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Synthesis and Antioxidant Properties of a New Lipophilic Ascorbic Acid Analogue

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Abstract—4-(4-Hydroxyphenyl)-5-(4-hydroxyphenylmethyl)-2-hydroxyfurane-2-one **1** was prepared by an acidic dimerisation of 4-hydroxyphenylpyruvic acid and some of its antioxidant and spectroscopic properties have been measured and compared to that of ascorbic acid. **1** is as good an antioxidant as ascorbic acid in the DPPH (2,2-diphenyl-1-picryl hydrazyl radical) test and the inhibition of hydroxyl radical and a powerful inhibitor of the Cu^{2+} or AAPH (2,2′-azobis-(2-amidinopropane) dihydrochloride) induced oxidation of human LDL. **1** gives a stable radical characterised by its ESR spectrum similarly to ascorbic acid but in lower concentration and with a different reactivity towards nitroxides. Theoretical calculations allow us to propose the structure for the radical formed from **1**, to explain its lower stability than ascorbyl radical and to evaluate the lipophilicity of **1**.

Introduction

Oxidative stress is thought to play an important contributory role in the pathogenesis of numerous degenerative or chronic diseases, such as atherosclerosis and cancer. Considerable experimental evidence links the production of reactive oxygen species to biological damage that can potentially provide a mechanistic basis for their initiation and/or progression. The human antioxidant protections include enzymes such as catalase, superoxide dismutase and glutathione peroxidase as well as water- and lipid-soluble antioxidants such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E).[1–4]

Ascorbic acid can be considered as one of the most potent naturally occurring antioxidants because it works by reacting with aqueous peroxyl radicals and by restoring the antioxidant properties of vitamin E.[5] However, its high hydrophilicity limits this latter property. The antioxidant and pro-oxidant activities of ascorbic acid remain controversal.[6] Similarly to animal data, the current evidence from epidemiological studies on the role of ascorbic acid in the prevention of cardiovascular disease is inconclusive, with some studies showing a very strong correlation between increased ascorbic acid intake and incidence of CVD events and other studies showing no correlation at all. Numerous in vitro studies have demonstrated that ascorbic acid inhibits LDL oxidation by a variety of mechanisms. Retsky et al.[7] have suggested two mechanisms by which ascorbic acid may protect LDL against oxidation. Ascorbic acid may scavenge free radicals in the aqueous phase or dehydroascorbic acid (the two-electron oxidation form of ascorbic acid), or its decomposition products may modify LDL leading to decreased copper binding to the LDL particle and increased resistance to copper-dependent oxidation. The recent efforts devoted to the development of new ascorbic acid analogues have led to the obtention of antioxidant,[8–10] antitumoural[11] and potentially anti-inflammatory[12] agents.

In connection with our interest in the synthesis of natural polyphenols[13,14] and our knowledge on the acid-catalysed dimerisation of arylethanoid and arylpropanoid derivatives,[15–17] we felt that arylpyruvic acids may lead to lipophilic ascorbic acid analogues by acidic dimerisation. Indeed, such dimerisation has been evoked in the biosynthesis of butyrolactone isolated from *Aspergillus terreus*[18] and numerous phenolic

