechin itself and some intermediate ones, expected to be generated in the alkaline medium, seems to indicate that radicals with the *cis* configuration (*cf* (–)epicatechin) have a much lower life time than those with the *trans* configuration (*cf* (+)catechin).

RESULTS AND DISCUSSION

Primary and secondary radicals from (+)catechin, (–)epicatechin, and catechinic acid. The above compounds were oxidized with air in aqueous alkaline dimethyl sulphoxide (DMSO) or aqueous alkaline ethanol (EtOH) solutions. For the primary radicals and those obtained from secondary products their stability and spectral parameters were dependent on the amount of water in the solvent and the pH. We were thus able to generate selectively primary and secondary radicals by choosing proper experimental conditions.

It is well known that flavonoid compounds with ortho dihydroxy substituents on the B-ring give rise to anion radical spectra similar to those obtained from pertinent monosubstituted catechol derivatives, i.e. the unpaired electron, mainly located in the B-ring, exhibits hyperfine couplings to the three B-ring protons and to one or more β- and γ-protons depending on the structure in question. From the hyperfine splitting constants (hfs) observed and shown in Table 1, we suggest the structures IV–VII, Scheme 1, for the generated secondary radicals, and from their mode of formation the reactions in Scheme 2. We shall apply Roman letters to designate radical structures as well as parent compounds whatever is appropriate.

Primary and secondary radicals from (+)catechin. (+)-Catechin autoxidizes to the primary radical I with a strong ESR spectrum at pH $\leq$ 12.70 in aqueous DMSO, while in aqueous EtOH only weak spectra of low resolution are observed. When pH $\geq$ 12.80 hydroxylation of (+)catechin leads to the observation of secondary radical species both in aqueous EtOH and aqueous DMSO solutions. For monosubstituted catechol derivatives hydroxylation takes place at C-2' or C-6' depending on the solvent and the substituent, R, at C-1' of the catechol unit, here numbered 3',4'-dihydroxybenzene, see Scheme 1. Thus, when R is an alkyl substituent the hydroxylation takes place at C-6',[7-9] and when R = CHO,COMe, COEt,COO mainly C-2' hydroxylation occurs.[10] For catechin considered as a monosubstituted catechol, however, hydroxylation takes place at C-6' as well as at C-2' leading to the two secondary radicals IV and V, respectively. To know solely from the spectral information whether hydroxylation at C-2' or at C-6' actually has occurred, recourse must be taken to results of pertinent trihydroxylated benzene radicals. Thus, radicals of 1'-substituted 2',3',4'-trihydroxybenzene constitute suitable models for radical V (hydroxylation at C-2') and radicals of 1'-substituted 3',4',6'-trihydroxybenzene those for radical IV (hydroxylation at C-6'). The former gives



I $R_1 = R_2 = H$

IV $R_1 = H, R_2 = O^-$

V $R_1 = O^-, R_2 = H$

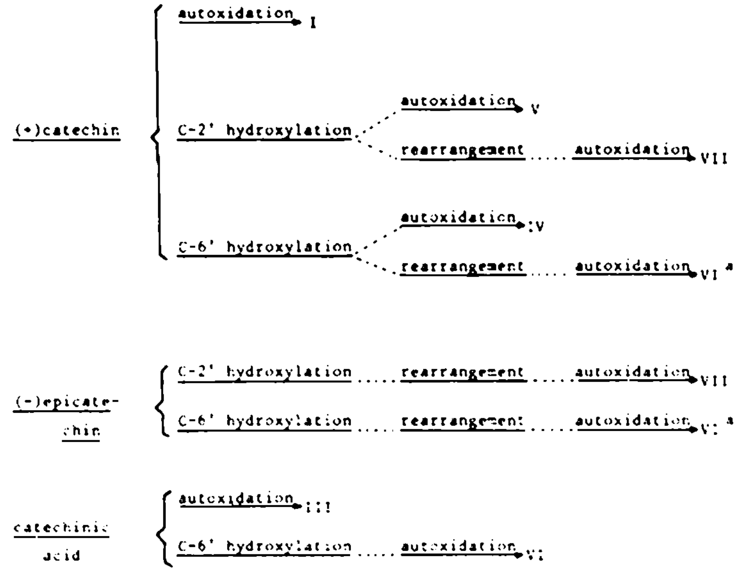
II $R_3 = R_4 = H$

III $R_5 = R_6 = H$

VI $R_5 = H, R_6 = O^-$

VII $R_5 = O^-, R_6 = H$

Scheme 1.



