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PAPER

Kinetic and thermodynamic aspects of the chain-breaking antioxidant activity of ascorbic acid derivatives in non-aqueous media†‡

Riccardo Amorati, Gian Franco Pedulli and Luca Valgimigli*

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Ascorbic acid (vit. C) is a cofactor whose reactivity toward peroxyl and other radical species has a key-role in its biological function. At physiological pH it is dissociated to the corresponding anion. Derivatives of ascorbic acid, like ascorbyl palmitate, are widely employed in food or in cosmetics and pharmaceuticals. While the aqueous chemistry of ascorbate has long been investigated, in non-aqueous media it is largely unexplored. In this work oxygen-uptake kinetics, EPR and computational methods were combined to study the reaction of peroxyl radicals with two lipid-soluble derivatives: ascorbyl palmitate and 5,6-isopropylidene-L-ascorbic acid in non-aqueous solvents. In acetonitrile at 303 K the undissociated $AscH_2$ form of the two derivatives trapped peroxyl radicals with k_{inh} of $(8.4 \pm 1.0) \times 10^4$ M^{-1} s^{-1}, with stoichiometric factor of *ca.* 1 and isotope effect $k_H/k_D = 3.0 \pm 0.6$, while in the presence of bases the anionic $AscH^-$ form had k_{inh} of $(5.0 \pm 3.3) \times 10^7$ M^{-1} s^{-1}. Reactivity was also enhanced in the presence of acetic acid and the mechanism is discussed. The difference in reactivity between the $AscH_2/AscH^-$ forms was paralleled by a difference in O–H bond dissociation enthalpy, which was determined by EPR equilibrations as 81.0 ± 0.4 and 72.2 ± 0.4 kcal mol^{-1} respectively for $AscH_2$ and $AscH^-$ in *tert*-butanol at 298 K. Gas-phase calculations for the neutral/anionic forms were in good agreement yielding 80.1/69.0 kcal mol^{-1} using B3LYP/6-31+g(d,p) and 79.0/67.8 kcal mol^{-1} at CBS-QB3 level. EPR spectra of ascorbyl palmitate in *t*BuOH consisted of a doublet with HSC = 0.45 G centred at $g = 2.0050$ for the neutral radical $AscH^{\bullet}$ and a doublet of triplets with HSCs of 1.85 G, 0.18 G and 0.16 G centred at $g = 2.0054$ for $Asc^{\bullet-}$ radical anion.

Introduction

Ascorbic acid (**1H$_2$**, vit. C) is a biological cofactor that plays a role in numerous biological pathways, fundamental to cellular function.[1] At physiological pH, **1H$_2$** is dissociated, ($pK_a = 4.1$), so the biological effects of vitamin C are usually ascribed to the ascorbate anion.[2] Being a strong reducing agent, ascorbate acts as radical quencher directly or by recycling other antioxidants such as α-tocopherol or glutathione.[1] In the presence of transition metals, vit. C shows pro-oxidant effects, as it reacts with O_2 to form H_2O_2 and dehydroascorbic acid, *via* the formation of $O_2^{\bullet-}$.[2] For this reason, high doses of intravenous ascorbate have been recently proven to deliver hydrogen peroxide to tissue fluids and to retard tumour growth in numerous animal models.[3]

Ascorbate salts of Na^+ and Ca^{2+} and the palmitate or stearate esters are commonly used as food additives. Ascorbyl palmitate (**2H$_2$**) is the antioxidant of choice to contribute specific functional properties *e.g.* in cosmetic[4] and pharmaceutical[5] products, or to stabilize oil-in-water emulsions, as it is localized in the lipid phase where oxidizable materials are usually contained.[6]

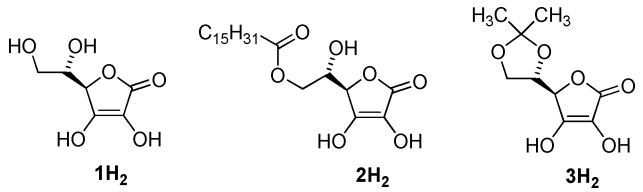
While the aqueous solution chemistry of ascorbic acid and ascorbate has long been studied,[7] many *in vivo* reactions of ascorbate occur in enzyme active sites or at membrane interfaces that have hydrophobic local environments, therefore knowledge of the chemistry of ascorbic acid or its derivatives in non-aqueous media is vital to the rationalization of their role. The non-protic polar solvent acetonitrile (MeCN) has been recently chosen as a suitable medium to study the non-aqueous chemistry of ascorbate salts from its isopropylidene ether **3H$_2$**.[8] As a complimentary model for non-aqueous media, the protic, moderately polar *tert*-butanol (*t*BuOH), which is sufficiently unreactive toward most radical species, appeared particularly suited. Since the pK_a of **3H$_2$** in MeCN is 18.3,[8] ascorbic acid derivatives in MeCN are almost exclusively in their protonated, neutral form, unless relatively strong bases are added to the solution. In this work, we studied in some detail the kinetic and thermodynamic aspects related to the antioxidant activity of ascorbic acid

Department of Organic Chemistry "A. Mangini", University of Bologna, Via San Giacomo 11, 40126, Bologna, Italy. E-mail: luca.valgimigli@unibo.it; Fax: +39 051 2095688; Tel: +39 051 2095683

† The manuscript is dedicated to the memory of Prof. Athel Beckwith (deceased May 2010) who illuminated the use of kinetic measurements in free radical chemistry.

‡ Electronic supplementary information (ESI) available: EPR spectra and simulations; results from theoretical calculations, autoxidation results. See DOI: 10.1039/c1ob05334e

lipid-soluble derivatives (**2H₂** and **3H₂**) including the influence of bases and acids on such properties. Particularly we focused on the reactivity toward peroxyl radicals that are responsible for oxidative damage in lipidic environments, such as membranes and proteins interior.