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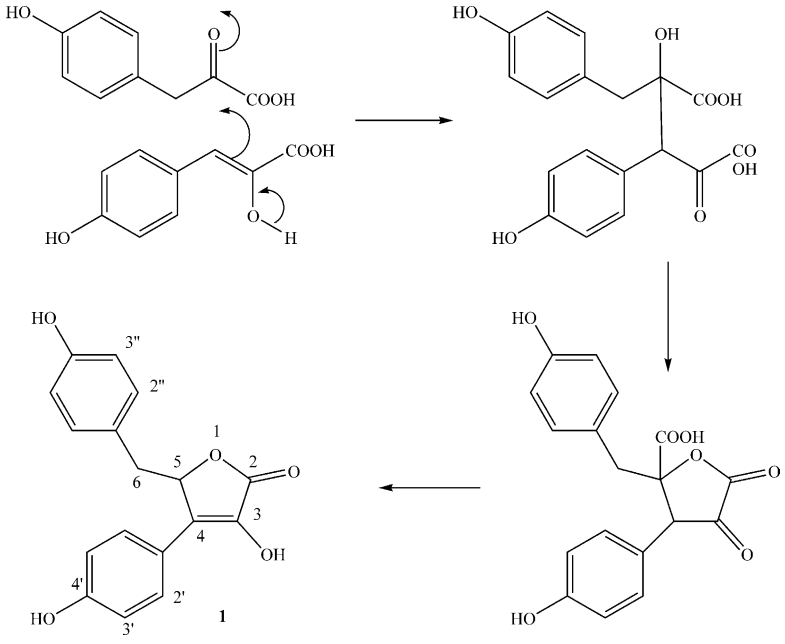
compounds from the mushroom *Suillus* spp.[19] In order to obtain true ascorbic acid analogues,[20] we studied the dimerisation of 4-hydroxyphenylpyruvic acid. To confirm the ascorbic acid character of the synthesised molecule, we evaluated its antioxidant activity by the DPPH test and the hydroxyl radical scavenging[21,22] and evidenced the generation of a stable radical under slightly alkaline conditions by ESR spectroscopy. The inhibitory effect of **1** on the Cu^{2+} or AAPH induced oxidation of human LDL was also evaluated. Additionally, theoretical calculations were performed to determine the structure of the radical formed and its stability has been compared with that of ascorbyl radical. The lipophilic character of the new molecule was also evaluated by the calculation of log *P*.

Results and Discussion

Our first attempts were directed towards the concomitant demethylative dimerisation of 4-methoxyphenylpyruvic acid using boron tribromide. Unfortunately, the demethylation occurred but the expected dimerisation failed.[23] So, we turned to the treatment of 4-hydroxyphenylpyruvic acid with concentrated hydrochloric acid. We serendipitously found that zinc amalgam in boiling hydrochloric acid gives the desired dimerisation product **1** in 20% yield beside the expected 4-hydroxyphenyllactic acid[24] in 50% yield. The supposed mechanism of formation of **1** involves an aldol condensation followed by a lactonisation and finally a decarboxylation (Scheme 1). Treating 2-(4-acetoxyphenylmethylene)-5-methyloxazol-3-one,[24] a precursor of 4-hydroxyphenylpyruvic acid, with 3 N HCl during 24 h can increase the yield in **1** up to 50% with the formation of 4-hydroxybenzaldehyde as an impurity.

The antioxidant properties of **1** and ascorbic acid were expressed by the traditional EC_{50} parameter describing the global reactivity of the tested compound towards DPPH and the kinetic parameter log *Z* influenced only by the first step of the reaction, that is, the hydrogen abstraction (from the donor to give the hydrazine and a radical) (see Experimental section for the calculation of log *Z*).[25] **1** And ascorbic acid did not show any difference (see Table 1) in this test, suggesting that the two molecules interact in the same manner with DPPH. The total stoichiometry ([DPPH]/2EC50) of **1** and ascorbic acid is about 2 and corresponds to the total number of labile hydrogen atoms transferred to DPPH.

The inhibition of hydroxyl radical ($OH^{\bullet}$) using DMPO (dimethylpyrroline-*N*-oxide) as spin trap was carried out only for **1** since ascorbic acid presents several problems in this test: (i) the second-order rate constants for the reaction of $OH^{\bullet}$ with ascorbic acid and DMPO are 7.2×10^9 and $3.4\times10^9\ M^{-1}\ s^{-1}$ respectively. Therefore the ESR signal of ascorbyl radical may superimpose on that of DMPO–OH and perturb the measurement of DMPO–OH signal; (ii) ascorbic acid can reduce



Scheme 1.

