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Chemical Studies of Proanthocyanidins and Hydrolyzable Tannins

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ABSTRACT

We investigated a number of natural polyphenols representing flavan-3-ols, gallotannins, and ellagitannins with regard to their antioxidant potential. For this purpose we used pulse radiolysis to determine scavenging rate constants with hydroxyl radicals and decay rates of the respective aroxyl radicals and EPR spectroscopy to identify the radicals after *in situ* oxidation. Using NMR spectroscopy, we could confirm phenolic coupling reactions of epigallocatechin gallate and pentagalloyl glucose after radical-induced oxidation. Antioxid. Redox Signal. 3, 995–1008.

INTRODUCTION

PROANTHOCYANIDINS OR CONDENSED TANNINS are oligomers of flavan-3-ols that are linked together by carbon-carbon bonds (B type with 4-6 and 4-8 linkages) and in some cases by additional ether bond (A type; 13, 18, 68, 75). The flavan-3-ols are dominated mainly by the catechins and gallocatechins, which may also exist as gallate esters of the C_3-hydroxy group. They have been isolated from nutritional plants with the more important sources including tea (*Camellia sinensis*; 31, 32, 39), grape seeds and skin (*Vitis vinifera*; 20, 44, 74, 76, 79), and cocoa (*Theobroma cacao*; 2, 54). Although they have been shown to exhibit various biological activities (7, 14), the main focus has been on their antioxidant capacity, which may be the basis of many of these properties. This capacity has been demonstrated repeatedly (6, 41, 50, 69, 87), and their high radical-scavenging potential that correlated linearly with the number of active hydroxyl groups (*i.e.,* catechol or pyrogallol moieties) has recently been verified by pulse radiolysis (4), confirming an earlier report using the TEAC assay (57).

Hydrolyzable tannins are principally glucose esters of gallic acid, its dimer hexahydroxydiphenic acid (HHDP), or the corresponding partly oxidized form dehydrohexahydroxydiphenic acid (DHHDP), which are often present in various combinations (33, 53). There are two types of hydrolyzable tannins, the gallotannins, which yield only gallic acid upon hydrolysis, and the ellagitannins, which produce ellagic acid as the common degradation product (9, 28, 62). They are not as widespread as the condensed tannins, but as they often oc-

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cur in wood or bark tissue including those from oak (43, 45, 60), they are of great importance for the production of high-quality barrel-aged red wines (58, 61, 73, 86). They have been shown to protect red blood cells against lipid peroxidation (78), in line with their effective bleaching of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (88)—an assay commonly used to evaluate antioxidant activity.

Oxidative condensation reactions have been investigated extensively with flavan-3-ols, a facile process that involves a phenolic coupling reaction (nucleophilic substitution, S_N2) between a quinoid structure and the parent phenol (15, 21, 27, 71, 72, 77). The related flavon(ol)s, in contrast, form unstable quinones, which are more likely to be cytotoxic via "futile redox cycling" (46, 48) or by nucleophilic attack of unstable quinones or quinone methides at macromolecules such as proteins or DNA. For quercetin, the hypothetical quinones were recently trapped—also by a nucleophilic substitution reaction—with glutathione (3).

The building blocks of ellagitannin oligomers, *e.g.,* HHDP, DHHDP, chebuloyl, gallagyl, sanguisorboyl, valoneoyl moieties, *etc.* (33, 53), are also considered the products of oxidative phenolic coupling reactions during their formation (33, 62). Although enzymes responsible for the esterification of glucose with gallate moieties to build β-D-pentagalloyl glucose (PGG) as the principal starting material have been isolated (24, 25, 51), thus far no enzymes are known that catalyze the stereospecific coupling of the individual galloyl moieties. What is known, however, is the preferential formation of 2-3 and 4-6 (*S*)-HHDP groups as opposed to the 2-4 and 3-6 (*R*)-HHDP orientation, which eventually also leads to DHHDP structures (33). Although Feldman and colleagues have recently been successful in synthesizing HHDP (17) and DHHDP (63) structures by coupling partially protected galloyl groups under strongly oxidizing conditions, the total synthesis of two ellagitannins, praecoxin B and pterocarinin C, was achieved by combining preformed HHDP with partially protected glucogallin (38).