Results and discussion

Kinetic measurements

The reactivities of **2H₂** and **3H₂** ($AscH_2$) toward ROO· in MeCN were investigated by studying the inhibition of the thermally initiated autoxidation of either styrene or cumene (RH) in acetonitrile (50% v/v), (eqn (1)–(7)).[9] This reaction, initiated by the thermal decomposition of azobis-(isobutyronitrile) (AIBN) at 30 °C, was followed by monitoring the oxygen consumption with an automatic-recording gas-absorption apparatus, built in our laboratory and described previously, which is based on a commercial differential pressure transducer.[10]

$$\text{Initiator} \xrightarrow{R_i} \text{R}^{\bullet} \quad (1)$$

$$\text{R}^{\bullet} + \text{O}_2 \rightarrow \text{ROO}^{\bullet} \quad (2)$$

$$\text{ROO}^{\bullet} + \text{RH} \xrightarrow{k_p} \text{ROOH} + \text{R}^{\bullet} \quad (3)$$

$$\text{ROO}^{\bullet} + \text{ROO}^{\bullet} \xrightarrow{2k_t} \text{Non-radical products} \quad (4)$$

$$\text{ROO}^{\bullet} + \text{AscH}_2 \xrightarrow{k_{inh}} \text{ROOH} + \text{AscH}^{\bullet} \quad (5)$$

$$\text{ROO}^{\bullet} + \text{AscH}^{\bullet} \rightarrow \text{ROOH} + \text{Asc} \quad (6)$$

$$2\ \text{AscH}^{\bullet} \rightarrow \text{AscH}_2 + \text{Asc} \quad (7)$$

The slope of the oxygen consumption trace during the inhibited period afforded k_{inh} values, while its length allowed the determination of the stoichiometric coefficient n, *i.e.*, the number of peroxyl radicals trapped by one molecule of inhibitor.[9] Results obtained by studying the inhibited autoxidation of cumene or styrene in MeCN at 30 °C are reported in Table 1. It can be seen that the rate constant for the reaction of **2H₂** and **3H₂** with ROO· is rather small (*ca.* 8×10^4 M^{-1} s^{-1}), as compared to that of 2,2,5,7,8-pentamethyl-6-chromanol (PMHC), a model for the physiological antioxidant α-tocopherol. The contribution of the deprotonated species $AscH^-$ to these rate constants is negligible, given that on the basis of the reported pK_a of **3H₂**, and the autoprotolysis constant of MeCN,[11] their concentration can be estimated as 10^{-12} M under our experimental setting (*ca.* 1 molecule out of 5×10^6 molecules of neutral form).

Table 1 Rate constants for the reaction of ascorbic acid derivatives and of PMHC with ROO· radicals (k_{inh}) in MeCN[a]

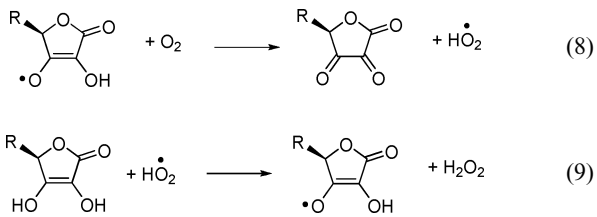
	$k_{inh}/10^4$ M^{-1} s^{-1}		
	Styrene/MeCN	Cumene/MeCN	n^b
2H₂	—	8.3 ± 0.9	0.8 ± 0.1
3H₂	5.8 ± 0.5	8.4 ± 0.8	1.0 ± 0.1
PMHC	68 ± 6	—	

[a] Mean of at least three measures, errors correspond to ±SD. [b] Determined in cumene.

Addition of a small amount of D_2O to MeCN slowed down the reaction of **3H₂** with ROO· radicals during cumene autoxidation, while H_2O did not have any effect on reaction rate. The deuterium kinetic isotope effect was determined as $k_H/k_D = 3.0 \pm 0.6$ and it is fully consistent with the transfer of the H-atom in the rate determining step. Therefore reaction 5 can be attributed to hydrogen atom transfer (HAT) or a proton-coupled electron transfer (PCET) mechanism.

It should be noted that in all cases the stoichiometric coefficient of **2H₂** and **3H₂** was lower than 2, that is the value of n expected for an antioxidant acting *via* eqn (5) and 6–7.[12]

This can be explained considering that reactions 6–7 compete with hydrogen transfer from the neutral ascorbyl radical to O_2 to yield the hydroperoxyl radical (eqn (8)), which in turn is able to propagate the oxidative chain or consume the antioxidant (eqn (9)). In the case of **1H₂**, reaction 8 is exoergonic by about 7 kcal mol^{-1} in H_2O, on the basis of the known bond dissociation free energies of AscH· and H–OO· of 53.8 and 60.4 kcal mol^{-1} respectively.[13] However, we have previously demonstrated that the barrier for H-atom donation to O_2 in *p*-semiquinone radicals is strongly enhanced by kinetic solvent effects, *i.e.* by H-bonding between the reactive phenolic -OH (the H-bond donor, HBD) and H-bond accepting (HBA) solvents, so reaction 8 may occur at a rate much slower than diffusion in MeCN.[14]



Upon addition of millimolar amounts of pyridine to the autoxidating mixture containing styrene, MeCN and 1% H_2O, the antioxidant behaviour of **3H₂** improves significantly, the inhibition being similar to that observed in the presence of the α-tocopherol analogue PMHC (see Fig. 1).

The rate of the O_2 consumption during the inhibited period depends on the inverse of the square root of the concentration of both pyridine (see Fig. 1B) and **3H₂** (see ESI‡), indicating that a H^+ transfer equilibration between the base and the antioxidant precedes the reaction with ROO· radicals (reactions 10–12).[15] Under the assumption that equilibrium 10 is fast, the oxygen consumption rate during the inhibited period is described by eqn (13). Although equilibrium 10 is mostly shifted toward the