Table 1. Antioxidant properties, ESR data and lipophilicity of **1** and ascorbic acid

	DPPH inhibition		$OH^{\bullet}$ inhibition	LDL peroxidation induced by		ESR data		Lipophilicity
	Log *Z*	EC_{50}	EC_{50}	Cu^{2+}	AAPH	*g*-Factor	a_{H5}	Log *P*
1	3.9	16 μM	2 μM	0.1 μM	0.18 μM	2.00529	1.32 G	+1.89
Ascorbic acid	3.9	13 μM	30 μM[10]	2.5 μM[26]	—	2.00518	1.72 G	−1.85

paramagnetic nitroxides such as DMPO–OH spin adduct; (iii) ascorbic acid can reduce Fe(III) to Fe(II) and in the presence of H_2O_2 can stimulate $OH^{\bullet}$ formation by the Fenton reaction. Whereas an IC_{50} of 30 μM has recently been reported for ascorbic acid,[10] we were unable to reproduce this result. The ESR signal of DMPO–OH is superimposed with that of ascorbyl radical. In the process of time, the ESR signal of DMPO–OH decreased with the concomitant increase of the ESR signal of ascorbyl radical.

The inhibitory effect of **1** on the Cu^{2+} or AAPH induced oxidation of human LDL was measured by the increase in absorbance at 234 nm caused by the formation of conjugated dienes.[26] Its activity was expressed as percentages of increase of their lag phase compared to the one of the control. As its antioxidant capability can be characterised by the evolution of the LDL oxidation curves (Fig. 1) with its concentration, **1** has been tested at concentrations of 0.1–100 μM. We considered that the drug concentration had 100% activity (efficiency dose 100%: ED_{100}) when it doubled the control lag phase duration and we defined the efficiency dose 50% (ED_{50}) as the concentration of the drug that increased this control lag phase by 1.5 times. Increased concentrations of **1** have been used with a logarithmic scale