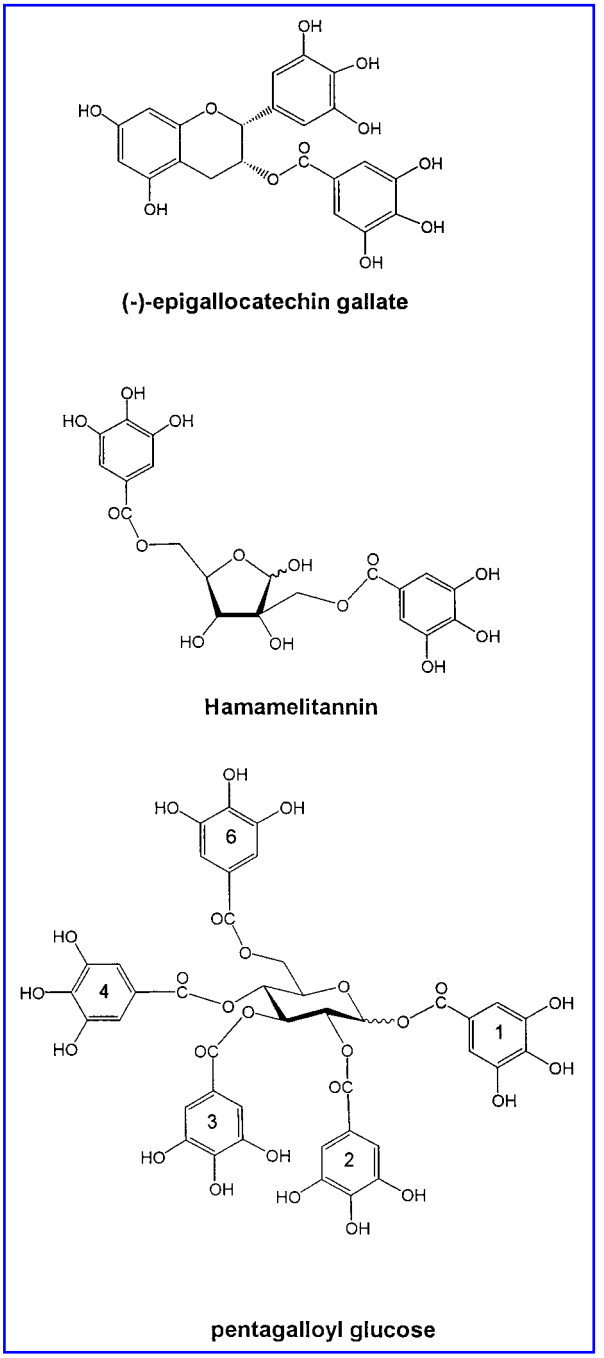
The distinction between the flavonoids and the flavan-3-ols residing in the ease of oxidative coupling of the latter compounds not only has bearings on the general chemistry of these compounds, but may also offer an explanation for their superior antioxidant capacity (4), as the active hydroxy groups are regenerated during the coupling reaction. Our interest therefore was to elucidate further the potential of phenolic coupling reactions: whether they are caused by initial radical reactions due to the antioxidant/radical-scavenging process; and whether a distinction can be found between intermolecular phenolic coupling of flavan-3-ols leading to biphenyl-linked oligomers of the B type, intramolecular coupling of proanthocyanidins leading to formation of an ether linkage [thus transforming a B- to an A-type proanthocyanidin for which process a radical reaction has recently been proposed (42)], and intramolecular coupling between galloyl moieties in the hydrolyzable tannins, potentially resulting in the formation of HHDP, a process that transforms a gallotannin into an ellagitannin.

To this end, we carried out pulse radiolysis experiments for the determination of rate constants with hydroxyl radicals to extend our previous correlations (4), and electron paramagnetic resonance (EPR) spectroscopy to determine the structure of the intermediate radicals formed by *in situ* oxidation (5). Nuclear magnetic resonance (NMR) spectroscopy (12, 84) was used to verify the carbon-carbon coupling during the nucleophilic substitution reaction. Earlier NMR studies along these lines have already been performed (27, 70), as this technique is also the standard tool to investigate the structures of the numerous plant constituents isolated over the years (11, 34, 84). The group of Vdovin, in particular, has to be mentioned in view of their comprehensive reviews on NMR studies of proanthocyanidins (81–83).

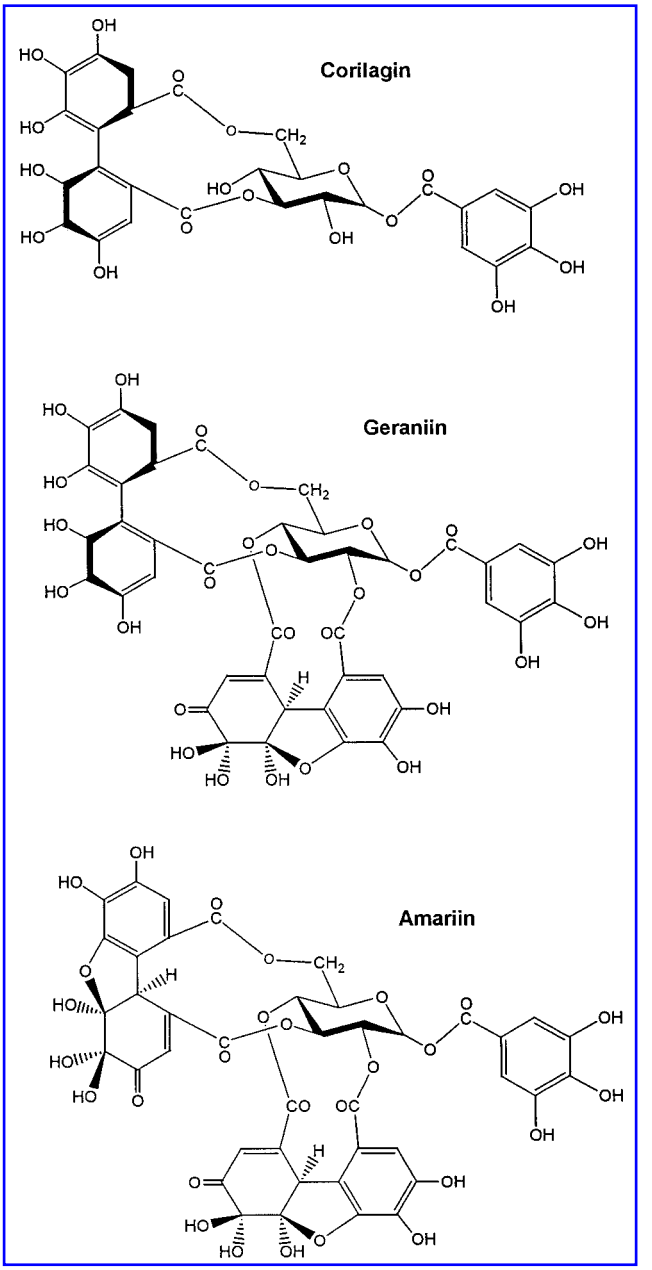
MATERIALS AND METHODS

Substances investigated consisted of the flavan-3-ol (−)-epigallocatechin gallate (EGCG) from AppliChem (Darmstadt, Germany), the hydrolyzable tannins hamamelitannin and PGG from Phytochem (Neu-Ulm, Germany), and the ellagitannins corilagin, geraniin, and amariin

(59); for structures see Schemes 1 and 2. EGCG and ellagic acid were also obtained from Calbiochem (Bad Soden, Germany). All other substances [N_2O, hydrogen peroxide (H_2O_2), horseradish peroxidase (HRP), buffer, NMR solvents—99.95% ^2H] were obtained at the highest available purity from local sources.



