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RADICAL INTERMEDIATES AND ANTIOXIDANTS: AN ESR STUDY OF RADICALS FORMED ON CARNOSIC ACID IN THE PRESENCE OF OXIDIZED LIPIDS

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Carnosic acid, an antioxidant extracted from rosemary, is shown to produce radicals when in contact with oxidized methyl oleate in the absence of air above 50°C. Two radical species are formed: the first one, stable up to ~110°C, is an hydroxy-phenoxy radical whose ESR spectrum was analyzed by studying its temperature dependence and its sensitivity to deuterium/proton exchange. The second species was observed above 110°C, its ESR spectrum was identical to the spectrum obtained when carnosol, another antioxidant extracted from rosemary, was heated at the same temperature in the presence of oxidized lipid. This observation is probably due to the transformation of carnosic acid into carnosol; the analysis of the corresponding ESR spectrum suggests the formation of a keto phenoxy radical exhibiting a great delocalization of the unpaired electron.

KEY WORDS: Antioxidant, free radicals, ESR, rosemary.

INTRODUCTION

The crucial role of antioxidants in various chemical and biochemical processes, such as lipid peroxidation and food preservation,[1,2] is now well recognized and considerable efforts have been made to understand the corresponding molecular mechanisms[3] and to discover natural compounds able to prevent oxidative degradation.[4] In this context, many experimental studies have been devoted, during the past ten years, to antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*.[5-11] Carnosic acid (1) is one of these constituents and, although the antioxidative properties of this molecule have been attributed to its ability to form radicals, as far as we know no radical intermediate due to the action of oxidized lipids on (1) has been reported. Furthermore, the ESR spectra previously obtained with an oxidized benzene extract of rosemary were poorly resolved and were due to at least 3-4 unstable free radical species which could not be identified.[12]

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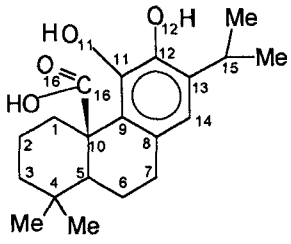


(1)

The aim of the present ESR study is twofold: 1) to explore the factors that, in a lipid environment, influence the formation of radicals centered on carnosic acid. 2) to get information about the nature, the stability and the structure of these radicals. In order to realize these objectives, the temperature dependence of the spectra as well as the role of the oxygen content during the preparation of the samples have been investigated.

EXPERIMENTAL

Chemicals. Carnosic acid[13] and carnosol[14] were obtained by purification of rosemary extracts according to previously described procedures. Carnosic acid, a slightly yellow crystalline powder (mp: 194–199°C), was found by ^{1}H-NMR spectroscopy and reversed phase HPLC to be 97% pure. Carnosol, colorless crystals (mp: 230–231°C), was found, by ^{1}H- and ^{13}C-NMR spectroscopy and TLC, to be 99% pure. Methyl oleate was purchased from Fluka.

Preparation of the samples. The samples were prepared in a specially designed glass apparatus composed of two compartments which allowed methyl oleate and the antioxidant to be degassed separately, and subsequently to be mixed under high vacuum. The device could be closed with high-vacuum stopcocks and the mixture could be directly studied by ESR without any further transfer. Degassing of the lipid sample was performed by successive freezing/vacuum (10^{-5} Torr)/melting cycles whereas solid compounds were degassed under high vacuum at room temperature. The hydrogen/deuterium exchange reactions were carried out in the apparatus described above by adding carefully degassed MeOD to the degassed solution of antioxidant in methyl oleate; after 30 min, methanol was eliminated under vacuum and the resulting sample carefully degassed.

ESR measurements and analysis. The ESR spectra were obtained on a VARIAN-E9 spectrometer equipped with a cylindrical JEOL cavity. The temperature dependence of the spectra was studied by using the JEOL variable temperature attachment. A NMR marker and a Hewlett-Packard microwave frequency meter were used for the calibration of the spectra. The ESR spectra, calculated with second order perturbation, were simulated on a AST microcomputer. Molecular mechanics calculations (MM^+ force fields)[15] were performed with a Silicon Graphics (Indigo) work station.