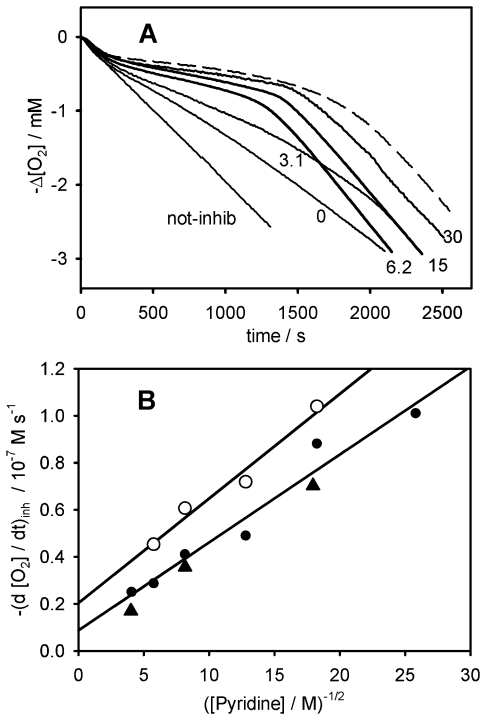


Fig. 1 **A**. Oxygen consumption during the autoxidation of styrene (4.3 M), in MeCN containing 1% of H_2O, initiated by AIBN (0.05 M) in the absence and in the presence of $\mathbf{3H_2}$ 1.2×10^{-5} M at various [pyridine, mM], or of PMHC 6.3×10^{-6} M (dashed). **B**. Dependence of the O_2 consumption rate during the inhibited period on the concentration of pyridine, in anhydrous MeCN (▲) and in the presence of 1% of H_2O (●) or D_2O (○).

reactants, reaction 11, very exergonic hence irreversible, is the driving force for the base-catalyzed antioxidant activity.

$$AscH_2 + Pyr \underset{}{\overset{K_{10}}{\rightleftharpoons}} AscH^- + PyrH^+ \qquad (10)$$

$$AscH^- + ROO^{\bullet} \xrightarrow{k_{11}} Asc^{\bullet -} + ROOH \qquad (11)$$

$$Asc^{\bullet -} + PyrH^+ \rightleftharpoons AscH^{\bullet} + Pyr \qquad (12)$$

$$-\left(\frac{d[O_2]}{dt}\right)_{inh} = \frac{k_p[RH]R_i}{k_{11}\sqrt{K_{10}[Pyr][AscH_2]}} \qquad (13)$$

From the retarded oxygen consumption recorded at the various concentrations of pyridine (Fig. 1B), and the measured value of R_i (5.9×10^{-9} M s^{-1}), the value of $k_{11} \times (K_{10})^{1/2}$ could be determined as $(6.5 \pm 1.1) \times 10^3$ M^{-1} s^{-1}. In the case of ascorbyl palmitate ($\mathbf{2H_2}$), experiments performed in the presence of 31 mM pyridine afforded $k_{11} \times (K_{10})^{1/2} = (5.9 \pm 0.9) \times 10^3$ M^{-1} s^{-1}.

When 1% H_2O was replaced with 1% D_2O, the reactivity of $\mathbf{3H_2}$ toward ROO· slightly decreased (Fig. 1B), and the value of $k_{11} \times (K_{10})^{1/2}$ was determined as $(4.7 \pm 1.8) \times 10^3$ M^{-1} s^{-1}. The modest isotope effect of 1.4 (H/D) might, in principle, suggest a different reaction mechanism for the ascorbate anion $\mathbf{3H^-}$ as compared to the neutral species $\mathbf{3H_2}$, not involving the proton transfer in the rate determining step. However it should be noted that the quantity $k_{11} \times (K_{10})^{1/2}$ is in fact the combination of a kinetic and an equilibrium constant that, most likely, are differently affected by deuterium substitution: indeed an inverse isotope effect on K_{10} might partly mask the real isotope effect on k_{11}. Therefore reaction 11 may well proceed by HAT/PCET mechanism. Indeed Warren and Mayer have recently shown that the reaction of $\mathbf{3H^-}$ with 2,4,6-tri-*tert*-butylphenoxyl radical or with 2,6-di-*tert*-butyl-4-methoxyphenoxyl radical or with 2,2,6,6-tetramethylpiperidinoxyl radical, all proceed by PCET mechanism (and have $k_H/k_D \geq 3$),[8a] in line with previous findings in aqueous solutions by Njus and Kelley.[7d,e]

In order to directly measure the reactivity of $\mathbf{3H^-}$ with peroxyl radicals, autoxidations were performed in the presence of the strong base DBU (1,8-diazabicyclo[5.4.0]undec-7-ene; $pK_{a\ MeCN}$ $DBUH^+$ = 24.3) which has been used to quantitatively generate $AscH^-$ anions.[8b] At the micromolar concentrations used in our experiments, DBU did not influence the kinetics of uninhibited autoxidation of styrene. On the other hand, in the presence of equimolar amounts of DBU, compound $\mathbf{3H_2}$ gave a strong inhibition of styrene autoxidation (Fig. 2). From these experiments, the value of k_{inh} for $\mathbf{3H_2}$ + DBU 1 : 1 was measured as $(5.0 \pm 3.3) \times 10^7$ M^{-1} s^{-1}. This rate constant should coincide with k_{11}, so the value of the equilibrium constant for the H^+ transfer between $\mathbf{3H_2}$ and pyridine (K_{10}) can be determined as 1.7×10^{-8}. Surprisingly, DBU did not increase the inhibiting activity of PMHC, although traces of PMHC anion should be present in solution, similarly to what was observed in the case of $\mathbf{3H_2}$ and pyridine ($pK_{a\ MeCN}$ PMHC ≈ 30).[13] The effect of the base was rather that of shortening PMHC inhibition period and increasing the rate of oxygen consumption (Fig. 2), most probably because of the reaction of PMHC anion with O_2 to yield $O_2^{\bullet -}$.

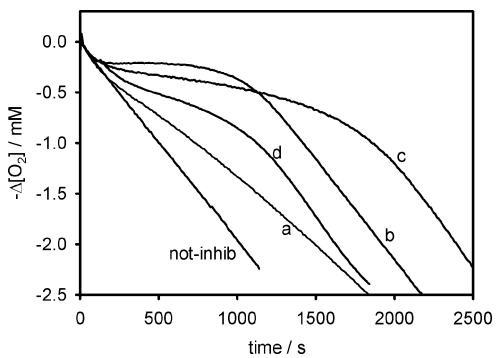


Fig. 2 Oxygen consumption during the autoxidation of styrene (4.3 M), in anhydrous MeCN initiated by AIBN (0.05 M) in the absence of inhibitors and in the presence of: (a) $\mathbf{3H_2}$; (b) $\mathbf{3H_2}$ + DBU 1 : 1; (c) PMHC; (d) PMHC + DBU 1 : 1. $[\mathbf{3H_2}] = 1.2 \times 10^{-5}$ M; [PMHC] = 6.3×10^{-6} M.