SCHEME 1. Structures of proanthocyanidins and simple gallate esters.



SCHEME 2. Structures of ellagitannins.

Pulse radiolysis was performed as described previously (4) to obtain both the rate constants with hydroxyl radicals, the UV-visible transient spectra, and, if possible, the decay rates of the primary radical intermediates. Prior to these studies, the p*K* values of the substances were determined by spectroscopic titration under nitrogen. The experiments were carried out at pH 9.3–9.5.

EPR spectroscopy was used to study the structure of longer-lived radicals (5), in which

case the radicals were generated either by oxidation with HRP/H_2O_2 at pH 8.0–8.5 or by alkaline autoxidation above pH 10 (26). Solutions were prepared with Milli-Q water (Millipore, Eschborn, Germany) at concentrations of 20–40 μM for the pulse radiolysis experiments and at 0.5–1 mM for the EPR studies. pH was adjusted with NaOH, avoiding the use of buffer.

The propensity of the various rings of PGG to dimerize to HHDP moieties would depend on their proximity to one another in a three-dimensional structure. We used MM2 calculations (Chem3D, CambridgeSoft, Cambridge, MA, U.S.A.) to obtain the various distances of the galloyl rings to predict potential condensation sites for comparison with the NMR studies, starting from both the axial and equatorial conformations of PGG. In a related approach, the chemical shifts for ^{1}H and ^{13}C based on these structures were calculated with the software program ACD Predictor, version 4.0 (ScienceServe, Pegnitz, Germany).

Solution (NaOD/D_2O, 303 K) NMR spectroscopy was performed on a Bruker DMX 500 NMR spectrometer operating at 500.13 MHz (^{1}H) and 125.76 MHz (^{13}C) utilizing an inverse 5-mm probe equipped with actively shielded gradient coils (gradient system BGU II, amplifier BPU 10; gradient pulse: 1 ms; gradient recovery: 500 μs). Bruker standard software was used for one-dimensional ^{1}H [first increment of the NOESY-presat sequence (pulse width, d1: 1.5 s; acquisition time, aq: 3.7 s; mixing time: 150 ms; 90°: 9.85 μs; exponential line broadening, LB: 1 s)] for water suppression at the residual HDO resonance (reference for all NMR spectra: $\delta_{HDO/NaOD} = 4.63$ ppm). Total correlation spectroscopy (TOCSY) NMR spectra ($t_{mix} = 70$ ms, d1 = 1.5 s, aq = 456 ms) were recorded up to 512 increments and multiplied by a $\pi/6$ shifted sine bell in experimental (F2) and calculated (F1) ^{1}H frequencies.

RESULTS

Pulse radiolysis

The rate constant of hamamelitannin (no. 2) with $^{\cdot}$OH radicals ($1.9 \times 10^{10} M^{-1} s^{-1}$) fits quite well into the linear correlation of the rate constants versus the number of reactive hydroxy groups for the other gallotannins (nos. 1, 3, and 7 in Fig. 1; ref. 4). Those hydroxy groups were defined as the catechol and/or pyrogallol moieties, and the deviation from that linearity by tannic acid was assumed to be due to steric hindrance of the gallate ester chains (4). As in the overall correlation the rate constants for the three ellagitannins, corilagin, amariin, and geraniin (1.21×10^{10}, 1.34×10^{10}, and $1.86 \times 10^{10} M^{-1} s^{-1}$, respectively), are below expectations counting all relevant hydroxy groups—yet above diffusion-controlled limits—we have to assume that, in view of the similarity of the UV-visible transient spectra, the gallate ester is the major site of attack and the pyrogallol/catechol moieties of the HHDP or DHHDP groups are less important. Figure 1 also contains the data of the same substances obtained with the DPPH assay (88), in which case the linearity is far better. As stated before, the transient spectra show little differences from the previously observed gallotannins, *i.e.*, absorption peaks at 275 nm (semiquinone) and 400–420 nm (ester bond) and a strong bleaching of the ester bond at 330 nm (4). With regard to the decay rates of these radicals, all three ellagitannin aroxyl rad-

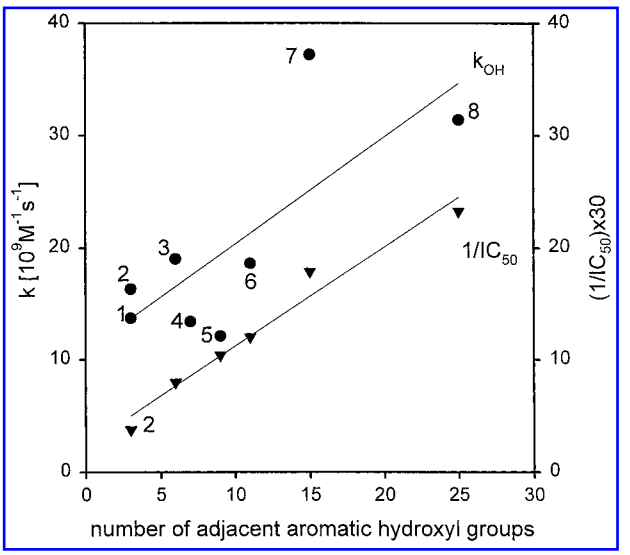


FIG. 1. Correlation of reactivities versus number of reactive hydroxy groups. Left ordinate: Absolute rate constants with $^{\cdot}$OH radicals at pH 9.3–9.5. **Right ordinate:** Inverse IC_{50} values (×30) from the reaction with the DPPH radical (88). **1,** propyl gallate; **2,** glucogallin; **3,** hamamelitannin; **4,** amariin; **5,** corilagin; **6,** geraniin; **7,** PGG; **8,** tannic acid.

icals disappeared with second-order rate constants of 0.8–2.3 $\times$ 10^6 M^{-1} s^{-1}, thus within the range of the more complex gallotannins previously studied: PGG (4.5 $\times$ 10^5 M^{-1} s^{-1}) and tannic acid (1.1 $\times$ 10^6 M^{-1} s^{-1}) (4).

