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Radical Reactions

Peroxyl Radical Reactions in Water Solution: A Gym for Proton-Coupled Electron-Transfer Theories

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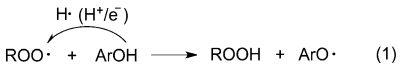
Abstract: The reactions of alkylperoxyl radicals with phenols have remained difficult to investigate in water. We describe herein a simple and reliable method based on the inhibited autoxidation of water/THF mixtures, which we calibrated against pulse radiolysis. With this method we measured the rate constants k_{inh} for the reactions of 2-tetrahydrofuranylperoxyl radicals with reference compounds: urate, ascorbate, ferrocenes, 2,2,5,7,8-pentamethyl-6-chromanol, Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-acetic acid, 2,6-di-*tert*-butyl-4-methoxyphenol, 4-methoxyphenol, catechol and 3,5-di-*tert*-butylcatechol. The role of pH was investigated: the value of k_{inh} for Trolox and 4-methoxyphenol increased 11- and 50-fold from pH 2.1 to 12, respectively, which indicate the occurrence of a SPLET-like mechanism. H(D) kinetic isotope effects combined with pH and solvent effects suggest that different types of proton-coupled electron transfer (PCET) mechanisms are involved in water: less electron-rich phenols react at low pH by concerted electron-proton transfer (EPT) to the peroxyl radical, whereas more electron-rich phenols and phenoxide anions react by multi-site EPT in which water acts as proton relay.

Introduction

Alkylperoxyl radicals are of fundamental importance in oxidative processes as they are key intermediates in the autoxidation of organic materials, a free-radical chain reaction with major implications in the oxidative transformation of biomolecules in aerobic organisms.[1–3]

The reactions of peroxyl radicals with phenols (ArOH) like tyrosine or with common radical-trapping antioxidants (RTAs) occur by formal transfer of a hydrogen atom, which is better described as a proton-coupled electron transfer (PCET) in which H^+/e^- are transferred to the peroxyl radical (Scheme 1).[4] Most recent theoretical and experimental studies converge to indicate that in non-polar organic solution the reaction is concerted, that is, $1H^+$ and $1e^-$ move, in a single kinetic step, from different orbitals on ArOH to different orbitals on ROO$^\bullet$;[4,5] therefore, it complies to the original definition of PCET given by Meyer and co-workers[6] and differs from the hydrogen-atom transfer (HAT) mechanism in which H^+/e^- are transferred concertedly between the same orbitals (or bonds).[7] Over the years, the term PCET has broadened its meaning to include single- and multi-step mechanisms involving the transfer of one or more H^+ and e^-;[7,8] therefore, more specific terms like concerted electron-proton transfer (EPT)[7] or concerted proton-electron transfer (CPET)[8,9] have been proposed to recapture the original meaning of PCET. Hence, EPT (CPET) and HAT are both examples of PCET.[10]



Scheme 1. Reaction of phenols with alkylperoxyl radical.

At variance with apolar media, in protic media other PCET mechanisms arising from the combination of electron-transfer (ET) and proton-transfer (PT) processes might become relevant for the reactions of phenols.[8] We have recently shown that their reactions are accelerated in polar organic solvents (MeCN or EtCN) containing protic acids, in which it was suggested that the reactions proceed by a stepwise mechanism involving rate-controlling ET to the protonated peroxyl radical.[11] Litwinienko and Ingold and also Foti and co-workers independently showed that, in alcohols, acidic phenols are oxidised by the 2,2-diphenylpicrylhydrazyl radical (DPPH$^\bullet$) at a much faster rate than expected from an EPT (CPET), following a stepwise PT–ET pathway named sequential proton loss electron transfer (SPLET), which involves the solvent as proton acceptor.[12] The key role of the solvent as proton acceptor was also highlighted by Savéant and co-workers in the electrochemical oxidation of phenols in water, although they described it as a CPET.[9] Somewhat similarly, the oxidation of tyrosine in the active site of

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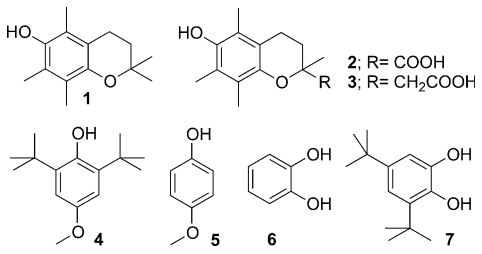
some enzymes has been described as a separated CPET (also called multi-site EPT, MS-EPT[7]) in which e^- moves from ArOH to the oxidant (e.g., chlorophyll) and H^+ is transferred in a concerted fashion to a different proton acceptor (e.g., a histidine residue).[8,13]

Which of the above mechanisms would be operating when the oxidising species are alkylperoxyl radicals in water? Arguably, the vast majority of phenols and RTAs in nature are water-soluble, yet remarkably little of their chemistry with peroxyl radicals is known in water, mostly owing to the limited availability of practical methods of investigation.

