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Original Scientific Paper

DFT Calculations of Isotropic Coupling Constants of Phenoxyl and Aroxyl Radicals

Wolf Bors,[a,*] *Christa Michel,*[a] *Kurt Stettmaier,*[a] *Snježana P. Kazazić,*[b] *and Leo Klasinc*[b]

[a]*Institut für Strahlenbiologie, GSF Research Center, D-85764 Neuherberg, Germany*

[b]*Ruđer Bošković Institut, P. O. Box 180, HR-10002 Zagreb, Croatia*

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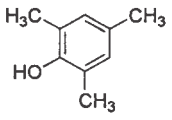
Hybrid density-functional calculations were carried out to corroborate the identity of phenoxyl radicals observed by EPR spectroscopy after oxidation of selected mono- and polyphenols with horseradish peroxidase/hydrogen peroxide or after alkaline autoxidation. Whereas quantitative correlations of experimental and theoretical coupling constants were less satisfactory, we could confirm formation of a biradical after initial oxidation of 2,4,6-trimethylphenol, the mesomeric structures of gallate ester aroxyl radicals and identify the radical site of a model gallotannin, hamamelitannin.

Key words: epr spectroscopy, coupling constants measurement, DFT calculation, polyphenols, antioxidant activity, phenoxyl and aroxyl radicals by phenol oxidation.

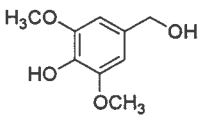
INTRODUCTION

Phenols, both mono- and polyphenols, comprise a large group of mainly plant-derived compounds which all to a certain degree exhibit antioxidant capacity. This entails both effective scavenging rates of oxidizing radicals and a reasonable stability of the resulting phenoxyl (from monophenols) or aroxyl radicals (from polyphenols). Antioxidant properties of phenolic compounds can be determined by various assay procedures, foremost among them pulse radiolysis to obtain absolute rate constants.[1–3] More common are assays yielding relative activities, such as the TEAC assay (trolox-equivalent anti-

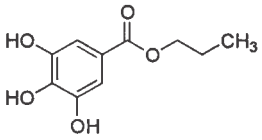
* Author to whom correspondence should be addressed. (E-mail: bors@gsf.de)



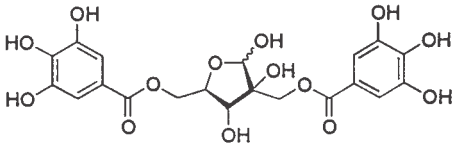
2,4,6-trimethylphenol



syringic alcohol



propyl gallate



hamamelitannin

Scheme 1. Structures of the phenols/polyphenols investigated.

oxidant capacity)[4,5] or the reaction with the stable radical 2,2-diphenyl-1-picryl hydrazyl (DPPH).[6,7]

With regard to identification of the structures of the antioxidant radicals, EPR spectroscopy is the method of choice. The assignment of the individual coupling constants may be rather straight-forward for most simple phenoxyl radicals, but problems arise for (a) hydrogen atoms in aliphatic side chains, (b) effects of adjacent substituents, and (c) structures of more complex polyphenols, in particular, if more than one radical site could occur within the molecule.

To overcome these problems, we compared experimentally obtained isotropic coupling constants for a variety of phenoxyl/aroxyl radicals with values resulting from hybrid density-functional calculations,[8] in continuation of earlier studies of syringic and sinapic acid.[9] Attempts using AM1 or PM3 calculations of spin densities proved to be far less accurate. In the following we shall demonstrate the applicability of this approach with four examples, a phenol shielded by two adjacent methyl groups (2,4,6-trimethylphenol), a phenol shielded by two methoxy groups (syringic alcohol), a simple gallate ester (propyl gallate) and a model gallotannin (hamamelitannin) – see Scheme 1 for the structures. DFT calculations of coupling constants at the B3LYP level have been carried out before for simple *p*-benzosemiquinone.[10–12] With regard to polyphenols, DFT calculations thus far were limited to determinations of thermodynamic parameters of flavonol quinones and quinone methides.[13]

MATERIALS AND METHODS

2,4,6-Trimethylphenol was from Lancaster (Mülheim/Main, Germany), syringic alcohol was synthesized from syringic aldehyde (Fluka, Deisenhofen, Germany) by I. Lukić (Zagreb), propylgallate was also from Fluka and hamamelitannin was from