Acid catalysis, particularly the effect of acetic acid on the antioxidant activity of $\mathbf{2H_2}$ and $\mathbf{3H_2}$, was also investigated, to explore the effects that carboxylic acids or functions may have on the radical chemistry of vitamin C in lipophilic environments. This interest stems from our recent discovery that both phenolic antioxidants[16] and nitroxides[17] have significantly improved antioxidant profiles in the presence of millimolar amounts of weak organic acids. In Fig. 3 it can be seen that small amounts of acetic acid added to the autoxidating mixture containing 1% H_2O significantly boosted

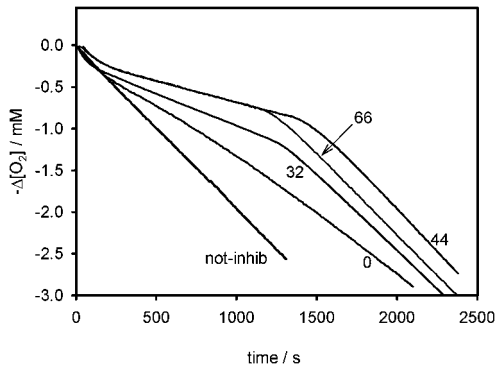


Fig. 3 Oxygen consumption during the autoxidation of styrene (4.3 M) in MeCN initiated by AIBN (0.05 M) in the absence of inhibitors and in the presence of $\mathbf{3H_2}$ (1.2×10^{-5} M) and increasing amounts of acetic acid (mM).

the antioxidant behaviour of $\mathbf{3H_2}$. Similar results (not shown) were also obtained for ascorbyl palmitate ($\mathbf{2H_2}$).

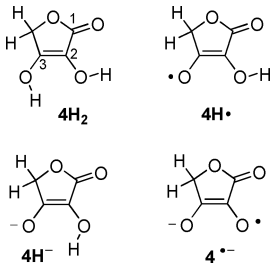
Acid catalyzed enhancement of the antioxidant activity, previously seen in styrene autoxidation inhibited by various phenols, can similarly be explained by considering that traces of protonated peroxyl radicals ($ROOH^{\bullet+}$) are formed upon addition of organic acids. Protonated peroxyls are very strong ET oxidizing agents, able to react at a diffusion controlled rate with electron donors.[16] In line with this proposed mechanism, based on pre-equilibration of peroxyl radicals with the acid (eqn (14)) followed by rapid reaction with ascorbic acid (eqn (15)), the slopes of O_2 consumption during the inhibited periods did not depend on the concentration of $\mathbf{3H_2}$ (see ESI‡), indicating that the rate limiting step is the protonation of the peroxyl radical.

$$CH_3COOH + ROO^{\bullet} \rightleftharpoons CH_3COO^{-} + ROOH^{\bullet+} \quad (14)$$

$$ROOH^{\bullet+} + \mathbf{3H_2} \rightarrow ROOH + \mathbf{3H^{\bullet}} + H^{+} \quad (15)$$

Theoretical calculations

To better understand the behaviour of ascorbic acid derivatives in MeCN, the dissociation enthalpies of the reactive OH bonds, as well as the ability of $AscH_2$ and AscH· to form H-bonds with MeCN, were calculated by computational methods. To do this, a vitamin C model lacking the lateral chain ($\mathbf{4H_2}$)[18] was employed to reduce the calculation time as it has been shown experimentally that $\mathbf{2H_2}$ and $\mathbf{3H_2}$ (which have different substituents in the side chains) have similar reactivity toward free radicals (*vide supra*). The most stable structures (optimized geometries at the B3LYP/6-31+g(d,p) level) for $\mathbf{4H_2}$, its anion $\mathbf{4H^-}$ and the respective radicals are reported in Scheme 1. The BDE(OH) for $\mathbf{4H_2}$ and for $\mathbf{4H^-}$ was calculated at the B3LYP/6-31+g(d,p) level from the difference between the enthalpies of the closed-shell molecules and those of the radicals, by using the BDE(OH) of TEMPOH in benzene as a reference.[19] These values collected in Table 2 are in reasonable agreement with those calculated at the CBS-QB3 level[20] in the gas phase. The BDE(OH) of the α-tocopherol analogue PMHC was also computed as a benchmark, and it was in good agreement with its corrected experimental BDE value of 77.2 kcal mol^{-1}.[21] The results reported in Table 2 show that the BDE(OH) of $\mathbf{4H_2}$ is about 11 kcal mol^{-1} higher than that of $\mathbf{4H^-}$, at both levels of theory.



Scheme 1

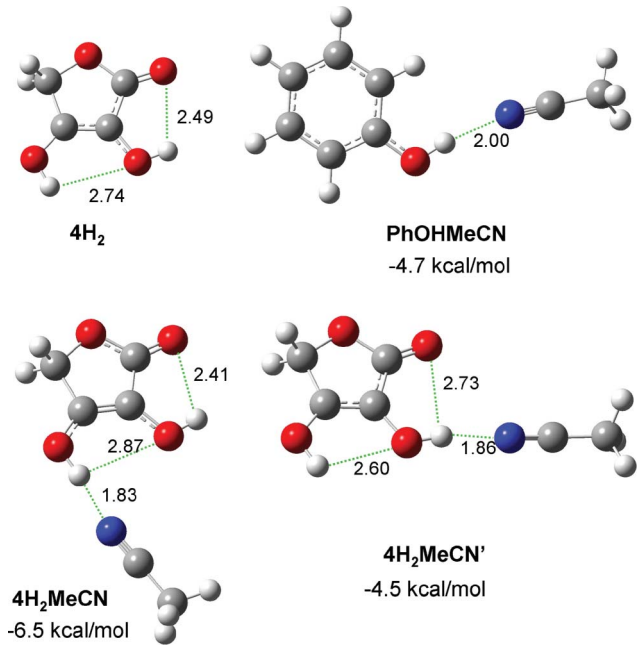
Table 2 Calculated BDE(OH) for the vitamin C analogue $\mathbf{4H_2}$ in the gas phase, in kcal mol^{-1}