[a] Sequence of hydroxylation and rearrangement, see text.

Scheme 2.

hfs constants from two aromatic protons with values, $a_{5'}^H \sim 0.8$–1.5 G and $a_{6'}^H \sim 4$–6 G, and β-proton couplings of 0.6–1.5 G, and the latter $a_{5'}^H \sim 0.40$–0.60 G and $a_{2'}^H \sim 0.7$–1.3 G, and β--couplings of 2–5 G.[10-12] The actual values observed depend on solvent, pH, counterion and substituent, and the ranges stated apply to alkaline aqueous DMSO and alkaline aqueous EtOH solutions with Na^+ as counterion.

In order to obtain the radical structure from the data of a given spectrum one must correctly assign the observed hfs constants. The important problem is to single out the coupling constant, which derives from the β-proton at C-2, since this constant is large in IV and small in V. Fortunately, this constant exhibits a stronger solvent and pH dependence than any of the other constants observed. This reflects partly changes in the dihedral angle, θ, partly changes in the spin density at C-1'.[11,12] The assignment of $a_2^H = a_\beta^H$ has therefore been based on pH and solvent studies of the secondary radicals. In Table 1 the hfs constants of the radicals IV and V are placed together with the constants of pertinent model radicals. For comparable systems we notice the assignments to be consistent.

We have observed that the hydroxylations taking place in the B-ring are dependent on the solvent used. In aqueous EtOH only IV (C-2′ hydroxylation) is observed, while both IV and V (C-2′ and C-6′ hydroxylations) are generated in aqueous DMSO. At pH = 12.80 and with DMSO/H_2O solvent mixtures the life time of IV is longer than the one of V, and the intensity of the spectrum of IV stronger than the one of V. The reverse is true at pH = 13.00 and the absolute solvent composition DMSO/H_2O = 3/1.

In aqueous DMSO at pH 12.80 the radicals IV and V disappear within minutes and two new radicals VI and VII appear within 5–15 min. In aqueous EtOH only VI appears. The absence in aqueous EtOH of radical VII is in line with the previously mentioned absence of radical V, as will be evident in what follows. When pH ≥ 13.10 the secondary radicals IV and V are unobserved, and VI and VII are detectable only. Raising the pH to more than 13.60 VI becomes the only radical observed in both solvents. We propose VI and VII to be the radicals generated from 2′-hydroxycatechinic acid and 6′-hydroxycatechinic acid, respectively. For VI this follows from the fact that a spectrum identical to the one of VI is observed in the direct hydroxylation of catechinic acid in alkaline solutions (see below). A further proof for the identity of radical VI is obtained by comparing its hfs constant in Table 1 with those of the related structure IV. Both give rise to two hfs constants of similar magnitude from the two aromatic protons (B-ring protons from IV) and both exhibit a large constant from an aliphatic β-proton and a smaller one from a γ-proton. From VI an extra splitting is discernible from the γ-proton at C-5. We conclude IV and VI share a common 3′,4′,6′-trihydroxy pattern and have the structures given in Scheme 1. In analogy with the above analysis we suggest V and VII share a common 2′,3′,4′-trihydroxy pattern and have the structures given in Scheme 1. Again an extra γ-coupling from the proton at C-5 is observed for VII in line with the result of VI.

For the radicals IV and VI we observe hfs constants for β- and γ-protons of the expected magnitudes. The observation of an unusual small hfs constant from the β-proton of V (or VII) can be explained partly by the lower spin density at C-1′ of these radicals compared with the spin density at C-1′ of IV (or VI), (*cf* the spin densities at the comparable positions of the anion radicals of 1,2,3-trihydroxybenzene and 1,2,4-trihydroxybenzene), and partly by difference in equilibrium conformations, which we shall discuss later.