Phytochem (Neu-Ulm, Germany). EPR spectra were obtained *in situ* either by oxidation of the precusor phenols with horseradish peroxidase (Sigma, Deisenhofen, Germany) and hydrogen peroxide (Fluka; HRP/ H_2O_2) in near neutral solution (pH 7.0–7.5) or by alkaline autoxidation (pH ~ 10). Concentration of the substrates was generally in the millimolar range, as was H_2O_2, and HRP was about 50–100 nM. X-band EPR spectra were recorded mostly at the following settings: modulation amplitude 1 G, sweep rate 10 s, sweep range 40 G, amplification $3.2x10^5$. In some cases weakness or instability of the EPR signals prevented the evaluation of the experimental coupling constants.

To assign the correct structure of the antioxidant radicals, we employed hybrid DFT calculations at the B3LYP level of theory (Gaussian98)[8] for the determination of the isotropic coupling constants. Three-dimensional structures, obtained with Chem3D (Cambridge Soft, Cambridge, MA) were imported into the Gaussian98 program as MOPAC RHF Z-matrices (the Z-matrices of biradicals were obtained with MOPAC UHF). The solvent effect was taken into account using both PCM and CPCM (Cosmo) options. Various basis sets were tested with the best results (both with regard of calculation time and correlation with the experimental data) obtained with the pairs 6-311G* and 6-311G**. We averaged theoretical values if the substituents were symmetric to each other, since the arbitrary numbering in the Z-matrices made the substituents interchangeable. In contrast, the theoretical results are listed separately for the individual basis sets, demonstrating their close similarity with few exceptions.

Since in our experience, both MOPAC and Gaussian98 DFT calculations reached limits at a molecule size of about 45 atoms per molecule, larger structures such as the ellagitannins could only be approximated by designing fragments for the assumed radical target structures. This approach is fraught with uncertainties but is presently considered the only viable alternative to ascertain radical target sites of such large structures (no example is given).

RESULTS AND DISCUSSION

Since DFT calculations are unaffected by the actual stabilities of the phenoxyl or aroxyl radicals, we obtained isotropic coupling constants also for very weak or unstable EPR signals. On the other hand, conversion of one radical structure to another, as observed quite often experimentally, cannot be simulated – except by presuming such potential conversion reactions and calculating the isotropic coupling constants of the new radical. A successful example was the radical observed after HRP/H_2O_2 oxidation of 2,4,6-trimethylphenol. While in benzene only the phenoxyl radical was observed,[14] the multi-line signal observed in neutral aqueous solutions could best be simulated as arising from a distorted biradical. In such a distorted biradical the planes of the two benzene rings coupled at the 5,5'-positions are twisted with respect to each other due to the sterical hindrance of the methyl groups (see Scheme 2). As shown by the comparison of the coupling constants of the biradical in Table I**,** the experimental values do not discriminate between the various methyl groups, whereas the DFT calculations distinguish between the

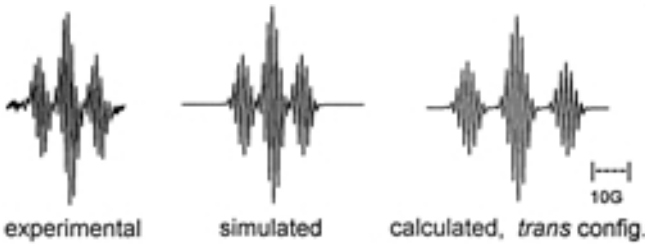
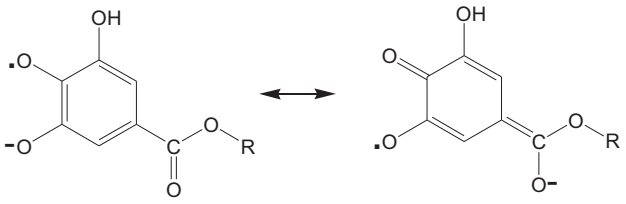