EPR spectroscopy

The earlier studies of the EPR spectra of the flavan-3-ols (almost exclusively performed after alkaline autoxidation) resulted in a rather confusing picture with quite diverse radical structures proposed, yet all agreeing on primary oxidation of the B-ring catechol or pyrogallol groups (26, 36, 90). Using zinc anions as a spin-stabilizing agent, we could distinguish between catechol and pyrogallol semiquinones (5). EPR spectroscopy likewise allowed a distinction between procyanidins A2 and B2 aroxyl radicals, concerning the structure and stability of the radicals (5).

With regard to the hydrolyzable tannins, in alkaline solution we obtained a closely similar spectrum for hamamelitannin as compared with propyl gallate, in both cases depicting the resonance interaction of the aliphatic hydrogens across the ester bond (Fig. 2). As PGG was our prime candidate to investigate intramolecular phenolic coupling, the relative stability of its radical (*i.e.*, EPR spectrum) was of interest. Figure 3 shows that after oxidation with HRP/H_2O_2 within a period of 16 min, the original semiquinone radical disappears and a smaller and different radical appears.

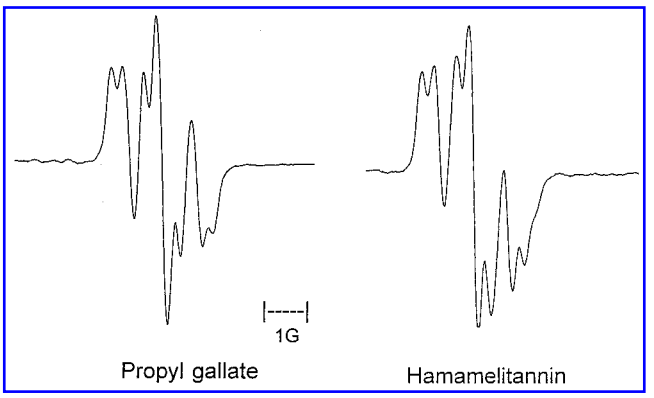


FIG. 2. EPR spectra of propyl gallate and hamamelitannin after alkaline autoxidation. Concentrations of both gallotannins: 0.5 m*M* in 0.05 *M* NaOH. EPR settings: X-band, modulation amplitude 0.6 G, sweep rate 0.5 G/s, gain 10^5, power 20 mW. Coupling constants for propyl gallate: a_{H2}/a_{H6} = 1.15 G, $a_{H\alpha}(2)$ = 0.40 G; for hamamelitannin: a_{H2}/a_{H6} = 1.15 G, $a_{H\alpha}(2)$ = 0.45 G.

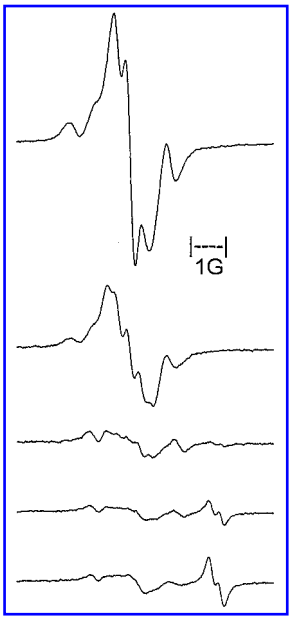


FIG. 3. Time-dependent change of the EPR spectrum of PGG. PGG (1 m*M*) was oxidized with HRP/H_2O_2 at pH 8.5. Traces were taken after mixing and 2, 6.5, 10.5, and 16 min later. EPR settings are as in Fig. 2.

The EPR spectra of the three ellagitannins, corilagin, geraniin, and amariin, show little differences if recorded after generation with HRP/H_2O_2 (Fig. 4, left panel), yet are sufficiently distinct but less stable in alkaline solutions (Fig. 4, right panel). The spectra in 0.05 *M* NaOH were taken at different times after initiation of the alkaline autoxidation. Thus far, unequivocal coupling constants could be determined only for amariin, which are very similar to those of the previously studied gallotannins with two equivalent a_H of 1.1 (5). For the two other ellagitannins, contribution of the HHDP moiety cannot be excluded. Unfortunately, model compounds, *i.e.*, glucose esters of HHDP alone, were unavailable, and we were therefore hampered in our identification of the radical structures.

NMR spectroscopy

EGCG. A solution of EGCG (1 m*M*) in D_2O provided a higher number of ^1H NMR resonances than expected from the molecular formula (one major set comprising ~80% and three other sets of resonances), which coalesced