Primary and secondary radicals from (−)-epicatechin. We did not succeed in obtaining a well resolved spectrum of the anion radical of (−)epicatechin, II, nor in obtaining spectra corresponding to the *cis*-analogues of IV and V. Our failure to observe these radicals seems to indicate a reduced life time for radicals having the *cis*-configuration. We expect the hfs constants of the B-ring protons of II to be of similar magnitude to those of I. The magnitude of $a_{\gamma}^{H} = a_{\beta}^{H}$ from II cannot be predicted with certainty, however, because it depends on the actual equilibrium conformation given by the dihedral angle, θ, which is unknown.

The only detectable radicals derived from (−)epicatechin are the radicals VI and VII in alkaline aqueous DMSO, and VI in aqueous EtOH. VI and VII thus turn out to be the common end product of the alkaline air oxidation of (+)catechin and (−)epicatechin. The generation of these radicals must obviously go through a hydroxylation reaction and a rearrangement reaction, the sequence of which is unknown, however. Therefore we have to consider the following pathways for the generation of the radicals VI and VII:

(+)catechin/(−)epicatechin →

$$(a)\xrightarrow{\text{C-6 hydroxylation}}\text{IV}\xrightarrow{\text{rearrangement}}\text{VI}$$

$$(b)\xrightarrow{\text{C-6 hydroxylation}}\text{V}\xrightarrow{\text{rearrangement}}\text{VII}$$

$$(c)\xrightarrow{\text{rearrangement}}\text{III}\xrightarrow{\text{C-6 hydroxylation}}\text{VI}$$

$$(d)\xrightarrow{\text{rearrangement}}\text{III}\xrightarrow{\text{C-2 hydroxylation}}\text{VII.}$$

In order to discriminate between these pathways, we have investigated the reactions of the key intermediate, catechinic acid, in alkaline medium.

Primary and secondary radicals of catechinic acid. In aqueous EtOH the primary radical of catechinic acid, III, is seen when pH = 12.80, while a secondary radical identical to the before mentioned radical VI is seen at higher pH. In aqueous DMSO the catechinic acid anion radical is observed when pH = 12.18, while a radical, again identical to VI is observed at higher pH. We therefore conclude that the secondary radical from catechinic acid and the radical VI are derived from the same compound, namely 6′-hydroxycatechinic acid. The anion radical of 2′-hydroxycatechinic acid is incompatible with the hfs constants observed. Only C-6′ hydroxylation occurs for catechinic acid, ruling out the reaction pathway (d). The reaction sequence leading to VII is (b) whereas both (a) and (c) are possible pathways for the generation of VI. Since VII is unobserved at higher pH we might consider the rearrangement and the C-2′ hydroxylation as competetive reactions, where the former is favoured at higher pH and the latter at lower ones.

Solvent, pH and temperature dependence of the splitting constants of the radicals I–VII. In general the splitting constants of anion radicals of catechol derivatives are dependent on solvent, pH, counterion and temperature.[1,5,7,10] In order to get insight into the mechanism leading to the β- and γ-couplings of the radicals observed, we investigated the solvent, pH, and temperature dependence of their hfs constants. Due to radical instability such studies were not performed for I.[5] The constants of III, V, VI and VII turned out to be nearly independent of changes in solvent and pH as indicated for III and VI in Table 1. Furthermore, for all compounds studied (excluding I) their hfs constants were found temperature independent within the range −25–40°. For the radicals III, V, VI and VII when combined with stereochemical considerations this can be interpreted as possession of frozen conformations, usually asserted as a necessary prerequisite for observation of sizeable long-range couplings on the time scale of the ESR experiment. In contrast, the β-coupling from C-2 of radical IV exhibits a marked solvent and pH dependence with the other hfs constants of the radical being constant as seen in Table 2.

We are presently engaged in studies of other flavonoids showing a similar "β-solvent/pH effect", e.g. dihydrofisetin exhibits a β-coupling increase from 1.75 to 3.75 G for a certain solvent and pH change with the other hfs couplings being constant. We believe the effect on the β-coupling is due to a simple change of the dihedral angle and the radical assumes a new equilibrium conformation under each experimental condition applied. The absence of a similar effect for radical VI, also having the 3,4,6-trihydroxy grouping, might be explained by the rigid structure of this radical.