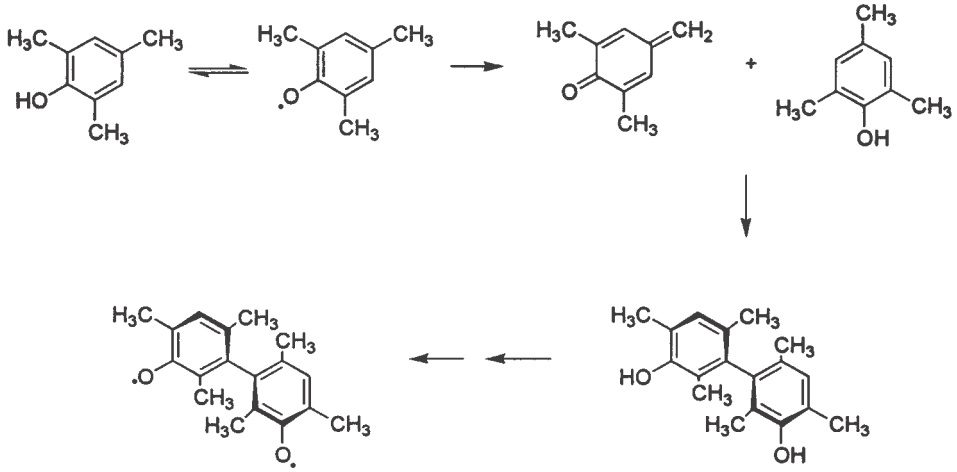
Figure 1. EPR spectra of the syringic alcohol phenoxyl radical. Oxidation by HRP/H_2O_2 at pH 7.5, concentration of syringic acid 1 mM, of H_2O_2 1 mM, HRP 100 nM; experimental, simulated and calculated spectra for the *trans*/*trans* configuration (for coupling constants see Table II).

2,6,2',6'-methyl groups in *ortho*- and the 4,4'-methyl groups in *para*-position. To account for these observations we assume that the initially formed phenoxyl radical disproportionates (as verified by pulse radiolysis experiments) and the resulting quinone methide reacts with excess phenol in a S_N2 reaction forming the dimer. As depicted in Scheme 2, only in the fourth and fifth subsequent oxidation steps can the biradical be formed, demonstrating the efficiency of such phenolic coupling reactions.[15–19]

It was observed that in almost all cases a reasonable correlation of experimental and calculated coupling constants existed only for the aromatic hydrogen atoms immediately adjacent to the radical site and the adjacent methoxy groups of the methoxyphenols. Coupling constants for more distant hydrogen atoms in the aromatic ring or aliphatic side chains deviated much more. In fact, simulated EPR spectra based on either experimental or DFT-calculated values practically never showed reasonable similarities except for the phenoxyl radical of syringic alcohol, seen in Figure 1. Here, only the $a_{H\alpha}(2)$ values disagreed considerably between experiment and calculation (Table II).[20,21] As shown as well, only the calculated values for *trans*-configuration of the methoxy groups adjacent to the phenoxyl radical corresponded reasonably well with the experiment.

With propyl gallate as model gallotannin, we could verify that the involvement of the ester bond observed pulse-radiolytically is indeed due to mesomery of both semiquinone anion structures:[22]





Scheme 2. Oxidation and phenolic coupling of 2,4,6-trimethylphenol to its biradical.

TABLE I

Experimental and theoretical isotropic coupling constants for the phenoxyl radical of 2,4,6-trimethylphenol and its dimeric biradical

	Assignments			Basis set
	phenoxyl radical			
	2-CH_3 (3)	4,6-CH_3 (6)	$H_{3,5}$ (2)	
benzene[a]	10.60	6.15	1.65	
calculated	11.12	7.02	2.46	6-31+G*
	10.97	7.10	2.29	6-311G*
	10.96	7.16	2.59	6-31G**
	11.87	7.54	2.36	EPR-II
	biradical			
	2,6,2',6'-CH_3 (12)	4,4'-CH_3 (6)	$H_{3,3'}$ (2)	
oxid. at pH 7[b]	5.78	5.78	1.43	
calculated	3.61	5.48	1.20	6-31+G*
(without solvent)	3.65	5.46	1.12	6-311G*
	3.66	5.43	1.26	6-31G**
	3.88	5.88	1.16	EPR-II
calculated	3.56	5.73	1.03	CPCM/6-311G*
(with solvent)	3.55	5.69	1.00	-/6-311G**
	3.57	5.72	1.02	PCM/6-311G*
	3.56	5.69	1.00	-/6-311G**

[a] Ref. 14.
[b] This work.