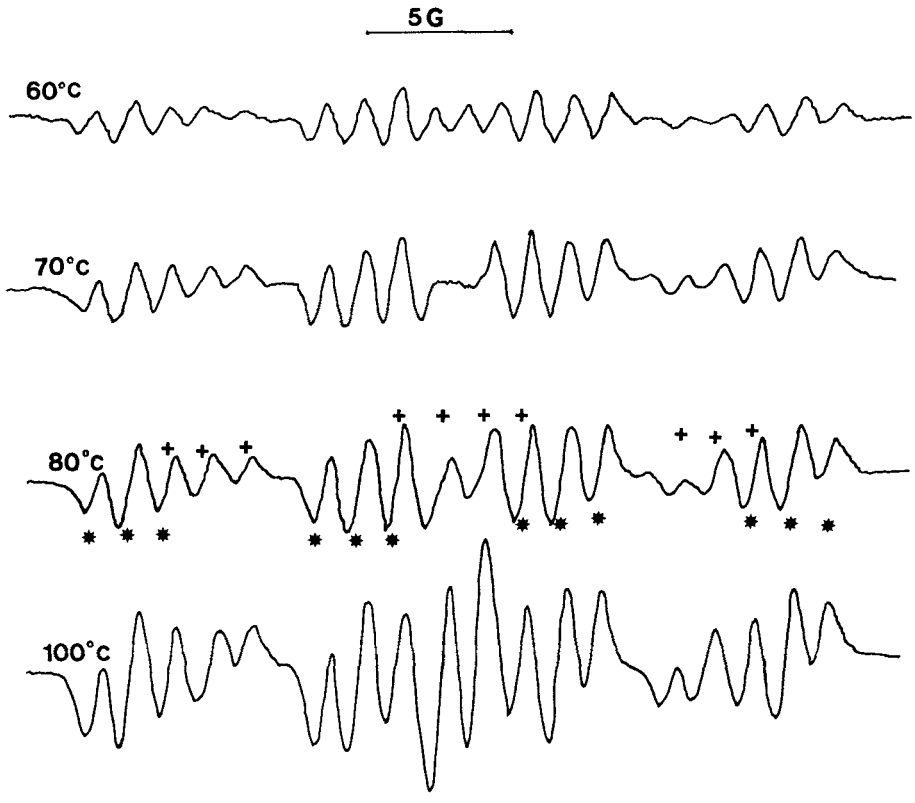


FIGURE 1 ESR spectra obtained at various temperatures with a solution of carnosic acid in oxidized methyl oleate. The signals marked * and +, on the spectrum recorded at 80°C, are attributed to conformations A′ and A″ respectively (see Figure 4).

RESULTS AND DISCUSSION

Radical Reactions Observed with Carnosic Acid

A solution of carnosic acid in an organic solvent (2-Methoxyethyl ether) yields no ESR spectrum whatever the temperature and the nature of the atmosphere above the solution (vacuum, argon or air). In the same way, no ESR signal was observed at room temperature with a freshly prepared solution of carnosic acid in methyl oleate previously degassed by argon bubbling; heating this sample slowly led, however, to the observation of some broad lines above 60°C. Better resolved ESR spectra were obtained by oxidizing methyl oleate (0.3 mL) in air at room temperature and mixing it with carnosic acid (5 mg) under high vacuum using the special device described above. Thus, a well resolved spectrum (referred to as A, Figure 1) was obtained when carefully degassed methyl oleate, previously stirred under air during two hours at room temperature, was mixed under high vacuum with degassed carnosic acid. Heating the resulting solution in the ESR cavity between 50°C and 90°C led to the observation of several ESR lines whose number

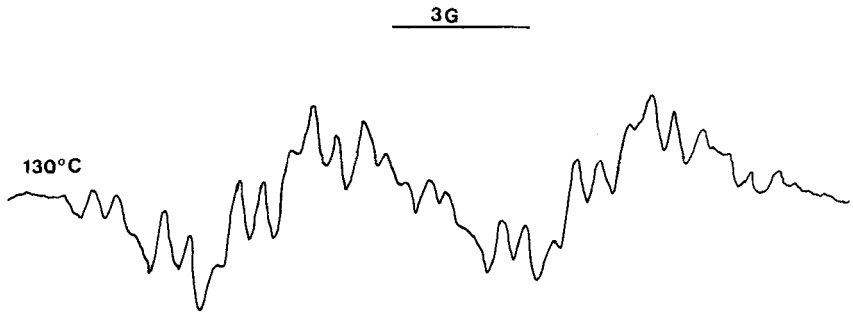
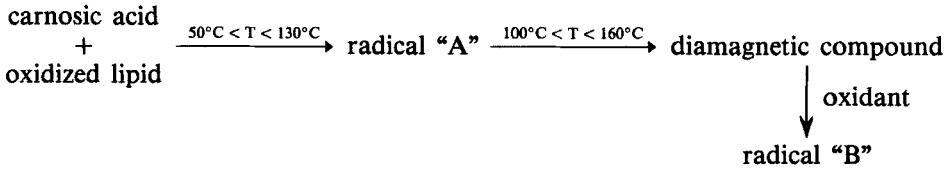