The constancy of the γ-couplings for IV, V, VI and VII indicates that the mechanism leading to these couplings are independent of the dihedral angle. Furthermore, the large reduction of the spin density at C-1 when going from IV to V or from VI to VII, reflected in the changes of the β-couplings, is not mirrored in the γ-coupling. Thus spin polarization rather than electron delocalization is apparently the important mechanism for the γ-coupling of these compounds. A similar mechanism has been claimed to operate in semiquinones of bicycloalkyl derivatives.[11] We have calculated the equilibrium conformations given by the dihedral angle, with aid of the Heller–McConnell relationship[14] for the β-proton splitting:

$$a_\beta{}^H = (B_0 + B_1 \cos^2\theta)\rho_{C'}^{\pi} = \langle Q(\theta)\rangle \rho_{C'}^{\pi}. \tag{1}$$

Here B_0 and B_1 are empirical parameters, $\rho_{C'}^{\pi}$ the spin density at C-1', and θ the dihedral angle. $\langle Q(\theta)\rangle$ is the quantum mechanical average of $\cos^2\theta$ over the appropriate rotational wave functions $\psi(\theta)$; B_0 is usually interpreted as a sum of indirect and direct spin polarizations. The indirect spin polarization corresponds to the consecutive polarization of the C–C σ-bond followed by spin decoupling of the C–H σ-electrons. The direct polarization refers to "through space" coupling. B_1 expresses the degree of charge transfer,—by some authors hyperconjugation. There is not complete agreement on the values which should be assigned to B_0 and B_1. Based on classical and quantum mechanical calculations Stone and Maki[15] considered the spin polarizations to be negligible. With this assumption eqn (1) is simplified to $a_\gamma{}^H = B_1 \cos^2\theta\rho_{C_{1'}}^{\pi}$. Stegman *et al.*[6] used this simplified equation in calculating the conformation of the catecholamine, (+)L-adrenaline, by setting $B_1 = 54$ G. On the other hand Adam and King[17] calculated $B_0 = 2.71$ G and $B_1 = 50.14$ G by means of the ASWO CI procedure for a C–C–H fragment. Their calculations included direct polarization and spin transfer, whereas indirect polarization was excluded due to its negligible effect according to Colpa and de Boer.[18]

The spin density in eqn (1) cannot be obtained directly, e.g. by the well known McConnel relationship since no proton coupling is available from the C-1' position. For each radical an individual procedure must be applied. In the case of I (or III) we may assume $\rho_{C_{6'}}^{\pi} \simeq \rho_{C_{1'}}^{\pi} = a_{6'}{}^H/|Q_{CH}^H|$ with $|Q_{CH}^H| = 23$ G.[16] For IV and VI we assume analogously that $\rho_{C_{1'}}^{\pi}(\mathrm{IV}) \simeq \rho_{C_{1'}}^{\pi}(\mathrm{VI}) = \rho_{C_5}^{\pi}$, where the last mentioned spin density is obtained from the anion radical of 5-methyl-1,2,4-trihydroxybenzene and the equation $\rho_{C_5}^{\pi} = a_{Me}^H/(B_0 + \frac{1}{2}B_1)$; ($\cos^2\theta = \frac{1}{2}$ assuming an average angle of 45°). For the 2',3',4'-trihydroxy derivatives, V and VII, we suggest the spin densities estimated by $\rho_{C_{1'}}^{\pi}(\mathrm{V}) \simeq \rho_{C_{5'}}^{\pi}(\mathrm{V}) = a_{5'}^H(\mathrm{V})/|Q_{CH}^H|$, and $\rho_{C_{1'}}^{\pi}$ (VII) $= \rho_{C_{5'}}^{\pi}$ (VII) $= a_{5'}{}^H$ (VII)/$|Q_{CH}^H|$ with $|Q_{CH}^H| = 23$ G. In Table 3 two sets of calculated equilibrium angles and spin densities, ρ, are given for the radicals in question, one set based on the parameters of Stegman[16] and one on the parameters of Adam and King.[17] The spin densities are calculated with the assumptions mentioned above.