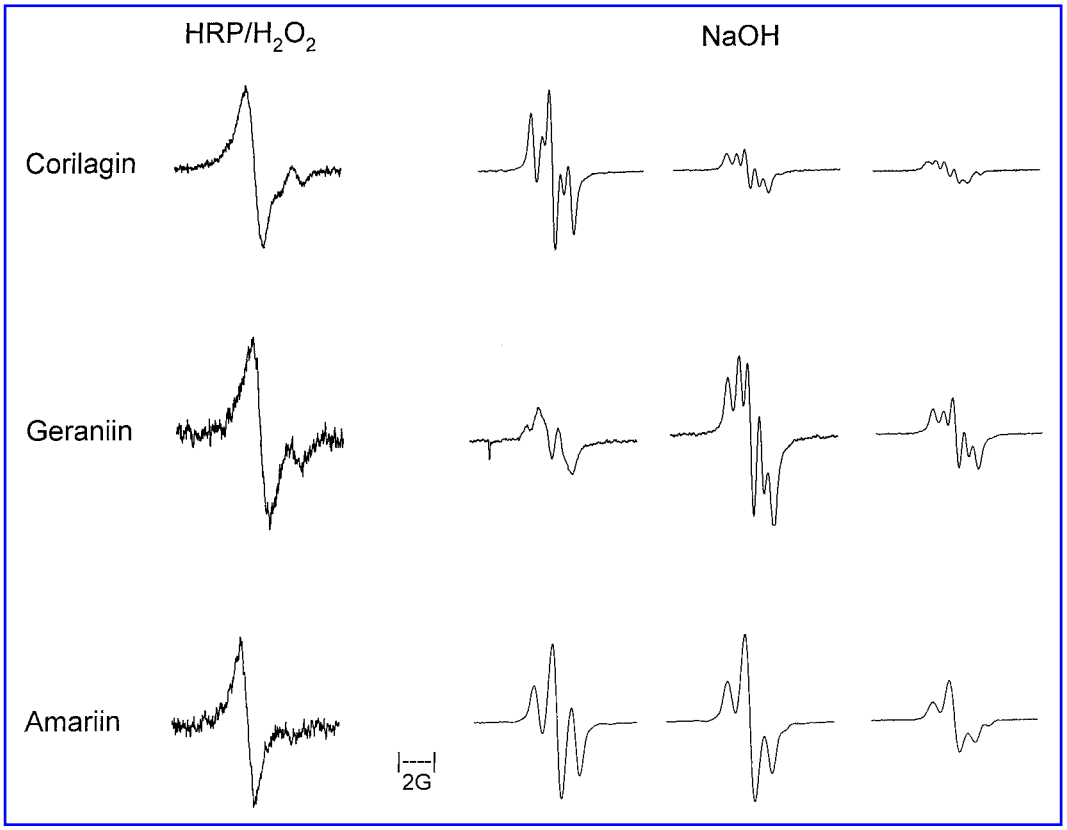


FIG. 4. EPR spectra of the ellagitannins. Left side: Oxidation with HRP/H_2O_2 at pH 8.5, concentration of ellagitannins at 1 mM. **Right side:** Alkaline autoxidation in 0.05 *M* NaOH, concentration of ellagitannins at 1 m*M*; signals were taken after mixing and 1 and 2.2 min later (note that signal intensity in arbitrary units is stronger in alkaline solution by about a factor of 2). EPR settings are as in Fig. 2.

in more concentrated and alkaline solutions into a uniform set of resonances. On dilution, the NMR signals sharpened, but did not split again. In alkaline solution, the H6 and H8 resonances disappeared within several hours due to H/D chemical exchange (the authenticity of EGCG was confirmed by ^{13}C NMR spectroscopy).

Addition of peroxidase alone to EGCG (1 m*M*) caused severe line broadening, many additional resonances appeared, and the original resonances of EGCG almost disappeared. These resonances are superimposed on a broad hump, possibly caused by the presence of transient radical species. Formation of intermediates is further indicated by continuous spectral changes over several minutes. In a higher concentrated solution of EGCG (6.2 m*M*), ~10% of a single coupling product is formed within 8 h at 303 K. When peroxidase is added, additional resonances appear instantaneously while 60% of EGCG remains, dropping to 40% after 2 days at 277 K.

Further addition of 4 m*M* H_2O_2 to the EGCG/HRP solution caused the appearance of new signals and broad humps centered at 5 and 8 ppm. Yet the original resonances of EGCG remain visible for several days, again accounting for 40% of the total ^1H NMR signal intensity. The additional resonances from two or three major and about five minor products show similar spectral properties, most of them retaining their geminal proton pair at C4. The chemical shift region ranging from 2.3 to 3.1 ppm accounts for 30% of the total aliphatic section (2.3–6.8 ppm).

The TOCSY spectrum reveals >15 correlations of the type H4-H3, but almost none of H3-H2. When the preferred conformation of the pyran ring is half chair, as found in the proanthocyanidin B-2 dimer (37), then the vicinal H3-H2 coupling constants are below 2 Hz and the corresponding TOCSY cross peaks will exhibit a low transfer amplitude. In addition, scaling with the low intensity of the one-dimensional

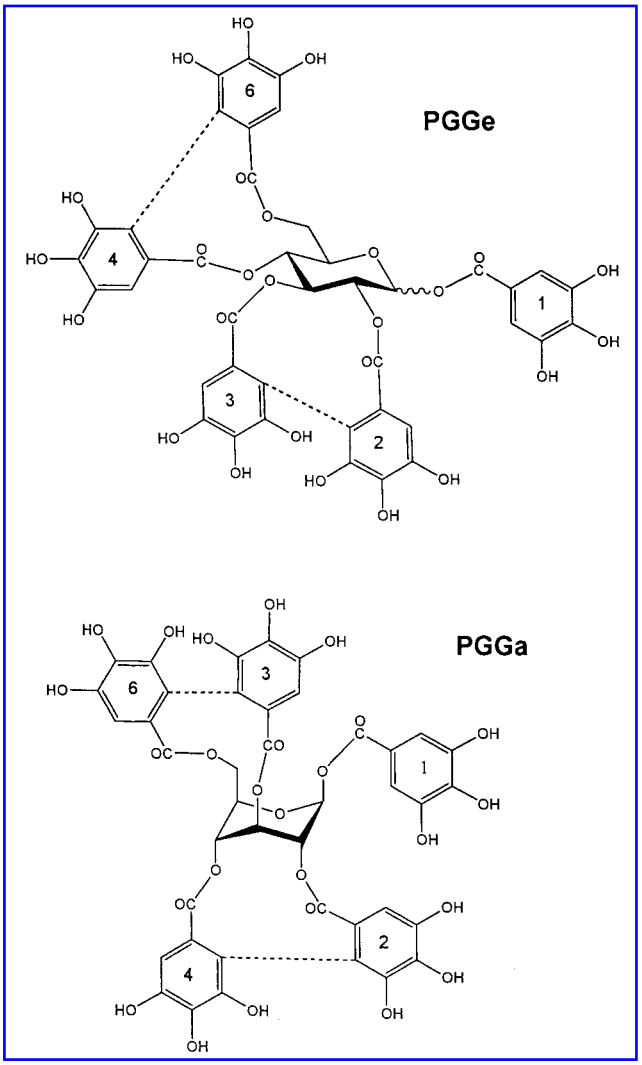
^{1}H NMR spectrum in the region of 4–6 ppm further diminishes cross-peak intensity of these units.