	B3LYP/6-31+g(d,p)	CBS-QB3
$\mathbf{4H_2}$	80.1	79.0
$\mathbf{4H^-}$	69.0	67.8
PMHC	77.9	

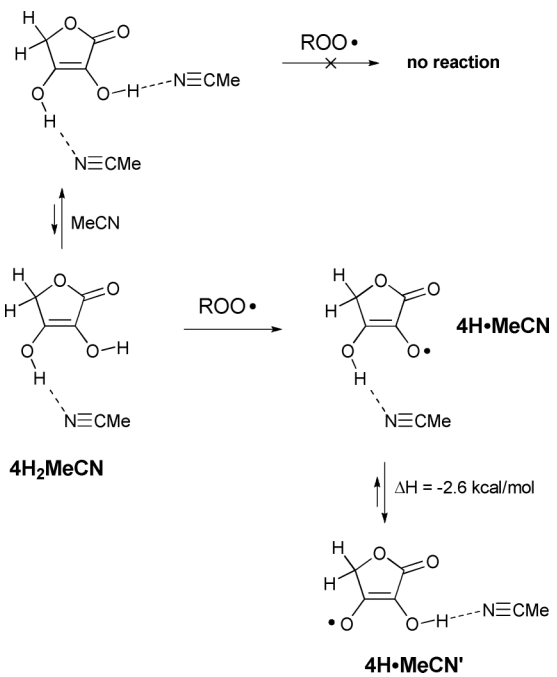
To keep into account solvation, the BDE(OH) values for $\mathbf{4H_2}$ and $\mathbf{4H^-}$ were also estimated in acetonitrile using the polarized continuum model implemented in the Gaussian03 program suite, as detailed in the ESI.‡ Calculations indicated that the BDE(OH) difference between the neutral and anionic form of $\mathbf{4H_2}$ is still 11 kcal mol^{-1}. This BDE (OH) difference is surprisingly much larger than the difference (5.1 kcal mol^{-1}) recently measured for $\mathbf{3H_2}$ using electrochemical cycles,[8a] which prompted further experimental investigation using a different approach (*vide infra*).

To rationalize the behaviour of neutral ascorbic acid derivatives in MeCN solution, the H-atom donation from $\mathbf{4H_2}$ was also investigated by explicitly considering the H-bonding of $\mathbf{4H_2}$ to one MeCN molecule, as shown by Scheme 2. Phenol was taken as a reference compound because it is a well known H-bond donor. Although in $\mathbf{4H_2}$ there are two intramolecular H-bonds (indicated in Scheme 2 by the dotted lines), it is also able to interact with MeCN molecules by forming bifurcated H-bonds.[22] The OH group in the 3 position is the preferential binding site, because it is the most acidic one,[18] and because it forms a weaker intramolecular interaction, as witnessed by the longer OH–O distance. The interaction between the -OH group in the 2 position of $\mathbf{4H_2}$ and MeCN, although weaker by 2 kcal mol^{-1} than that formed by the -OH in the 3 position, is still quite strong, being very similar to that formed between phenol and MeCN (compare $\mathbf{4H_2MeCN'}$ in Scheme 2 with **PhOHMeCN**).

In MeCN solution $\mathbf{4H_2}$ is expected to bind two MeCN molecules, the first one very tightly on the -OH in the 3 position, the second one less tightly on the -OH in the 2 position. The reactivity of $\mathbf{4H_2}$ in MeCN solution toward ROO· radicals can therefore be ascribed to the species $\mathbf{4H_2MeCN}$, as shown in Scheme 3.[23] The BDE for the free OH of $\mathbf{4H_2MeCN}$ calculated at the B3LYP/6-31+g(d,p) level is 81.8 kcal mol^{-1}, that is 1.7 kcal mol^{-1} larger than the calculated value for the cleavage of the O–H group in the 3 position of $\mathbf{4H_2}$ in the gas phase. The radical $\mathbf{4H{\cdot}MeCN}$ then transforms into the more stable isomer $\mathbf{4H{\cdot}MeCN'}$.



Scheme 2 Optimized geometries (H-bond lengths are in Å) and enthalpy change for the formation of the H-bond between the analogue of vit. C or phenol and acetonitrile, at the B3LYP/6-31+g(d,p) level.

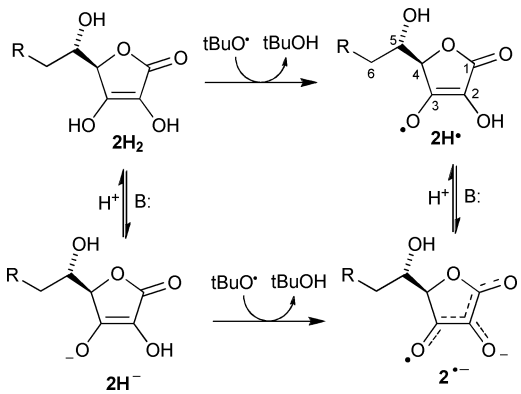


Scheme 3 Reaction of $\mathbf{4H_2}$ with peroxyl radicals in the presence of MeCN.

EPR and experimental thermodynamics

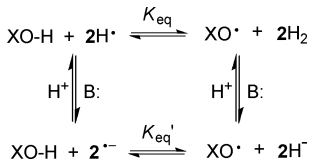
In order to find experimental confirmation of our computational efforts and to rationalize the kinetic behaviour recorded for ascorbic acid derivatives, we subjected ascorbyl palmitate ($\mathbf{2H_2}$) to a detailed EPR investigation, using *t*BuOH as a non-aqueous medium due to higher solubility and lower dielectric constant as compared to acetonitrile.