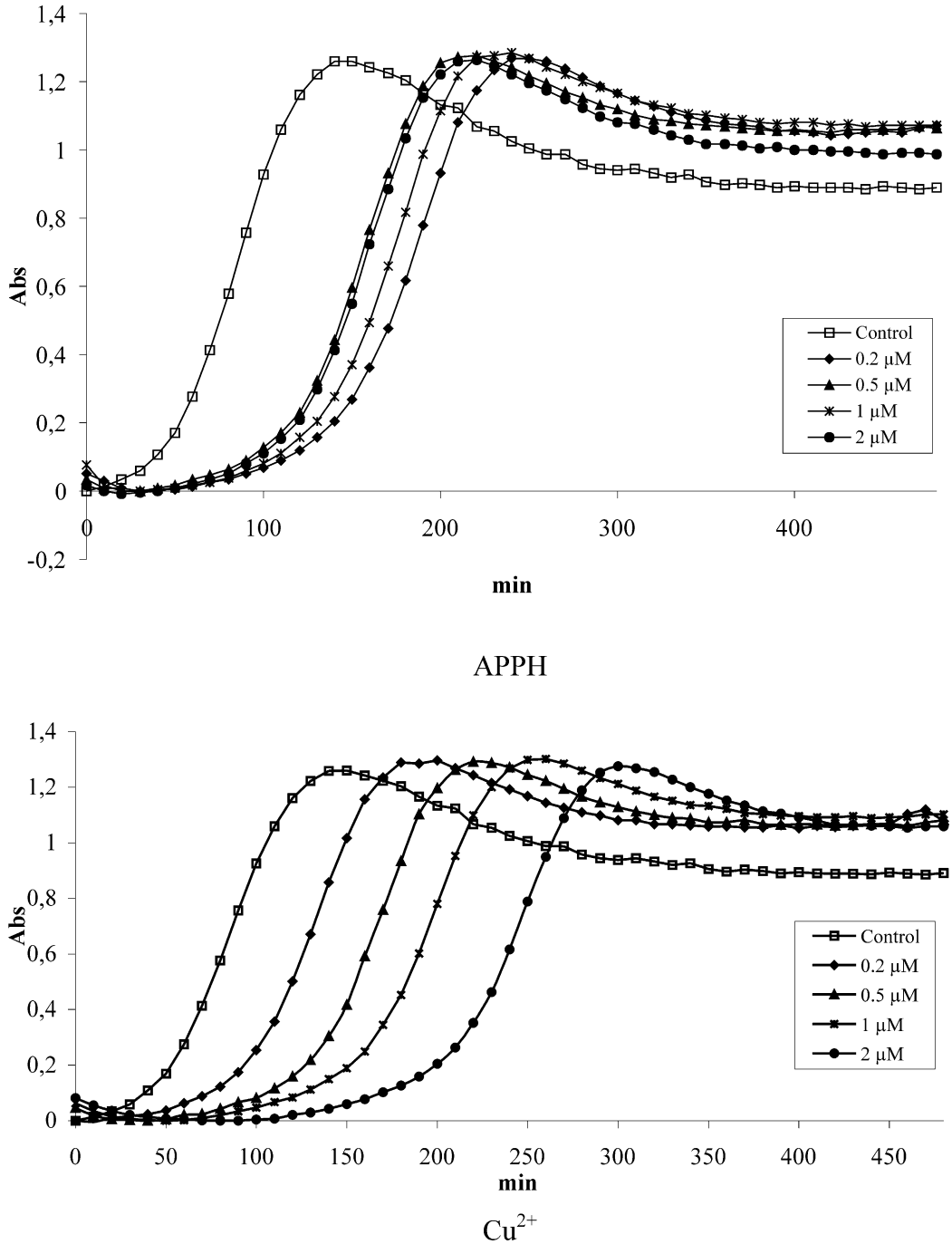


Figure 1. Effect of **1** on the lag phase of LDL oxidation by copper (II) and AAPH by increase of the diene absorption. The LDL solution (125 μg protein/mL) was supplemented with 0, 0.2, 0.5, 1, 2 μM of **1** in 0.01 M PBS.[26] The reference cuvette contained LDL and **1** without copper or AAPH.

to calculate the ED_{50} of antioxidant activity. The ED_{50} values of **1** in the various peroxidation experiments are reported in Table 1 and compared to that of ascorbic acid.[27] The ED_{50} of **1** on the induction of LDL oxidation by Cu^{2+} and AAPH are 0.10 and 0.18 μM, respectively, whereas Esterbauer et al.[27] reported for ascorbic acid an ED_{50} of about 2.5 μM for the oxidation of LDL induced by Cu^{2+}. The similar ED_{50} of **1** whatever the peroxidation inducer shows that the activity of **1** is expected to be linked to a radical scavenging property. Nevertheless other mechanisms, that is, copper complexation or reduction, may not be rejected. Therefore the interaction of **1** with Cu^{2+} was measured by UV spectroscopy. The UV spectrum of **1** (Fig. 2) was not modified by adding Cu^{2+} as well as after the addition of EDTA, denoting an absence of interaction or reaction of **1** with Cu^{2+}. As a control, the UV spectrum of **1** in alkaline solution was also recorded (Fig. 2). The powerful activity of **1** compared to ascorbic acid may be due to a higher lipophilicity associated with its antioxidant properties.

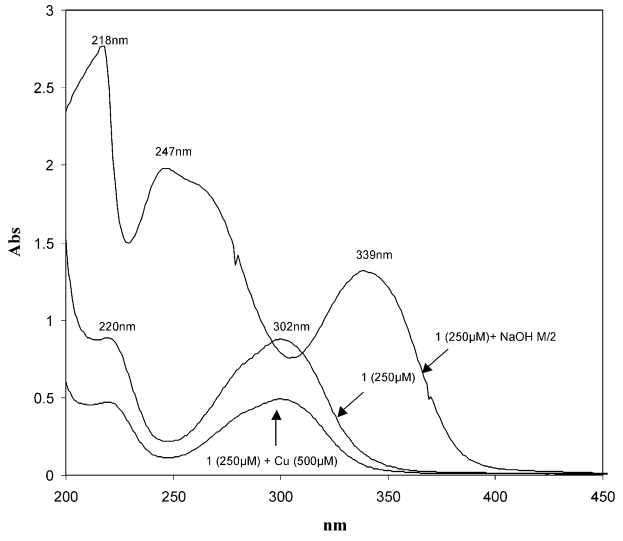


Figure 2. UV spectra of **1** in aqueous solution, after addition of Cu^{2+} or after addition of NaOH M/2.