PGG. According to the calculations of the chemical shifts with the ACD Predictor software, it appears very difficult to discriminate between the various conformations depicted in Scheme 3 on the basis of the chemical shift of the galloyl resonances alone. The carbohydrate resonances are therefore more diagnostic.

Solutions of PGG show a concentration-dependent line broadening in their ^{1}H NMR spectra, which indicates either conformational changes of the carbohydrate ring (28) or chemical exchange depending on the dissociation of individual phenolic groups (or any combination of both effects). A pronounced sharpening of resonances occurs in alkaline solution accompanied by up to 0.15 ppm downfield chemical shift of the carbohydrate and galloyl ring resonances (this was not observed with EGCG after addition of NaOD). The electrostatic interaction of fully ionized, negatively charged phenolic anions may induce further variation in the average conformation of the glucose ring.

Increase of pH also causes an enhanced hydrolysis rate of PGG, resulting in free glucose and gallic acid, both clearly discernible in the NMR spectra of solutions left standing at room temperature for several days. The ^{1}H resonances in alkaline solutions remain sharp when peroxidase is added; they also stay visible and become dominant again after a complex ^{1}H NMR spectrum indicates the formation and decomposition of transient species. Solutions of PGG (1–5.5 m*M*) with HRP/H_2O_2 at pH ~10 retain 30–70% of the PGG signals with three other main compounds visible in the ^{1}H NMR spectrum. These new products, however, are unstable species and decay within 1 day. An initially very complex ^{1}H NMR spectral pattern in the region of 3–3.8 ppm eventually transforms into the NMR spectrum of glucose and a multiplet centered at 3.5 ppm. Further resonances are visible at 4.17 (m), 5.74 (dd), and 5.81 (t) ppm.

The ^{1}H NMR spectrum of a higher concentrated (5.5 m*M*) solution of PGG in less alkaline solution (pH ~8–8.5) is broadened to ~10 Hz linewidth, with the glucose resonances showing a chemical shift more closely resembling the PGGe than the PGGa conformation (see Scheme 3). Addition of HRP/H_2O_2 causes severe line broadening with no recognizable resonances. After 2 days a considerable number of sharp resonances indicates the presence of several coupling products. This spectrum, when multiplied with 15-Hz line broadening, closely resembles the NMR spectrum appearing shortly after addition of HRP/H_2O_2 and lasting for several hours. The TOCSY spectrum shows a great number of cross peaks over a considerable spectral range, indicating a high number (>10) of different coupling products initially formed.



SCHEME 3. Potential intramolecular phenolic coupling sites of PGG.

DISCUSSION

With regard to the radical-scavenging properties of the ellagitannins, the pulse-radiolytic experiments again verified their very efficient scavenging of ·OH radicals, with rate constants above the diffusion-controlled limit. In our previous study (4), we considered this as evidence for multiple sites of attack, *i.e.*, simultaneously at the various catechol and/or pyrogallol groups. Based on our earlier observation of a linear correlation of the number of reactive hydroxy groups of gallotannins with the rate constants with ·OH radicals (4), the addition of three ellagitannins decreased the coefficient of correlation, but made the exceptional reactivity of PGG even more pronounced. We were somewhat surprised about the relative low rate constants for the ellagitannins, and have to assume that the two hydroxy groups (the catechol structure) of the DHHDP moiety are less prone to attack by ·OH radicals than the gallate ester in C1′—as reflected in the similarity of the pulse-radiolytic transient spectra. A good linearity, however, is retained for all substances if the inverse IC_{50} value of the reaction with the DPPH radical (88) is plotted. As this hydrogen transfer from the phenolic antioxidant to a much more bulky DPPH radical is kinetically less favored than reaction with the far smaller ·OH radical, the relatively poor reactivity of the ellagitannins (and tannic acid) with the latter radical, in our view, could possibly reflect some steric hindrance—in that sense, ·OH is a surprisingly selective radical!