FIGURE 2 ESR spectrum obtained at 130°C with a solution of carnosic acid in oxidized methyl oleate.

and relative intensities were reversibly dependent on temperature. Around 100°C, the spectrum disappeared and above *c.a* 110°C a new spectrum (referred to as B, Figure 2) was observed, the corresponding signals could be detected until 160°C. The oxidation state of methyl oleate was found to play an important role in the radical reactions of carnosic acid. Thus, if methyl oleate was oxidized for a shorter period of time (e.g. 15 min), only spectrum A could be recorded, but reopening the sample tube and reoxidizing the solution with air led, after degassing, to successive observations of spectra A and B. In the same way, if the oxidized lipid/carnosic acid ratio was low and the sample very slowly heated, only spectrum A could be observed, and this up to 130°C. Finally, in order to confirm the role of oxidized lipid in the radical process, we tried to generate radicals by using a more "inert" lipid (paraffin) instead of methyl oleate: under these conditions no ESR signal could be detected.

These results suggest that the detection of radical B requires to have enough oxidized methyl oleate first to transform carnosic acid into radical A and then to further oxidize radical A. All these observations are consistent with the mechanism shown in scheme 1 and the problem is now to identify the species A and B.



Radical Reactions Observed with Carnosol (2)

No ESR spectrum was obtained below 110°C with carefully degassed solutions of carnosol in oxidized methyl oleate. Above this temperature, however, two species leading to the intense well-resolved spectrum shown in Figure 3 were formed. This spectrum disappeared only above 160°C, but above 140°C additional narrow lines were observed; due to the complexity of the spectrum these extra lines could not be analyzed.

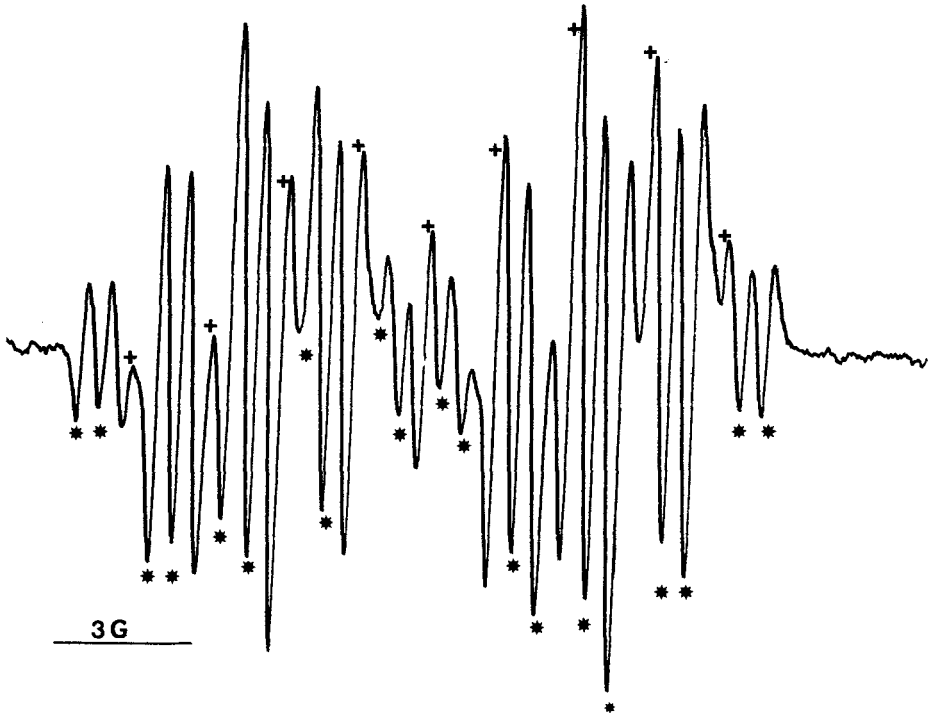
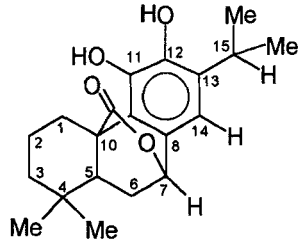


FIGURE 3 ESR spectrum obtained at 130°C with a solution of carnosol in oxidized methyl oleate. The signals marked * and + are due to species B_1 and B_2 respectively. (see Figure 6).