The lipophilicities of **1** and ascorbic acid were evaluated by the calculation of the log *P* values using the Villar method implemented in Spartan packages. Whereas the calculated (and the experimental) value for ascorbic acid is −1.88 (−1.85) the calculated one for **1** is 1.89. A good correlation between the calculated and the experimental values of log *P* of ascorbic acid validates the use of the Villar method. The calculated positive sign for the log *P* value indicates that **1** is much more lipophilic than ascorbic acid but its intrinsic moderate lipophilicity suggests that it may be localised at the interface of the bilayers (water and lipid phases).

The ESR spectra displayed in Figure 3 show the ascorbyl radical and the radical of **1** obtained under mild alkaline solution. On both spectra, a doublet can be observed arising from the hyperfine coupling with the H5 proton. The hyperfine splitting constant a_{H5} and the *g*-value for both molecules are given in Table 1 and are very similar for both molecules with a small decrease of 0.4 G for **1**. No additional proton feature was detected for **1**.

It is well known that ascorbic acid can reduce paramagnetic nitroxides into diamagnetic hydroxylamines: the ESR signal of TEMPO totally disappears after the addition of ascorbic acid whereas when **1** was added to a 1 mM solution of TEMPO, we have not observed any modification of ESR signal even for a high concentration (i.e., 80 mM). On the contrary, DPPH was reduced either by ascorbic acid or by **1**. These results suggest that the reduction potential of **1** is expected to be lower than that of TEMPO ($E^0 = 880$ mV versus NHE)[28] and therefore **1** is unable to reduce it and to be higher than that of DPPH ($E^0 = 560$ mV versus NHE).[29] The oxidation of ascorbic acid at pH 5 has been reported to occur at 902 mV versus NHE with a modified carbon electrode.[30]

Theoretical calculations were employed first to understand the most favourable structure of radical formed under auto-oxidation conditions. Effectively the three structures that can be considered are the radical **1**•, the radical anion $\mathbf{1}^{-\bullet}$ and the radical dianion $\mathbf{1}^{2-\bullet}$.

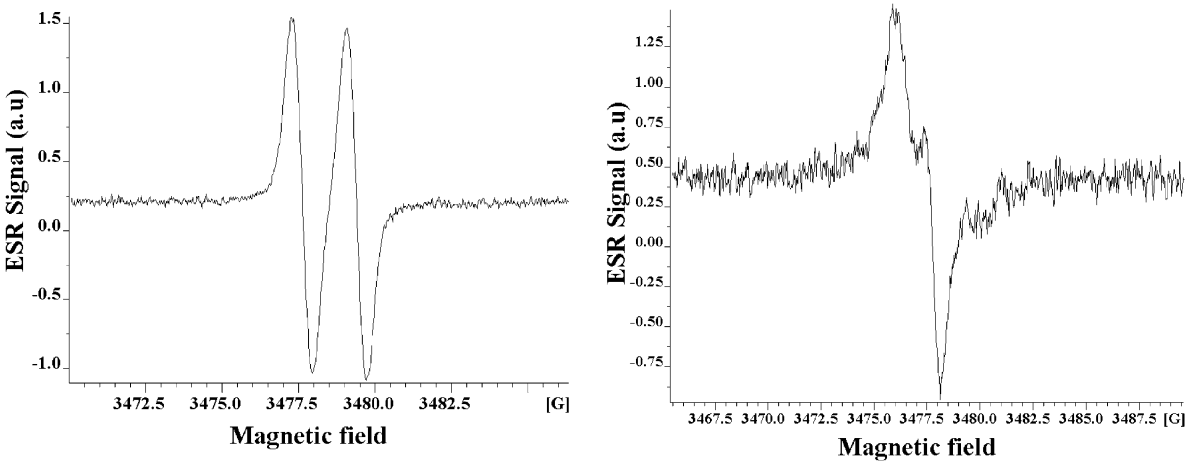


Figure 3. ESR spectra of ascorbyl radical (left) and $\mathbf{1}^{-\bullet}$ (right).