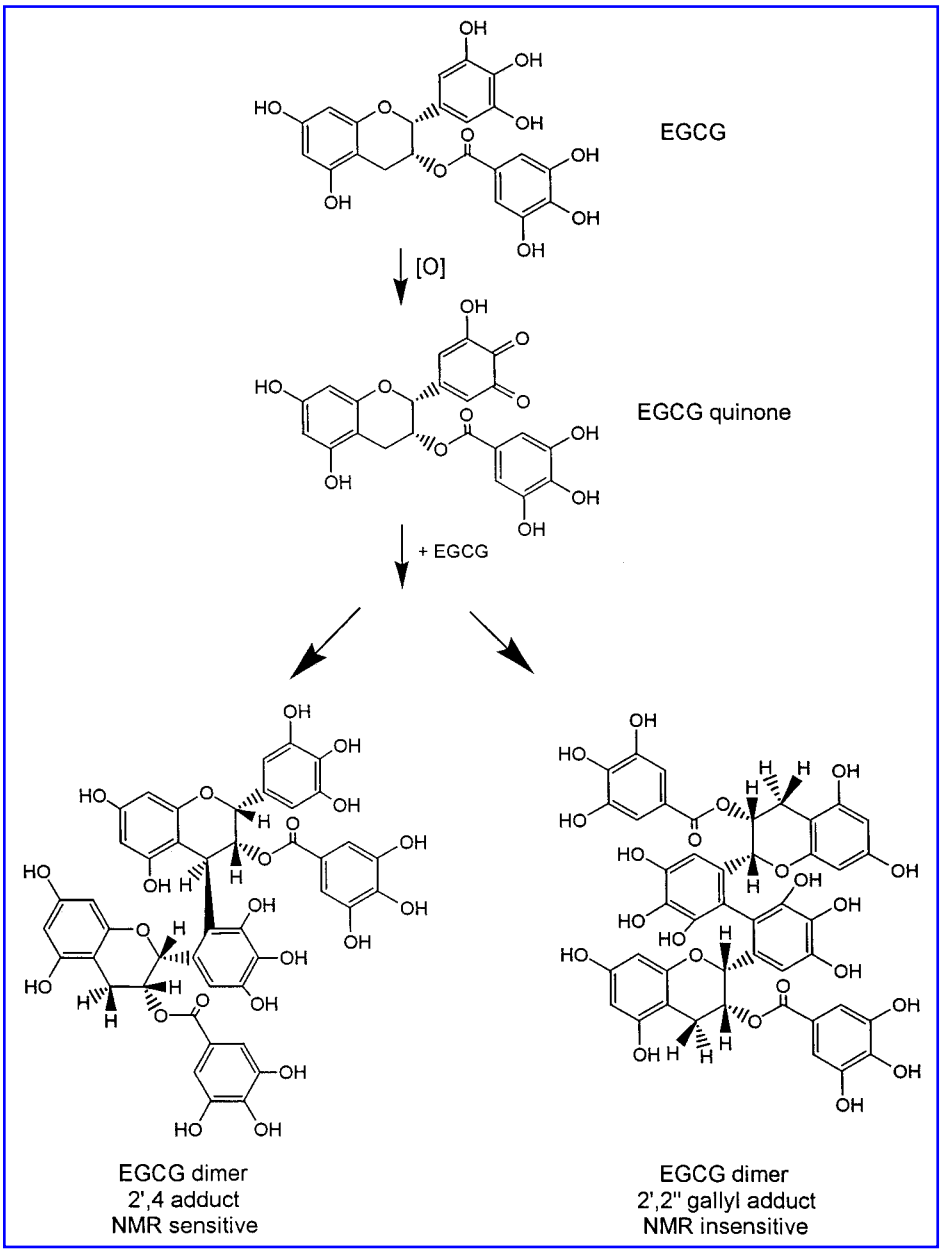
Looking at the types of radicals generated by either pulse-radiolytic oxidation with ·OH radicals or after oxidation with HRP/H_2O_2 in the EPR experiments, despite the quite different time resolution of at least three orders of magnitude, we assume that initially the same types of semiquinones are produced. As expected, the aroxyl radicals of the ellagitannins all decayed in second-order processes with bimolecular decay rates of $0.8–2.3 \times 10^6\ M^{-1}\ s^{-1}$. This is in the same range as the decay rates for the gallotannins (4) and is in line with the general decay mechanism of the semiquinones as a disproportionation process. The assumption that the predominant radical species observed in EPR are indeed semiquinone compounds, furthermore, is corroborated by the assignment of the hyperfine coupling constants. For the gallotannins such as propyl gallate, the coupling constants and assignment of the respective hydrogen atoms could be compared with literature values (1)—and hamamelitannin showed a closely analogous EPR spectrum and coupling constants (Fig. 2).

The EPR spectrum of amariin in alkaline solution with two equivalent a_H of 1.1 is so similar to those of the gallotannins that one can draw but one conclusion: only the galloyl moiety in C1′ and not the catechol group of the DHHDP moiety is being oxidized to the respective semiquinone. This is in line with the transient spectrum after pulse-radiolytic oxidation, which also reflects the galloyl moiety exclusively (4). For the other two ellagitannins with HHDP groups (corilagin and geraniin) (Fig. 4), the EPR spectra could not yet be resolved and may probably represent both structures. HHDP has only two isolated aromatic hydrogens and two hydrogens in the α-position of the ester bond, as the two rings are twisted with respect to each other and would not show any resonance overlap, even if a biradical is the hypothetical product. Identification of these radical structures is presently carried out by density-functional calculations of model compounds, as the regular molecules are too large for the density-functional calculations.

As mentioned in the beginning, a major goal of our investigations was to elucidate whether the phenolic coupling reactions (i) can be initiated by radical reactions and (ii) occur intramolecularly in the gallate esters, *e.g.*, with PGG, as well—the latter reaction possibly pointing to a synthetic pathway for the formation of the HHDP moiety from two adjacent galloyl groups (33). With regard to phenolic coupling of flavan-3-ols, our earlier studies yielded indirect evidence for phenolic coupling of EGCG: the pulse-radiolytic results established a bimolecular decay, which as a disproportionation process would result in both the phenolic parent compound and the quinoid oxidation product in equal parts (4), the latter as substrate for the S_N2 reaction. At the time resolution of the EPR experiments, we observed a change of the spectrum, indicative of an oligomerization

process (5). The potential reaction products of 2′,2″-coupling (of the respective B rings) of two EGCG molecules (Scheme 4, right bifurcation) have in fact been described as constituents of partially fermented Oolong tea and are called theasinensins (31, 52, 89). However, the ADC calculations clearly showed that this type of coupling does not result in significant changes of ^{1}H chemical shifts. Only coupling reactions involving the geminal hydrogens at C4 (*cf* the B-series oligomers with 4-6 and 4-8 covalent bonds) give very pronounced changes of the ^{1}H NMR spectra. Extrapolating from our pulse radiolysis and EPR experiments, the logical coupling product would then be a 4-2′ adduct, as depicted on the left pathway of Scheme 4. An interesting aspect is the lability of the C6 and C8 hydrogens toward H/D exchange in view of the dominance of these B-type structures in proanthocyanidin oligomers (18, 75).

Any coupling of the aromatic positions C6, C8, and C2′ to the position C4 will induce a considerable downfield chemical shift in the proton (from 3.0 to 4.8 ppm) and carbon (from



SCHEME 4. Intermolecular phenolic coupling of EGCG.