(2)

Spectral Analysis

Spectrum A. The ESR spectra of the A radical observed between 50°C and 130°C are shown in Figure 1. It is clear that these spectra, particularly their central pattern, are sensitive to temperature. A careful analysis of these signals (Figure 4) showed that each of these spectra is composed of two sub-spectra A′ and A″ and that four proton couplings participate in the hyperfine structure of each sub-spectrum: two of these protons – H_a and H_b – are equivalent and exhibit a coupling of 1.3 G (for

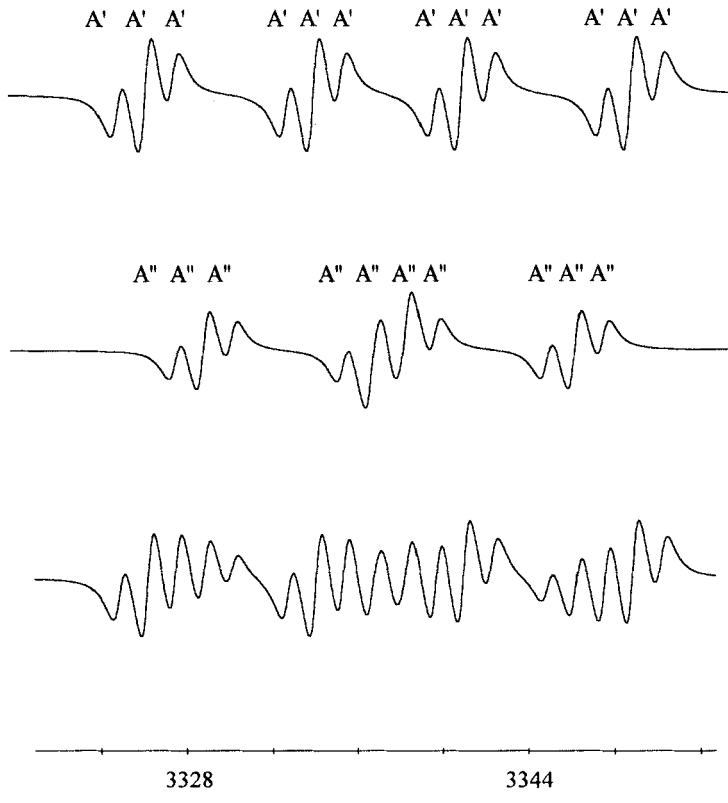


FIGURE 4 Simulation of the ESR spectrum obtained with carnosic acid at 80°C: the first and second spectra are due to the isolated conformations A′ and A″ respectively. The third one results from a combination of these two spectra.

A′ and for A″) and a third proton - H_c - shows a coupling of 7.9 G (for A′ and for A″). In contrast to these three coupling constants, the hyperfine interaction measured for the fourth proton - H_d - is different for A′ and A″ and varies with temperature: on passing from 50°C to 130°C, $a(H_d)$ decreases from 15.2 G to 14.4 G for A′ and from 10.1 G to 8.8 G for A″. The relative abundance of the two "species" A′ and A″ is also temperature dependent and the ratio of intensities I(A″)/I(A′) used for the simulation of the spectra increases from 0.55 to 1 when temperature increases from 60°C to 130°C. In order to confirm this analysis and to get an insight into the origin of the couplings the spectra were analyzed after deuteration with MEOD (see experimental). This deuteration caused a broadening of the lines (Figure 5) due to unresolved coupling with ^2D which prevented us from measuring the temperature dependence of the coupling constants with satisfactory precision. It appears that the coupling constants observed with the non-deuterated species have practically not been affected by deuteration, except for one of the two constants of ~1.3 G which is probably replaced by an unresolved ^2D splitting (expected coupling ~0.2 G).