When a solution of $\mathbf{2H_2}$ in dry *t*BuOH containing 5–15% di-*tert*-butylperoxide (*t*BuO*t*Bu) is irradiated at 298 K inside the cavity of an EPR spectrometer, the resulting signal is a broad (line-width ~0.4 G) doublet centered at g = 2.0054 with HSC of *ca.* 1.7 G (see ESI‡), which can be attributed to the radical anion $\mathbf{2^{\bullet-}}$ by comparison with literature data recorded for $\mathbf{1^{\bullet-}}$ in water (HSC = 1.76 G; g = 2.0052)[24] and for $\mathbf{3^{\bullet-}}$ in MeCN (HSC = 1.91 G; g = 2.0059).[8a] Such a relatively persistent species originates by rapid deprotonation of the acidic short-lived radical $\mathbf{2H^{\bullet}}$ (pK_a ~14 in MeCN and ~0 in water),[8] formed by H-atom transfer to *t*BuO• radicals, as depicted in Scheme 4. Upon addition of excess trifluoroacetic or hydrochloric acid, the EPR spectrum simplifies to a broad (LW ~0.5 G) singlet at g = 2.0050, indicative of lesser spin delocalization on oxygen atoms, assigned to the neutral species $\mathbf{2H^{\bullet}}$. Upon moderate heating the signal resolves into a doublet with HSC of 0.45 G (Fig. 4A) due to coupling at H(4), similarly to the spectrum of ascorbyl radical in water/$HClO_4$.[24] Conversely, addition of 1 equivalent of the strong base DBU (before irradiation) quantitatively yields the palmitoyl ascorbate anion $\mathbf{2H^-}$ that is converted to $\mathbf{2^{\bullet-}}$ upon H-transfer to *t*BuO•. As shown in Fig. 4B, besides coupling to H(4) with HSC of 1.85 G, upon heating the spectrum reveals hyperfine interaction with H(6), HSCs 0.18 G and 0.16 G, while unresolved coupling with H(5) is responsible for residual line broadening (see also ESI‡).



Scheme 4

Following a well-established procedure developed in our group,[21] we used EPR spectroscopy to measure the O–H bond dissociation enthalpy in $\mathbf{2H_2}$ and $\mathbf{2H^-}$ by equilibration of the species under investigation with a suitable reference compound (XO–H), in the presence of photolytically generated *t*BuO• radicals in the spectrometer's cavity. Recorded EPR spectra are due to the superimposition of signals from the two equilibrating radical species (Req-EPR) and afford their relative concentrations by numerical fitting. Under appropriate setting so to avoid significant consumption of the starting compounds during the experiment, the equilibrium constant, K_{eq}, can be determined (Scheme 5), and,



Scheme 5

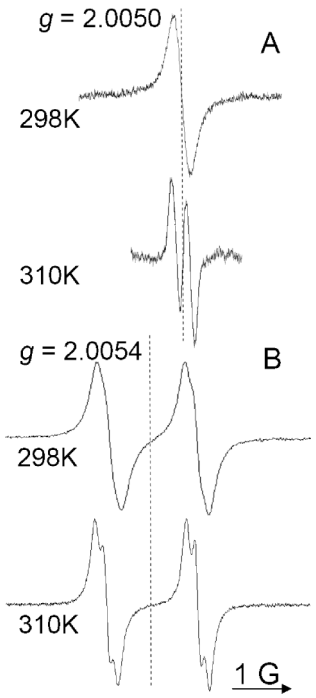


Fig. 4 X-Band EPR spectra recorded at 298 K and 310 K during UV-irradiation of a solution of 25 mM ascorbyl palmitate (**2H$_2$**) in *tert*-butanol containing 10% (v/v) di-*tert*-butylperoxide and: (A) 5% (v/v) trifluoroacetic acid; or (B) 25 mM 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 1.0 equiv.).

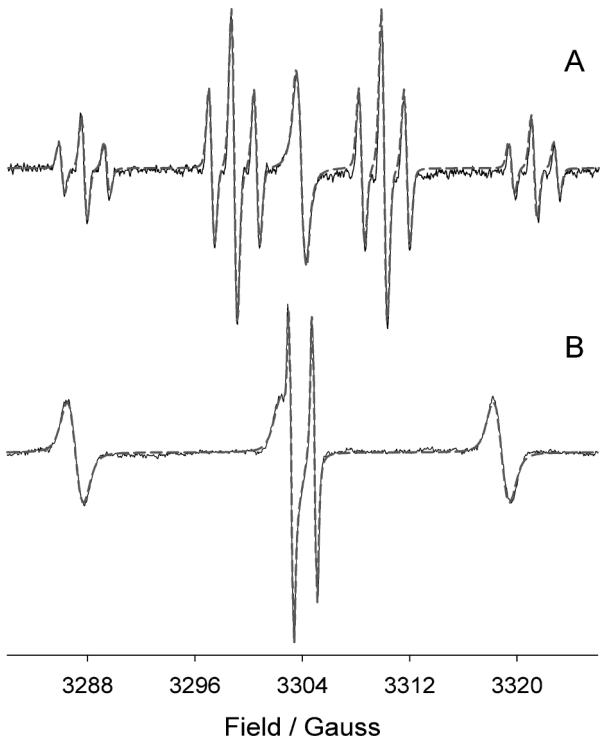


Fig. 5 Simulated (---) and experimental (—) spectra recorded at 298 K during UV-irradiation of a mixture of 35 mM ascorbyl palmitate (**2H$_2$**) in *t*BuOH containing 10% (v/v) *t*BuOO*t*Bu and: (A) 17 mM BHT, and 5% (v/v) conc. HCl; or (B) 5 mM TEMPOH and 35 mM DBU (1.0 equiv.).

under the assumption that the entropy change is negligible,[25] it yields the enthalpy change associated with the H-atom exchange (ΔBDE), according to eqn (16).

$$\Delta\mathrm{BDE} = \mathrm{BDE}(\mathbf{2H_2}) - \mathrm{BDE}(\mathrm{XOH}) \sim \Delta H - T\Delta S = -RT\ln K_{eq} \quad (16)$$