The majority of kinetic data currently available has been obtained by pulse radiolysis, in which peroxyl radicals are generated through a complex radical cascade, by irradiating with accelerated electrons an aqueous solution containing organic substrates, such as DMSO.[14] The difficulty in controlling the radical species generated by high-energy irradiation limits the versatility of this technique, which over the years offered mainly the kinetics of reactions with methylperoxyl and trichloromethylperoxyl radicals (these last being too reactive to be representative of alkylperoxyl radicals).[15] Furthermore, the reactions of alkylperoxyl radicals with relevant biomolecules are often too slow to be followed accurately by this technique, and typically only rate constants higher than $10^5\,M^{-1}s^{-1}$ are reported.

The best-established method for gaining kinetic data on the reactions of RTAs or biomolecules with peroxyl radicals is to study their perturbation of the controlled autoxidation of a standard oxidisable substrate, which can conveniently be done by following the kinetics of oxygen consumption.[2,11,15] At variance with the good availability of suitable substrates for investigation in organic solution, in water this method is limited by the poor solubility of typical oxidisable substrates, which therefore have to be incorporated into micelles or liposomes.[16] Unfortunately, in such heterogeneous systems the measured rate constants reflect the rate of exchange of reactants between suspended particles,[17] and the reactivity of different biomolecules mostly depends on their lipophilicity.[18]

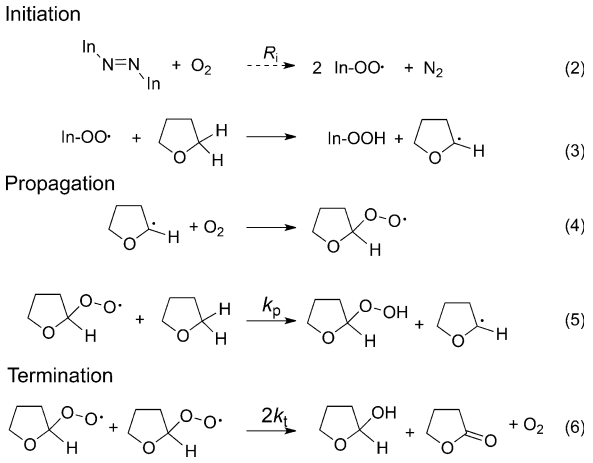
With the aim of clarifying the kinetics and mechanisms involved in the reactions of alkylperoxyl radicals with biologically relevant molecules in homogenous water solution, we carefully standardised a simple and versatile method based on the inhibited autoxidation of THF in water and applied the method to investigate the reactions of phenols and peroxyl radicals for the representative set of phenolic compounds **1**–**7**. We will show that the reactions occur by different PCET mechanisms in water. Most notably, electron-poorer phenols react by EPT (CPET), similarly to the mechanism known in organic solvents, whereas electron-richer phenols react by multi-site EPT (separated CPET), in which water acts as proton relay in the rate-determining step, a mechanism that is reminiscent of the chemistry of tyrosine in some radical enzymes. Our findings expand the current understanding of this fundamental reaction in water and have important implications for the biochemistry of peroxyl radicals.



Results and Discussion

THF as the oxidisable substrate for autoxidations in water

Searching for a suitable oxidisable substrate that would have sufficient solubility in water, it occurred to us that tetrahydrofuran (THF) presents most of the ideal features: 1) it is miscible with water in any ratio; 2) it is non-ionic, so to avoid interference in the kinetics by ionic interactions; 3) it is readily available in high purity; 4) it undergoes rapid autoxidation in the presence of air. Its autoxidation proceeds by hydrogen abstraction from the α position as exemplified in reactions 2–6 in Scheme 2.



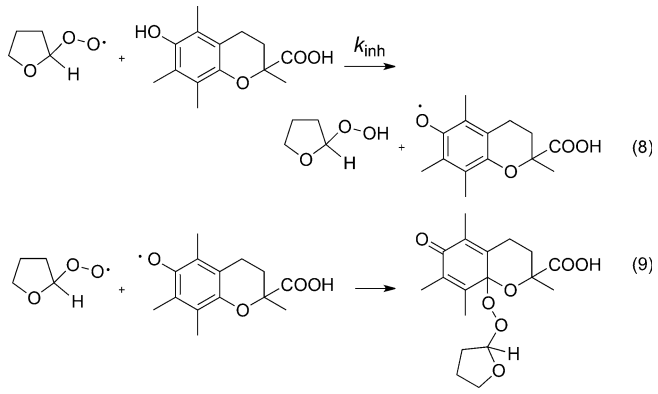
Scheme 2. Main reactions involved in the radical-initiated autoxidation of THF.