The estimated equilibrium conformations given by θ_{eq} for I, IV and V are in accordance with equilibrium conformations of similar radicals.[16] The dihedral angles of III, VI and VII are lower than the 90° that represents the equilibrium angle for radicals having a tertiary β-carbon (e.g. the isopropyl catechol anion radical).[11] Thus the different substituent pattern at C-5 and C-7 must explain this deviation.

ESR of naturally occurring flavon-3-ols. In our continued application of the ESR technique to detect phenolics from crude plant extracts[4,19] we have observed a spectrum indistinguishable from the one of radical VI in several plant specimens. Thus, the spectrum has been obtained from leaf extracts of *Thuja* (*Cypressaceae*), *Cassiope*, *Ledum*, *Rhododendron* (*Ericaceae*), *Juglans* (*Juglandaceae*) and catkins of *Betula* (*Betulaceae*). From several specimens of *Salix* and *Populus* (*Salicaceae*) we observed the spectrum from extracts of buds, all collected in February/March on Danish locations, including botanical gardens. We think VI is an artifact generated in the alkaline medium from (+)catechin or (−)epicatechin present in the plant extract.

CONCLUDING REMARKS

The present ESR studies have shown how alkaline air oxidation of (+)catechin and (−)epicatechin lead to generation of the 2'-hydroxy- and 6'-hydroxycatechinic acids detected as corresponding anion radicals. The generation proceeds via a rearrangement reaction and a hydroxylation reaction, the latter being substantiated by the observation of intermediate radicals of 2'-hydroxy- and 6'-hydroxy-(+)catechin. Many flavonoids are known to degrade to simpler phenolics, e.g. protocatechuic acid, phloroglucinol, p-hydroxybenzoic acid and caffeic acid.[20-21] None of these compounds were observed in the degradation of the flavan-3-ols indicating the produced catechinic acids to be stable against further alkaline transformations.

The estimated dihedral angle of compound I is significantly larger than the recently calculated angle of the related compound tetra-O-methyl-(+)catechin.[25] Mattice *et al.*[25] found their calculated conformation inadequate, however, when compaired with the one they obtained from dipole moment measurements. In line with this finding they suggested an alternative conformation in which the B-ring of the catechin compound adopts an axial rather than an equitorial conformation. Our

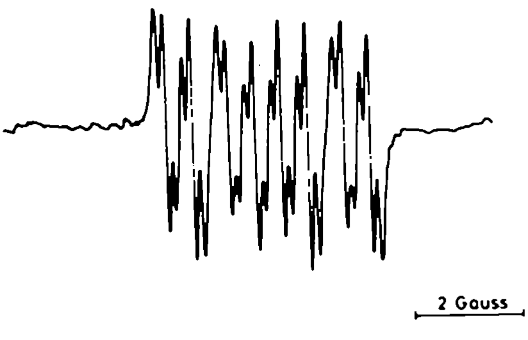


Fig. 1. ESR spectrum of 6'-hydroxy catechinic acid (VI) from an aqueous ethanolic solution (v/v 50%), pH = 13.30.

Table 1. Radicals observed during the autoxidation of (+)catechin, (−)epicatechin and catechinic acid with hyperfine splitting constants (Gauss).

Radical	Solvent	pH	$a^H_{2'}$	$a^H_{5'}$	$a^H_{6'}$	a^H_2/a^H_6	a^H_3/a^H_5	a^H_7	g-factor
I[a]	DMSO/H_2O 4/1	12.68	1.08	1.23	3.45	1.90	-	-	
III	DMSO/H_2O 2/1	12.18	0.65	0.90	3.75	2.20	-	-	2.00445
	EtOH/H_2O 1/1	12.60	0.65	0.90	3.75	2.20	-	-	2.00445
IV	DMSO/H_2O 3/1	13.00	1.30	0.60	-	3.75	0.30	-	2.00439
V	DMSO/H_2O 3/1	12.80	-	0.55	3.70	0.27	0.23	-	2.00439
VI	DMSO/H_2O 1/1	13.30	1.13	0.45	-	2.25	0.15	0.15	2.00438
	EtOH/H_2O 1/1	13.35	1.15	0.50	-	2.20	0.15	0.15	2.00437
VII	DMSO/H_2O 2/1	13.30	-	0.60	5.85	0.15	0.15	0.15	2.00437
b	DMSO/H_2O 1/1	13.30	0.75	0.55	-	5.15[d]	-	-	2.00434
	EtOH/H_2O 1/1	13.30	0.70	0.58	-	5.08[d]			2.00434
c	DMSO/H_2O 4/1	13.30	-	0.85	5.13	-	-	-	2.00458

[a] Ref. 5.