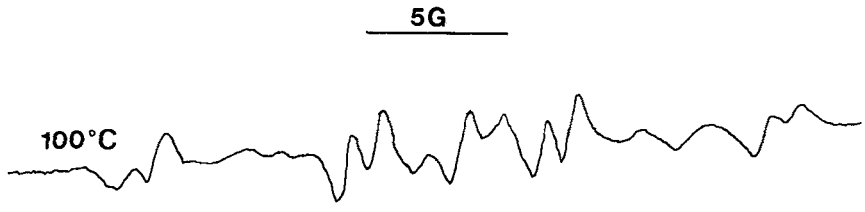


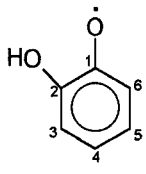
FIGURE 5 ESR spectrum obtained with a solution of carnosic acid in oxidized methyl oleate after reaction with MeOD.

Spectrum B. In contrast to A, spectrum B was temperature independent and was imperfectly resolved. Moreover deuteration with MeOD had no effect on this spectrum. The observed pattern was similar to that found with carnosol in oxidized methyl oleate; furthermore other characteristics of both spectra - spectrum B of carnosic acid and spectrum of carnosol - were identical: temperature range of formation of the radical, insensitivity to deuteration by exchange with MeOD. The main difference between the B spectrum and the carnosol spectrum lies in the considerably higher intensity and better resolution of the carnosol spectrum. The spectrum obtained with carnosol was simulated (Figure 6) by adding the contributions of two species B_1 and B_2 characterized by the following constants: $a(H_1) = 8$ G (one proton), $a(H_2) = 1.65$ G (four protons), $a(H_3) = 0.45$ G (one proton) for the B_1 species and $a(H_1') = 6.5$ G (one proton), $a(H_2') = 1.62$ G (four protons) for the B_2 species. At 130°C, the ratio of intensities $I(B_2)/I(B_1)$ was equal to 0.35. By slightly increasing the linewidth these coupling constants led to a spectrum which fits the spectrum B obtained with carnosic acid very satisfactorily.

INTERPRETATION

Species A

The oxidation of simple o-catechols into 2-hydroxy-phenoxy radicals has been the



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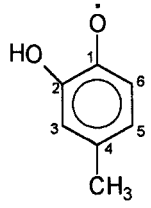


(4)

object of numerous studies.[16-18] At −103°C, the proton located in para position from the C—O· moiety in the unsubstituted o-semiquinone radical (3), has the maximum absolute coupling (~8 G), whereas that of the o-proton and of the hydroxyl proton were equal to 4 G and 1.7 G respectively.[16-17] The hyperfine constants found for the m-protons were small (1.8 G and 0.1 G for the protons 3 and 5 respectively). The corresponding spectrum showed a pronounced temperature

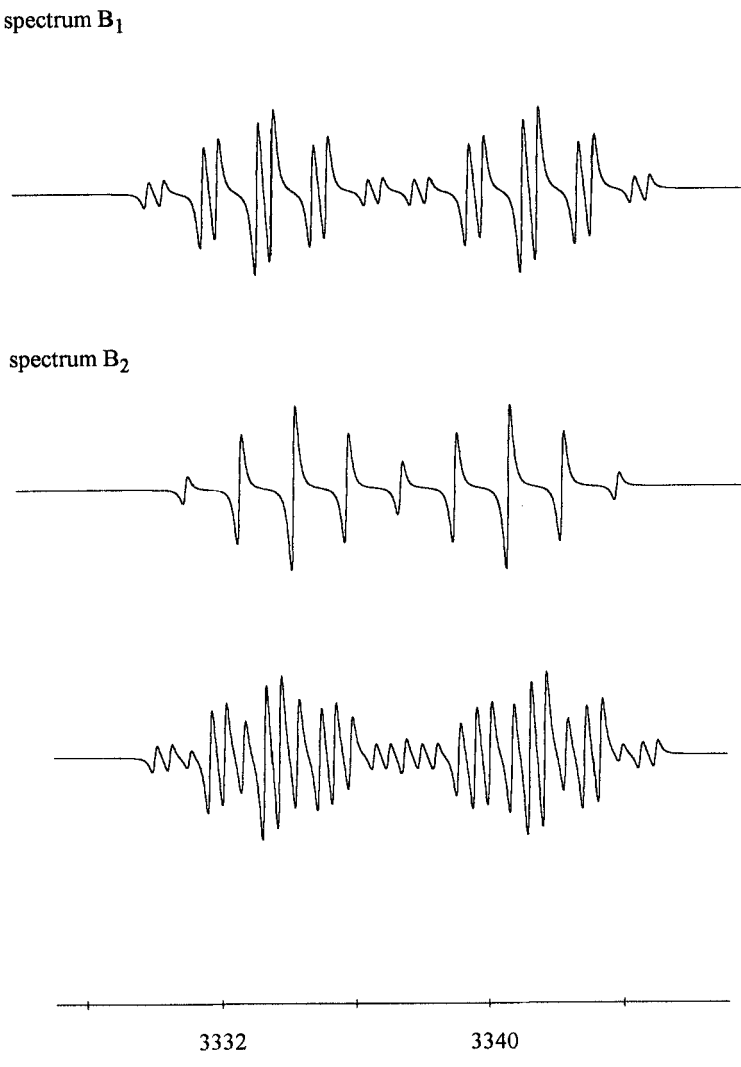
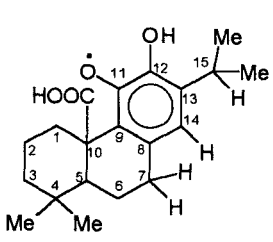
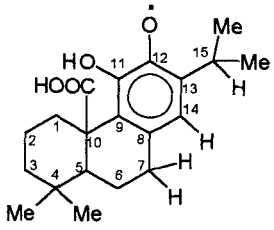