UV-irradiation of a mixture of ascorbyl palmitate with a reference phenol, such as 2,6-di-*tert*-butyl-4-methylphenol (BHT) or 2,6-di-*tert*-butyl-4-methoxyphenol (BHA), in *t*BuOH/*t*BuOO*t*Bu solvent mixture, produced equilibrium radical concentrations which were largely affected by the addition of weak bases such as pyridine or triethylamine (see ESI‡), This can be explained on the basis of Scheme 5: weak bases (B:) like pyridine, are expected to deprotonate more easily the very acidic **2H$^{\bullet}$**, to form **2$^{\bullet-}$** (the only species visible in Req-EPR spectra), than **2H$_2$**,[26] thereby shifting the equilibrium and producing an apparent decrease in the BDE(OH) of **2H$_2$**. The solvent itself, unable to deprotonate **2H$_2$**, will allow for some deprotonation of the acidic **2H$^{\bullet}$**, decreasing the apparent BDE(OH). Therefore, to distinctively assess the BDE(OH) of **2H$_2$** and **2H$^-$**, Req-EPR measurements were performed in the presence of excess HCl or of 1 equivalent DBU, respectively. BHT and *N*-hydroxy-2,2,6,6-tetramethyl-piperidine (TEMPOH) were chosen respectively as reference compounds for **2H$_2$** and **2H$^-$**, and representative Req-EPR spectra are displayed in Fig. 5. The corrected[21c] BDE(OH) of reference BHT is 79.9 kcal mol^{-1} in benzene[21a] and this value should be corrected for solvent *t*BuOH. Due to the modest α_2^H and β_2^H values for BHT,[27] its H-bonding interaction with *t*BuOH is negligible. Regarding the corresponding phenoxyl radical, its HSCs in *t*BuOH (11.20 G and 1.67 G) are identical within experimental error to those measured in benzene,[21a] indicating similar interactions with the solvent. The BDE(OH) of BHT in *t*BuOH is therefore expected to be nearly identical to that measured in benzene (79.9 kcal mol^{-1}). In the case of TEMPOH, its α_2^H is 0.39[26] while the β_2^H of TEMPO· is 0.46,[28] so the H-bonding stabilization (Δ*G*) of the parent hydroxylamine in *t*BuOH is –0.4 kcal mol^{-1}, while that of the radical is negligible. The BDE(OH) of TEMPOH in *t*BuOH can therefore be corrected to 70.0 kcal mol^{-1}. The resulting BDE(OH) values for **2H$_2$** and **2H$^-$** are collected in Table 3.

The BDE(OH) value measured here for **2H$^-$** in *t*BuOH is in fair agreement with the value previously reported by Warren and Mayer for **3H$^-$** in MeCN (70 ± 1 kcal mol^{-1}),[8a] as the difference (*ca.* 2 kcal mol^{-1}) is partly to be attributed to specific solvation effects associated with higher H-bonding ability of *t*BuOH compared to MeCN.[8b] Conversely, the Req-EPR BDE(OH) value measured here for undissociated **2H$_2$** largely exceeds (by about 6 kcal mol^{-1}) the value determined, in the same study, for the analogous **3H$_2$** from electrochemical cycles (75 ± 1 kcal mol^{-1}),[8a] a difference that can hardly be attributed to solvation. Importantly, our measured BDE difference between the neutral and the anionic form of **2H$_2$** in *t*BuOH is 9 ± 1 kcal mol^{-1}, which favourably compares to the ΔBDE(OH) of 11 kcal mol^{-1} calculated for **4H$_2$** in the gas phase

Table 3 Req-EPR equilibrium constants and corresponding BDE(OH) for ascorbyl palmitate (**2H$_2$**) and its mono anion (**2H$^-$**) in *tert*-butanol at 298 K. Errors correspond to ±SD and do not include error in the reference compound

Compound	Reference	K_{eq}	BDE (kcal mol^{-1})
2H$_2$	BHT	5.8 ± 2.8	81.0 ± 0.4
2H$^-$	TEMPOH	41 ± 19	72.2 ± 0.4

or in MeCN. Furthermore our experimental ΔBDE(OH) fully justifies the recorded very large difference in antioxidant activity between $AscH_2$ and $AscH^-$ forms of ascorbic acid derivatives.

As a final note, we wish to compare our results with some of those reported for aqueous solutions. The BDE(OH) values for **1H$_2$** and **1H$^-$** in water, obtained by electrochemical cycles, have been reported as 78.0 and 71.8 kcal mol^{-1} respectively.[7d,13] The rate constants for the reaction between ROO· radicals and ascorbic acid in the undissociated or in the dissociated forms have been estimated as 3×10^5 and 1.7×10^6 M^{-1} s^{-1} for R = CH_3,[7b] and 1.6×10^4 and $\approx 10^7$ M^{-1} s^{-1} for R = H.[7a] Our results confirm the large reactivity difference between **1H$_2$** and **1H$^-$** found in the studies with HOO· radicals, and suggest that the BDE(OH) of neutral ascorbic acid obtained by electrochemical cycles may have been significantly underestimated.

Conclusions

Our experimental and theoretical results on lipophilic ascorbic acid derivatives give important insight into the chemistry of vitamin C, and, possibly into its biological role. The antioxidant activity of ascorbic acid derivatives in non-aqueous media is due to formal hydrogen atom transfer (HAT or PCET) reaction with chain-carrying peroxyl radicals, which experiences dramatic basic catalysis and largely depends on the protonation state of ascorbate. Indeed the neutral $AscH_2$ and anionic $AscH^-$ forms have O–H bond dissociation enthalpies that differ by about 10 kcal mol^{-1} reflecting in a difference in reactivity of about 3 orders of magnitude. Interestingly the antioxidant activity of ascorbic acid is also sensitive to catalysis by carboxylic acids, which might have interesting biological implications in the case of localization in lipophilic compartments close to proteins' acidic (or basic) side chains.

The results obtained in this study will complement the few available data of the behaviour and properties of ascorbic acid in non-aquous media and the vast body of knowledge in water, creating a hopefully clearer picture of its chemistry and biology.