Modelling these structures using semiempirical UHF/PM3 formalism has shown that the most favourable structure was the radical anion form with a heat of formation of −184 kcal/mol whereas the two other ones have respective heats of formation of −134 kcal/mol and −167 kcal/mol for radical and radical dianion forms. This first approach does not take account of the solvent and can be used only to estimate electrostatic potentials and to provide a reasonable spatial representation of the species. The structure of $\mathbf{1}^{-\bullet}$ is presented in Fig. 4. The electrostatic potential contours show the most favourable hydrogen bond donation sites for the radical anion. As expected the densest regions of spin are located at the O3 atom and the O4′ atom (see Scheme 1 for numbering of **1**) that conform to the concept of ascorbic acid analogues. Deprotonation of the phenolic group that is conjugated with the heterocycle probably stabilises the radical because of an extended electron delocalisation. Then, deprotonation of the remaining and less acidic phenolic group does not further enhance radical stability since it is not conjugated with the heterocycle. The apparent in vacuo stabilisation probably reflects the repulsion between the two negative charges, a phenomenon greatly overestimated in the absence of solvent. In order to overcome this problem, the solvent effect (water or methanol) was computed using PCM models[31,32] at the DFT B3LYP levels using 6-31G** basis set. Calculated $\Delta\Delta G$ between the radical $\mathbf{1}^{\bullet}$ and the radical anion $\mathbf{1}^{-\bullet}$ and dianion $\mathbf{1}^{2-\bullet}$ are respectively 48 and 624 kcal/mol higher whereas the solvation energies are −25 kcal/mol for the radical $\mathbf{1}^{\bullet}$, −56.7 kcal/mol for the radical anion $\mathbf{1}^{-\bullet}$ and −147 kcal/mol for the radical dianion $\mathbf{1}^{2-\bullet}$. These results indicate that the phenolic group conjugated to the heterocycle can be easily deprotonated and therefore the order of stability is $\mathbf{1}^{\bullet} \approx \mathbf{1}^{-\bullet} > \mathbf{1}^{2-\bullet}$. Additional isotropic Fermi contact coupling term was computed and yielded the hyperfine splitting constants $a_{H5} = 10.46$ G ($\mathbf{1}^{\bullet}$), 2.13 G ($\mathbf{1}^{-\bullet}$) and 2.45 G ($\mathbf{1}^{2-\bullet}$). From the calculated and experimental hyperfine constants, we can conclude that the radical observed during the auto-oxidation of **1** is the radical anion $\mathbf{1}^{-\bullet}$. Moreover E_{hf} of ascorbyl radical (−278 kcal/mol) is much lower than those calculated for $\mathbf{1}^{-\bullet}$ of about 90 kcal/mol that can explain the lowest stability of the radical formed. Measuring the steady state concentrations of the two radicals under the same experimental conditions may easily check this assertion. As expected the double integration of this signal shows that the concentration of ascorbyl radical is 10-fold higher than in radical anion $\mathbf{1}^{-\bullet}$.

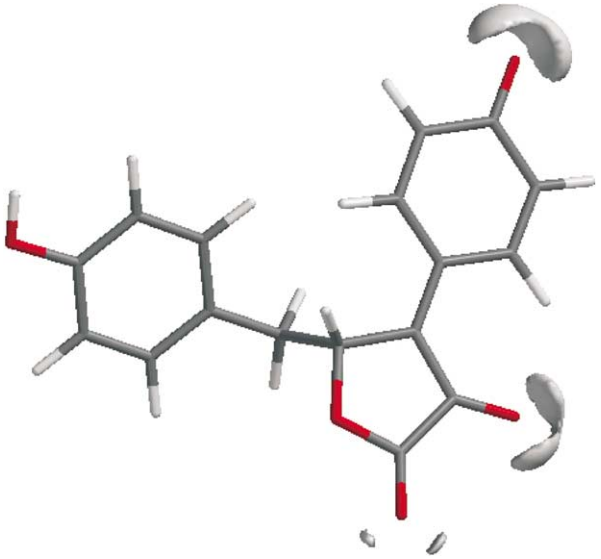


Figure 4. Structure of $\mathbf{1}^{-\bullet}$.