[b] Anion radical of 1'-methyl-3',4',6'-trihydroxybenzene.

[c] Anion radical of 1,2,3-trihydroxybenzene, - smaller splittings from a^H_4 and a^H_6 and larger splitting from a^H_5.

[d] Splitting methyl group protons.

observation of a large dihedral angle might support this suggestion.

EXPERIMENTAL

All radicals were generated by oxidation in alkaline solns using the static method and ESR spectra recorded on Varian E-3 and Bruker ER 200 ESR spectrometers at room temp. Desired pH values were obtained from standardized NaOH solns. The accuracy of the coupling constants was estimated to ± 0.03 G and of the *g*-factor to ± 0.00002. The anion radical of 1,2,4-trihydroxy-5-methylbenzene was used as an internal standard with $g = 2.00434$.

(+)Catechin (I) and (−)epicatechin (II) were of reagent grade and used as obtained. Catechinic acid (III) was synthesized from II by the method of Sears,[6] and its structure verified by UV, IR, MS and ^{13}C-FT NMR (shift values (ppm) in aqueous solution at neutral pH value: 221.99(C9), 193.1(C2), 144.4(C3',C4'), 131.6(C1'), 121.8(C6'), 117.2(C4), 117.1(C5'), 116.9(C2'), 66.85(C7), 66.4(C6), 58.2(C1), 53.5(C5), 36.6(C8)), chemical shift from C3 was not observed. The assignment of the chemical shifts were based partly on the SEFT-FTNMR technique and partly on empirical additivity rules.[24] The kinetics of the rearrangements of (+)catechin and (−)epicatechin to catechinic acid have been reported recently by Kiatgrajai *et al.*[26]

Table 2. pH and solvent effects of 6'-hydroxy-(+)catechin (IV)

$DMSO/H_2O$	pH	$a^H_{2'}$	$a^H_{5'}$	a^H_2	a^H_3	g-factor
4/1	12.90	0.60	1.35	4.15	0.30	2.00439
4/1	12.95	0.65	1.40	4.00	0.35	2.00439
4/1	13.00	0.60	1.40	3.75	0.30	2.00439
4/1	13.10	0.55	1.40	3.75	0.30	-
3/1	13.00	0.60	1.30	3.75	0.30	2.00439
2/1	13.00	0.55	1.35	3.25	0.35	2.00439

Table 3. Calculated spin densities at C-1' and equilibrium dihedral angles of the radicals I-VII

Radical	Solvent	pH	ρ^π_C a)	ρ^π_C b)	θ a)	θ b)
I	$DMSO/H_2O = 4/1$	12.68	0.150	0.150	61.0°	63.5°
III	$DMSO/H_2O = 2/1$	12.18	0.163	0.163	60.0°	62.4°
	$EtOH/H_2O = 1/1$	12.60	0.163	0.163	60.0°	62.4°
IV	$DMSO/H_2O = 4/1$	12.90	0.195	0.190	51.1°	51.8°
	$DMSO/H_2O = 2/1$	13.00	0.188	0.183	55.5°	57.5°
V	$DMSO/H_2O = 3/1$	12.80	0.024	0.024	62.8°	65.6°
VI	$DMSO/H_2O = 1/1$	13.35	0.195	0.190	62.5°	64.7°
	$EtOH/H_2O = 1/1$	13.30	0.188	0.183	62.3°	64.5°
VII	$DMSO/H_2O = 2/1$	13.35	0.026	0.026	71.0°	75.8°

a) $B_0 = 0$, $B_1 = 54$ G, Stegman *et al.*[16]

b) $B_0 = 2.71$ G, $B_1 = 50.14$ G, Adam and King.[17]

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