Experimental section

Materials

Anhydrous solvents (MeCN and *t*BuOH) were of the highest grade commercially available (Aldrich–Fluka) and were used as received. Ascorbyl palmitate (**2H$_2$**), 5,6-isopropylidene-L-ascorbic acid (**3H$_2$**), 2,2,5,7,8-pentamethyl -6-chromanol (PMHC), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) were purchased from Sigma-Aldrich and used without further purification. 2,6-Di-*tert*-butyl-4-methylphenol (BHT) and 2,6-di-*tert*-butyl-4-methoxyphenol (BHA) (Aldrich) were recrystallized from hexane. 2,2′-Azodiisobutyronitrile (AIBN) was recrystallized from hexane and stored at –20 °C. Cumene and styrene (Fluka) were distilled under reduced pressure and percolated twice through silica and alumina prior to use. 1-Hydroxy-2,2,6,6-tetramethylpiperidine (TEMPOH) was available from previous studies.[17] Solutions were prepared fresh immediately prior to use.

Autoxidation studies

The chain-breaking antioxidant activity of the title compounds was evaluated by studying the inhibition of the thermally initiated autoxidation of either styrene or cumene (RH) in acetonitrile.

In order to have good chain-length during the inhibited period, the oxidizable substrate was used at 50% by volume of the final solutions, therefore the solvent (acetonitrile) was only 50%. Autoxidation experiments were performed in a two-channel oxygen-uptake apparatus, based on a Validyne DP 15 differential pressure transducer, already described elsewhere.[10] The entire apparatus was immersed in a thermostatted bath to ensure a constant temperature within ±0.1 °C. In a typical experiment, an air-saturated acetonitrile solution of styrene or cumene containing AIBN (0.05 M) was equilibrated with an identical reference solution containing also an excess of PMHC (from 1×10^{-3} to 1×10^{-2} M) in the same solvent at 30 °C. When a constant oxygen consumption was observed, a concentrated solution of the antioxidant was injected into the sample flask, and the oxygen consumption was measured, after calibration of the apparatus, from the differential pressure recorded with time between the two channels. From the slope of the oxygen consumption during the inhibited period (R_{inh}), k_{inh} values were obtained by using eqn (17),[10] where R_0 is the rate of oxygen consumption in the absence of antioxidants, R_i is the initiation rate, $2k_t$ is the bimolecular termination rate constant of styrene or cumene (4.2×10^7 and 4.6×10^4 M^{-1} s^{-1} respectively)[9,29] and n is the stoichiometric coefficient of the antioxidant. The coefficient n was determined experimentally from the length of the inhibited period (τ) by eqn (18).[9]

$$\frac{R_0}{R_{inh}} - \frac{R_{inh}}{R_0} = \frac{nk_{inh}[\mathrm{AH}]}{\sqrt{2k_t R_i}} \quad (17)$$

$$n = \frac{R_i \tau}{[\mathrm{AH}]} \quad (18)$$

Theoretical calculations

Geometry optimization and frequencies were computed in the gas phase at the B3LYP/6-31+g(d,p) level using Gaussian03,[30] and stationary points were confirmed by checking the absence of imaginary frequencies. Frequencies were scaled by 0.9806.[31] BDE values were obtained by using the isodesmic approach,[32] that consists of calculating the ΔBDE between the investigated compounds and TEMPOH, and by adding this value to the known experimental BDE(OH) of TEMPOH in benzene (69.6 kcal mol^{-1}). The BDE(OH) were also computed at the CBS-QB3 level,[20] by subtracting the enthalpy of the reactants to the sum of those of the radicals and of H·. The enthalpy change for the H-bond formation in the gas-phase was calculated from the differences between the enthalpy of the products and those of the reactants.

EPR spectroscopy

Spectra were recorded in 4 mm I.D. quartz tubes at 298 K on a Bruker Elexsys 500 X-band spectrometer equipped with a Bruker VT-1000 variable temperature unit. Spectral analysis was optimized by means of computer simulations and subjected to a

least-squares fitting procedure based on the systematic application of the Monte Carlo method, available in WinESR Commander V.1.0 software, developed by Prof. Marco Lucarini (University of Bologna). Spectra were recorded in deoxygenated *t*BuOH solutions containing 5–10%(v/v) *t*BuOO*t*Bu at 298 K or 310 K, by irradiating the samples with a 500 W high-pressure Hg lamp and using calibrated metal sectors to modulate the intensity of irradiation. Measured *g*-factors were corrected using those of reference compounds: BHT, $g = 2.0046$ in *t*BuOH (HSCs 11.20 G and 1.67 G); and TEMPO $g = 2.0061$ in *t*BuOH (HSC 15.89 G). In the case of $\mathbf{2H}^-$, strong EPR signals were obtained already after short (5 s) irradiation of the solution.

Thermodynamic measurements

Deoxygenated *t*BuOH solutions containing ascorbyl palmitate (20–100 mM), BHT or TEMPOH as reference compound (10–100 mM), di-*tert*-butyl peroxide (10%, v/v) and either 10 μL of concentrated HCl (or neat CF_3COOH), or 1 equivalent (with respect to ascorbyl palmitate) of DBU was sealed under nitrogen in a Suprasil quartz EPR tube placed inside the thermostatted (298 K) cavity the EPR spectrometer. In order to prepare and transfer *t*BuOH solutions, they and *t*BuOH itself were maintained in the liquid form by incubation in a thermostatted oven at 30 °C, until they were mixed in final solutions, which where thermostatted directly within the EPR cavity at the desired temperature. Photolysis was carried out in the cavity by using the unfocused light from a high-pressure mercury lamp. The temperature was monitored before and after each run with a copper-constantan thermocouple. The molar ratio of the two equilibrating radicals was obtained from the EPR spectra by computer simulation and interactive least square fitting. It was then used to determine the equilibrium constant $K\text{eq} = [\text{AscH}]_0[\text{Ref}\cdot]/[\text{RefH}]_0[\text{Asc}\cdot]$, where the subscript zero refers to the initial concentrations. Initial concentrations were chosen so as to avoid significant reagent consumption during the experiment. To confirm that the two radicals were at their equilibrium under the experimental conditions, different initial absolute concentrations of the equilibrating species and different light intensities were investigated.

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