26.4 to 36.5 ppm) frequency (37). TOCSY cross peaks showing H4-H3 correlations in the region 4–5 ppm are very scarce, but this may be caused by the typically very small vicinal H4-H3 coupling constants. As meaningful heteronuclear correlation spectra were not accessible, no final conclusion can be drawn about the amount of the various coupling products, which nevertheless exist.

Studies to elucidate the oligomerization process of proanthocyanidins have been manifold (18, 75), and likewise phenolic coupling of hydrolyzable tannins has been a topic of research for >40 years (33). Although enzymatic processes governing these latter reactions are still unknown, a preference for structures derived from (*S*)-HHDP groups is readily apparent (33). In line with these observations is a recent synthetic study, in which the stereoselective biaryl coupling of carbohydrates with two 2-iodo-3,4,5-trimethoxy gallates was investigated—which turned out to be highly selective for 2,3- and 4,6-glucopyranosides in this (*S*)-configuration (10). According to Scheme 3, the parent compound PGG consequently existed in the equatorial $^4C_1$ conformation. A more detailed conformational analysis on the relative stabilities of the respective (*S*)- and (*R*)-diphenoyl structures of all four potential dimers was carried out very recently (35). These calculations of methyl glucoside model compounds explained both the (*S*)-stereoselectivity and the configurations of axially orientated HHDP moieties of natural ellagitannins.

With regard to the NMR experiments with PGG, the results from the calculations of the chemical shifts for the various structures derived from the equatorially orientated gallate esters in PGGe and the axially orientated PGGa (see Scheme 3), pointing out the difficulty of discriminating between these two conformations from the chemical shift of the galloyl resonances alone, present a fundamental problem. Nevertheless, any intramolecular coupling reactions of galloyl units will cause the singlet resonances of the starting materials (five and two aromatic resonances of PGG and EGCG, respectively) to decrease in intensity, whereas new pairs of singlet resonances with identical intensity corresponding to new coupling products should grow according to their rate of formation. An accurate integration of these resonances is impeded by their low spread in chemical shift resulting in significant overlap of old and new signals. More importantly, subtle changes in chemical shift induced by pH and concentration effects throughout the reaction preclude an unambiguous assignment by curve-fitting procedures of these strongly overlapping and moving signals. Despite these drawbacks, individual changes of resonance frequencies are clearly visible throughout the continuous coupling reaction of PGG at the concentrations used. The structure of the coupling products depends on the concentration of the PGG solutions and the pH value. At high pH, the original resonances of PGG have all disappeared, and besides gallic acid and glucose only minor amounts of other coupling products remain. The initial line broadening of the NMR resonances is caused by the formation of EPR-visible transient radical species. The conformity of radical and mathematically broadened NMR spectra indicates only minor changes in the structure and ratio of the quickly formed coupling products.

Conclusions

Polyphenolic compounds such as the condensed and hydrolyzable tannins have shown in various antioxidant assays their high potential, the most comprehensive study being the reaction with the DPPH radical (88). Although the majority of the studies were confined to proanthocyanidins (condensed tannins; 50, 57, 69, 87), our intention to include hydrolyzable tannins in the pulse-radiolytic and EPR studies of the respective aroxyl radicals was based on the following argument. The so-called "French paradox" is now a well known phenomenon (16, 23, 40, 66), pertaining to the relatively low incidence of cardiac problems in the south of France as compared with other industrial countries, despite the fact that the French diet is rather similar. The effect seems to have a specific French connotation, and is distinct from the related phenomenon of the "Mediterranean diet" (29, 64, 80, 85). The presently favored explanation is the content of proanthocyanidins

in red wine (30, 47, 65, 67). On the other hand, the French were the most consistent to produce high-quality red wines in barrels (73), whereas Italy and Spain as the other major wine-producing countries of that period were more apt to produce high-volume/low-quality table wines in steel tanks. It is therefore quite conceivable that, depending on the type of oak used for cooperage (8, 61), the tannins from the oak leaching into the wine (60) might have an effect not only on the flavor (58, 61), but also on the antioxidant capacity of red wines. At the present, the effect of tannins from oak barrels on wines is discussed primarily with respect to retardation of the oxidation processes occurring during fermentation and aging (56, 86).

Other aspects to be considered are the distinctive pathways of phenolic coupling reactions observed for enzymatic or chemical oxidation reactions of proanthocyanidins (27, 72, 75) versus those with hydrolyzable tannins. Although for proanthocyanidin formation various synthetic coupling sites have been observed, the enzymatic reactions leading to the predominant A and B series of oligomers are still unknown (18, 75). Regarding the formation of the HHDP and DHHDP structures in ellagitannins, the observed highly specific stereoisomerism obviously calls for some enzymatic control, which is also lacking (33). As quinones are obligatory intermediates during these reactions (33, 62), initiation by semiquinone formation by the antioxidative scavenging of radicals and subsequent disproportionation of the semiquinones is likewise a reasonable assumption. In fact, our results with NMR spectroscopy, although ambiguous with regard to the actual structures of the dimeric compounds, highlight the overall sensitivities of these polyphenols toward such oxidative coupling reactions.

An unresolved question is also the function of the oligomeric polyphenols in the plants themselves such as are they merely oxidation products formed, *e.g.*, during the fermentation of tea leaves (31, 32) and maceration/fermentation of wine (19, 22, 49, 55, 65), because any enzymatic system for their biosynthesis is yet to be discovered.

ABBREVIATIONS

DHHDP, dehydrohexahydroxydiphenic acid; DPPH, 1,1,-diphenyl-2-picrylhydrazyl radical; EGCG, (−)-epigallocatechin gallate; EPR, electron paramagnetic resonance; HHDP, hexahydroxydiphenic acid; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; NMR, nuclear magnetic resonance; PGG, β-D-pentagalloyl glucose; TOCSY, total correlation spectroscopy.

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