TABLE II

Experimental and theoretical isotropic coupling constants for the phenoxyl radical of syringic alcohol

	Assignments			Basis sets
	3/5-OCH_3 (6)	H_2/H_6 (2)	H_α (2)	
		experimental		
HRP/H_2O_2[a]	1.45	1.45	9.80	
HRP/H_2O_2[b]	1.31	1.31	9.53	
		calculated		
trans/*trans*	1.65	1.87	15.74	CPCM/6-311G*
	1.65	1.82	15.78	-/6-311G**
	1.64	1.75	16.84	PCM/6-311G*
	1.63	1.69	16.89	-/6-311G**
cis/*cis*	3.75	1.75	12.40	CPCM/6-311G*
	3.72	1.69	12.42	-/6-311G**
	3.56	1.66	13.27	PCM/6-311G*
	3.53	1.60	13.30	-/6-311G**

[a] Ref. 20.
[b] Ref. 21.

As shown in Table III, these two structures of course yielded almost identical theoretical coupling constants for the monoanions, which were quite similar to the experimental values, but not for the dianions. Since very similar transient spectra and EPR spectra were seen also with other gallo- and ellagitannins, the same mesomery has to be assumed for these substances.

In the case of the more complex structures of the oligomeric tannins, these had to be represented by smaller model structures of assumed radical target sites to enable the MOPAC and DFT calculations. For hamamelitannin, this approach suggests that the radical site is more likely the C2' gallate ester rather than the C4' ester, again with a stronger deviation of the aliphatic hydrogen coupling constants (Table III). Studies with larger ellagitannin structures, containing either gallate ester dimers (hexahydroxydiphenic acid structures) with nominally six reactive hydroxy groups or the further oxidized dehydrohexahydroxydiphenic acid moiety with only a catechol structure as potential radical target are hampered by the size limitations of the MOPAC and Gaussian calculations.[22]

In our experience, hybrid DFT calculations as supplementation to EPR experiments are useful for assignment of the individual hydrogen coupling constants of phenoxyl and aroxyl radicals. They are also particularly sensitive to structural modifications in aliphatic side chains, and allow to pin-

TABLE III

Experimental and theoretical isotropic coupling constants for the semiquinone/quinoid structure of the propyl gallate radical anions, and the anion radical of hamamelitannin and the C2' and C4' furanose gallate radicals

	Assignments		Basis sets
	$H_{2,6}$ (2)	H_{α} (2)	
	propyl gallate		
experimental results[a]	1.15	0.40	
semiquinone anion	1.20	0.75	CPCM/6-311G*
	1.21	0.78	PCM/6-311G*
quinoid anion	1.17	0.75	CPCM/6-311G*
semiquinone dianion	1.51	0.35	CPCM/6-311G*
	1.62	0.35	PCM/6-311G*
quinoid dianion	1.32	0.83	CPCM/6-311G*
	hamamelitannin		
experimental results[a]	1.15	0.45	
furanose gallate, C2' ester bond	1.29	0.67	CPCM/6-311G*
	1.21	0.74	PCM/6-311G*
furanose gallate, C4' ester bond	0.89	0.25	CPCM/6-311G*
	0.88	0.24	PCM/6-311G*

[a] Ref. 22.

point radical sites in more complex structures of polyphenols. They may thus be quite helpful to distinguish between the different catechol and pyrogallol structures even of such complex structures as ellagitannins, provided truncation of such large molecules into molecule fragments representing likely radical target sites can be validated.

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SAŽETAK

Račun izotropnih konstanti sprezanja za fenoksilne i aroksilne radikale primjenom hibridnih metoda teorije funkcionala gustoće

Wolf Bors, Christa Michel, Kurt Stettmaier, Snježana P. Kazazić i Leo Klasinc

Kvantno-kemijskim računom, tj. primjenom hibridnih metoda teorije funkcionala gustoće željelo se potvrditi prisutnost fenoksilnih radikala u EPR spektrima mono- i polifenola oksidiranih bilo smjesom peroksidaze hrena i vodikova peroksida ili alkalnom autooksidacijom. Iako kvantitativna koorelacija eksperimentalnih i teorijskih izotropnih konstanti sprezanja nije u potpunosti zadovoljavajuća, ipak je bilo moguće potvrditi nastajanje biradikala nakon početne oksidacije 2,4,6-trimetilfenola, kao mezomernu strukturu aroksilnih radikala galatnog estera, te odrediti radikalsko mjesto u modelnim molekulama galotanina i hamamelitanina.