FIGURE 6 Simulation of the ESR spectrum obtained with carnosol. The two first spectra show the contribution of each radical species (species B_1 and B_2 respectively).

dependence due to a proton exchange between the two oxygen atoms (formation of an intramolecular O-H-O bridge). If we assume, for the ($1_a^{\cdot}$) and ($1_b^{\cdot}$) isomers, a spin delocalization similar to that reported for the 2-hydroxy-phenoxy radical (3), we expect, for ($1_a^{\cdot}$), a coupling of ~ 8 G for the proton in position 14 and of ~ 1.5 G for the hydroxyl proton. No additional large coupling can occur for this isomer since the other protons are located in β-position to carbon atoms $C_{(8)}$ and $C_{(13)}$ whose spin densities are very small.

For the isomer ($1_b^{\cdot}$), on the contrary, the unpaired electron is mainly localized on carbons $C_{(8)}$ and $C_{(13)}$, and hyperconjugation can lead to large coupling constants with protons in β-position to these two atoms. In contrast to the experimental



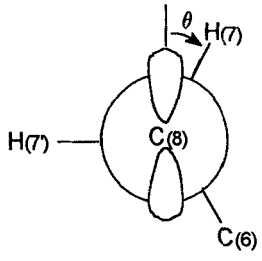


$(1^{\cdot}_a)$ $(1^{\cdot}_b)$

results for A′ and A″, the spectra of the isomers $(1^{\cdot}_a)$ and $(1^{\cdot}_b)$ are therefore expected to be very different from each other. Both sub-spectra A′ and A″ exhibit two coupling constants larger than 7 G and cannot, therefore, be attributed to the coexistence of these two isomers. Moreover, the large experimental coupling constants allow us to rule out $(1^{\cdot}_a)$ as a reasonable candidate, whereas $(1^{\cdot}_b)$ seems to be consistent with both A′ and A″. Indeed, the large coupling constant (15.2 G for A′, 10 G for A″) can be reasonably assigned to one of the two protons $H_{(7)}$ in β position to the carbon $C_{(8)}$, while the 7.9 G constant is consistent with the proton $H_{(15)}$ in β position to $C_{(13)}$. The constant of 1.3 G is associated with the proton linked to $C_{(14)}$ or, less probably, with the second cyclohexenyl proton, $H_{(7')}$, in β position to $C_{(8)}$. Finally, the other constant of 1.3 G can be unambiguously attributed to the hydroxyl proton since it disappeared in the presence of MeOD. This analysis is in good accord with the coupling constants[18], measured at −74°C, in the 2-hydroxy-4-methylphenoxy (4): $a(H_{(3)}) = 1.84$ G, $a(CH_3) = 9.68$ G, $a(H_{(5)}) = 0.38$ G, $a(H_{(6)}) = 3.89$ G, $a(OH) = 1.35$ G.