Conclusion

In this paper, we have prepared a true ascorbic acid analogue that presents antioxidant activities very close to that of ascorbic acid. A radical anion that gives very similar data has also been generated under the same conditions than for ascorbic acid but its stability is lower and its redox potential lower than those of ascorbyl radical. Due to its higher lipophilicity, **1** has proved to be a good candidate in the prevention of lipid peroxidation and is expected to be a good relay between ascorbic acid (in the water phase) and α-tocopherol (in the lipid phase). We are currently attempting to prepare the isomer of **1** where the 4-hydroxyphenyl moiety replaces the 2-hydroxy group of the molecule in order to evaluate the relative importance of the hydroxy groups in ascorbic acid.[33]

Experimental

Chemistry

All reagents and solvents were purchased from Aldrich-Chimie (Saint-Quentin-Fallavier, France) of ACS reagent grade and were used as provided. TLC analyses were performed on a 3×10 cm plastic sheet precoated with silica gel 60F254 (Merck) (Solvent system: ethyl acetate/hexane 1:4). SiO_2, 200–400 mesh (Merck), was used for column chromatography. Melting points were obtained on a Reichert Thermopan melting point apparatus equipped with a microscope and are uncorrected. NMR spectra were obtained on an AC 200 Bruker spectrometer in the appropriate solvent with TMS as internal reference.

Synthesis of 1

A mixture of 2-(4-acetoxyphenylmethylene)-5-methyl-oxazol-3-one (10 mmol) in 3 N HCl (60 mL) was refluxed during 24 h. The resulting mixture was filtered while it was still hot. The filtrate was allowed to cool to room temperature and was extracted with EtOAc (5×20 mL). The combined filtrate was then dried (Na_2SO_4) and was evaporated. The residue was purified by column chromatography on silica gel (cyclohexane/EtOAc 3:7) ($R_f = 0.71$), yield = 50%; mp = 248–249 °C (lit. 246–249 °C); ^{1}H NMR (200 MHz, Acetone-*6d*): 2.84 (1H, dd, $^2J = 14.5$ Hz; $^3J = 6.0$ Hz, H6), 3.27 (1H, dd, $^2J = 14.5$ Hz; $^3J = 3.3$ Hz, H6), 5.59 (1H, dd, $^3J = 3.3$ Hz; $^3J = 6.0$ Hz, H5), 6.66 (2H, d, $^3J = 8.5$ Hz, H3″), 6.87 (2H, d, $^3J = 8.5$ Hz, H2″), 6.98 (2H, d, $^3J = 8.8$ Hz, H2′), 7.61 (2H, d, $^3J = 8.8$ Hz, H3′), 8.60 (1H, br s); ^{13}C NMR

(50 MHz, Acetone-*6d*): 39.4 (t), 79.3 (d), 115.6 (d), 116.5 (d), 127.0 (s), 129.3 (s), 130.1 (d), 131.6 (d), 137.4 (s), 157.0 (s), 158.7 (s), 169.7 (s), 172.1 (s).

ESR spectroscopy

ESR measurements: ESR experiments were performed on a Bruker Elexys 580 spectrometer operating at 9.7 GHz with a 100 kHz modulation frequency and 0.8 G of amplitude modulation. Microwave power detection was set to 5 mW. Samples were systematically prepared as follows: 10 μL of sodium hydroxide solution (0.1 M) was added to 500 μL of a solution of the compound in methanol (0.01 M).