The two questions which have now to be answered are: why can only one isomer be observed?, what is the origin of the difference between A′ and A″? The fact that $(1^{\cdot}_a)$ is not formed is probably due to the presence of the carboxyl group near the hydroxyl proton in position 11. The bonding interaction between these two groups is expected to prevent the hydroxyl hydrogen from migrating and explains why there is no hydrogen bond with $O_{(12)}$ during the oxidation of carnosic acid. This interpretation is quite in accordance with the MM^{+15} optimized structure of $(1^{\cdot}_b)$, shown in Figure 7, which indicates that the hydroxyl bond $O_{(11)}H_{(11)}$ is indeed oriented towards the oxygen of the carbonyl group $C_{(16)}O_{(16)}$ (the $H_{(11)} \cdots O_{(16)}$ distance is equal to 2.1 Å whereas the $H_{(11)} \cdots O_{(12)}$ distance is equal to 3.5 Å).

The single difference between the sub-spectra A′ and A″ can be interpreted in terms of the $H_{(7)}$ coupling. Indeed this splitting is expected to follow the well known $(B_o + B_1 \cos^2\theta)\rho_\pi$ rule[19] and therefore to be very sensitive to small changes in the conformation of the $C_{(5)}$–$C_{(10)}$ cyclohexene ring. It is therefore quite possible



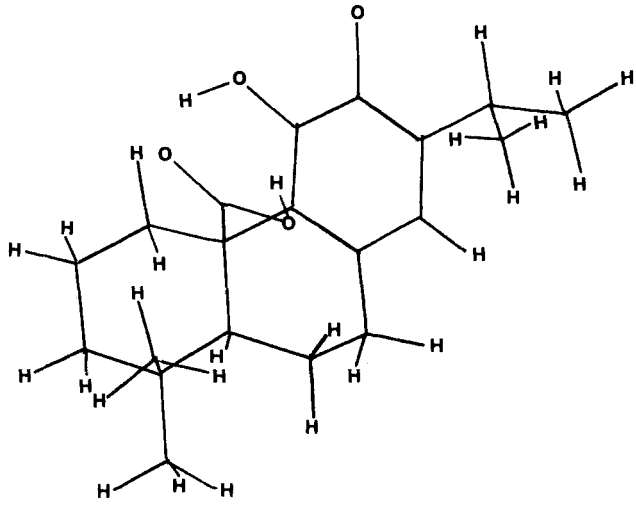


FIGURE 7 Optimized structure (MM+) of the $C_{(12)}$-phenoxy radical derived from carnosic acid.

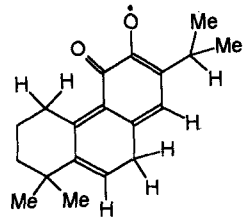
that A′ and A″ correspond to two slightly different conformations of the phenoxy radical ($1_b^{\cdot}$) (a rough estimation of θ for A′ and A″ leads to values of 23° and 40° respectively while the θ value predicted by molecular mechanics calculations[15] for the optimized structure is equal to 50°).

The temperature dependence of the spectrum between 50°C and 110°C can be explained, on the one hand, by a variation in the ratio of the populations of these two conformers, and, on the other hand, by the temperature sensitivity of the $H_{(7)}$ coupling.

Species B

The radicals, observed above 100°C from carnosic acid as well as from carnosol, exhibits couplings with five (species B_2) or six protons (species B_1). So many couplings indicate that the unpaired electron is probably delocalized over a great number of carbon atoms and suggest that the corresponding radical contains several conjugated double bonds. The oxidation reactions of carnosic acid which leave the carboxyl group untouched[20] are not expected to increase the number of conjugate double bonds. Furthermore, the fact that the spectrum is insensitive to deuteration by exchange with MeOD is indicative of the disappearance of one hydroxyl group. Radical (5) is shown as an example for a reasonable candidate for the B species.

The present interpretation is consistent with previous studies which showed that oxidation of carnosic acid results in the formation of carnosol.[6,10] It is therefore not surprising that the same ESR spectrum can be obtained from both carnosic acid and carnosol. The fact that we could not observe the spectrum B from (1) when the intermediates A′ and A″ had not been previously formed indicates that these species are likely to be involved in the transformation of (1) into (2). Moreover the detection of a highly delocalized radical during the oxidation of (2) is reminiscent of recent reports which showed that oxidation of some aromatic diterpenes can lead to molecules containing a great number of conjugated bonds.[20]



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Acknowledgements

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