Hydroxyl radical inhibition

Produced by the Fenton reaction, the hydroxyl radical was detected by the ESR spin-trapping method. The hydroxyl radical reacts with 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) to give DMPO–OH, which is ESR detectable. Reaction mixtures contained ferrous sulfate (0.5 mM), H_2O_2 (0.5 mM), DMPO (10 mL, 0.4 M) and various amounts of the tested molecule in 0.21 mL of 0.1 M sodium phosphate buffer, pH 7.4. The reaction was initiated by the addition of H_2O_2. ESR spectrum was taken 2 min after addition of H_2O_2.

DPPH inhibition

Spectrophotometric measurements were performed with a Uvikon 932 spectrophotometer. Calibration curves for DPPH were performed in triplicate and the initial DPPH concentration was then calculated from eq (1).

$$A_{517\ \mathrm{nm}} = 0.109 \times C_{\mathrm{DPPH}} \times 10^5 + 0.0013 \quad (1)$$

Standard solutions of the antioxidants were prepared in ethanol and rapidly mixed (volumes from 0.02 to 0.4 mL) with an ethanol solution of DPPH taken between 50 and 63 μM. The decrease in absorbance was recorded every 6 s for 5 min. Seven measurements per potential antioxidant were recorded with [antioxidant]/[DPPH] ratios varying from 0.19 to 1.7. In parallel a blank solution of DPPH was screened to estimate DPPH decomposition during the time of measurement. The decrease in absorbance was then plotted against time, after correction for DPPH decomposition. The effective concentration ratio (EC_{50}) is the concentration of antioxidant producing a 50% decrease in DPPH at steady state. The initial reaction followed second-order kinetics; the rate constants k (obtained from plots of 1/[C] versus time) were plotted against the ratio [antioxidant]/[DPPH]. Linear regression ($r^2 > 0.9$) gave the parameter Z (slope of the line in L mol^{-1} s^{-1}) which was examined as a quantification of radical scavenging activity.

UV spectroscopy

UV spectra were recorded on a thermostated Kontron Uvikon 932 spectrometer.

Theoretical calculations

Geometry optimisation calculations were performed with Spartan Pro 1.0 package software for semi-empirical at the UHF/PM3 level for heat of formation and log P calculations. Solvation calculation was performed using Gaussian 98.[32]

LDL preparation

Human LDL was isolated from freshly drawn blood from healthy, normolipidaemic, and fasting volunteers. Blood was collected into EDTA and the plasma was separated by low speed centrifugation. LDL was isolated by sequential density gradient ultracentrifugation in sodium bromide density solutions in the density range 1.019–1.063 g/mL as previously reported.[26] Then, LDL was dialysed against 0.01 M PBS (phosphate buffered saline: 0.15 M NaCl, 0.01 M Na-phosphate, pH 7.4) containing 0.01% EDTA and sterilised by filtration through a 0.22 μm pore-size filter and stored at 4 °C before use. The protein concentration was determined by Peterson's method[34] using bovine serum albumin as standard. Total and free cholesterol, triglycerides (Boehringer kit) and total phospholipids (bioMerieux kit) were analysed by enzymatic methods.

Induction of LDL oxidation by copper or AAPH

Ethanolic solutions of derivatives were prepared at 10^{-2} M in ethanol and diluted in 0.01 M PBS to give final concentrations of 0.1–100 μM in a total ethanol concentration of 1% (v/v). The same amounts of pure ethanol were added in blanks.

Prior to oxidation, EDTA was removed by extensive dialysis of LDL solution against 0.01 M PBS under N_2, then oxidation was induced at 30 °C by adding 100 μL of 16.6 μM $CuSO_4$ or of 2 mM 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) to 800 μL of LDL (125 μg protein/mL) and 100 μL drugs in 0.01 M PBS. Since the tested compounds absorb at 234 nm, the diene formation has been studied with a control containing LDL and the compound to be tested without copper or AAPH. During copper- or AAPH-induced LDL oxidation the diene conjugate formation was followed by measuring the optical density (OD) at 234 nm every 10 min for 8 h with a thermostatted Kontron Uvikon 930 spectrophotometer equipped with a 10 positions sample changer. Analyses were performed in triplicate.

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