The chain reaction kinetics of THF in the neat form (no solvent) was first investigated in detail by Howard and Ingold and reported to follow the typical radical-initiated autoxidation kinetics represented by Equation (7), in which R_i is the rate of initiation. The rate constants for chain propagation and termination were determined to be, respectively, $k_p = 4.3\,M^{-1}s^{-1}$ and $2k_t = 3.1\times10^7\,M^{-1}s^{-1}$ (at 30 °C), thereby affording an oxidisability value $k_p/(2k_t)^{1/2}$ of $7.8\times10^{-4}\,M^{-1/2}s^{-1/2}$.[19] On the basis of these initial inputs we studied the autoxidation of water/THF mixtures by monitoring the rate of oxygen consumption with a differential oxygen uptake apparatus, according to well-established practice.[2,11,15,19] For any water/THF ratio in the range 7:1 to 1:1, we recorded well-behaved linear traces of oxygen consumption, as dictated by Equation (7),[19] however, the ratio 3:1 was preferred as it allows good solubility of inorganic buf-

fers to control the pH along with a convenient rate of O_2 uptake.

$$-\frac{d[O_2]}{dt} = \frac{k_p}{\sqrt{2k_t}}[THF]\sqrt{R_i} \quad (7)$$

Examples of typical oxygen uptake plots recorded in the absence and presence of Trolox (**2**) as reference inhibitor (Scheme 3) are displayed in Figure 1.



Scheme 3. Inhibition of THF autoxidation by Trolox (**2**).

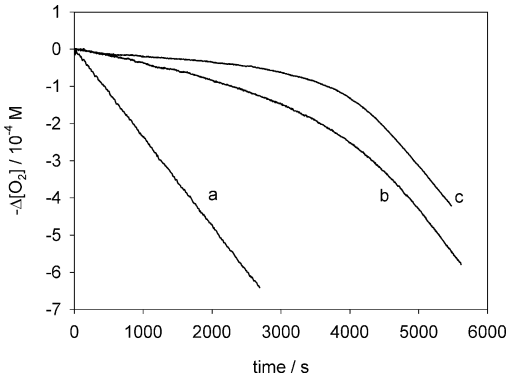


Figure 1. Oxygen consumption during the autoxidation of THF (3.1 M) initiated by AAPH (50 mM) at 30 °C in buffered water without inhibitor at pH 7.4 (a) or in the presence of Trolox (**2**; 4.0×10^{-5} M) at pH 2.1 (b) or pH 7.4 (c).

Effect of pH on radical-chain initiation by azo compounds

The autoxidation reaction was initiated at 30 °C by using the positively charged azo initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and, for comparison, also by using the negatively charged 4,4′-azobis(4-cyanovaleric acid) (ABCV), which was used as the sodium salt after titration with NaOH.

The rate of initiation (R_i) was determined by measuring the length of the inhibition period (τ) during the autoxidation of THF in the presence of Trolox (**2**) as reference antioxidant according to Equation (10),[2,15] in which n is the number of ROO$^\bullet$ radicals trapped by each antioxidant (AH) molecule, the stoichiometric factor, which is known to be 2 for Trolox,[15] this being consistent with the inhibiting mechanism shown in Scheme 3.

$$R_i = \frac{n[AH]}{\tau} \quad (10)$$

Measurements were performed in buffered water at pH 2.1, 7.4 and 12. All buffers were based on phosphoric acid and phosphate salts, as detailed in the Experimental Section, so to avoid buffers containing potentially oxidisable organic molecules.

Figure 2 shows that R_i is linearly dependent on the concentration of the azo initiators. For both AAPH and ABCV as the initiator, R_i was approximately constant on increasing the pH of the solution. This result differs from what is reported in the literature for initiation by AAPH in micelles. In negatively charged sodium dodecylsulfate (SDS) micelles, at pH 7 or 12, the value of R_i was found to be two- to three-fold lower than that at pH 4.[20] On the other hand, in uncharged Triton X-100 micelles, R_i was reported to increase up to 10-fold upon changing the pH from 5 to 9.[21] Instead, in our homogeneous system, only at pH 12 were the values of R_i with AAPH found slightly lower than those recorded at lower pH values, as shown in Figure 2. The slightly lower R_i at pH 12 can be explained as due to the occurrence of partial hydrolysis of the amidinium group of AAPH, which is relevant in basic media and leads to the formation of an amide derivative that generates radicals more slowly than AAPH.[22] Therefore, the anionic azo initiator ABCV was tested as a possible substitute for AAPH at basic pH. Similarly to AAPH, initiation by ABCV was well behaved and, indeed, the values of R_i recorded at basic pH were superimposable on those determined at pH 7.4. However, from Figure 2 it can be observed that ABCV is a much slower initia-

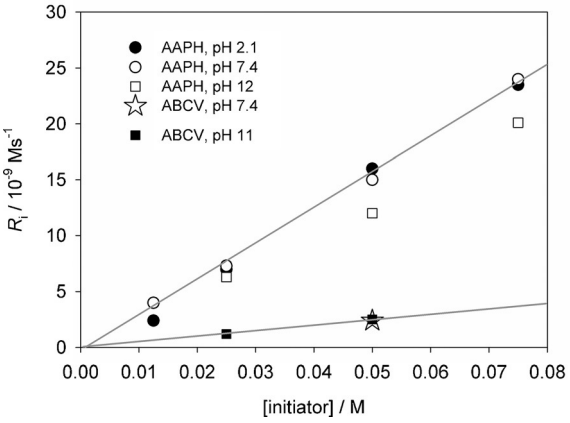
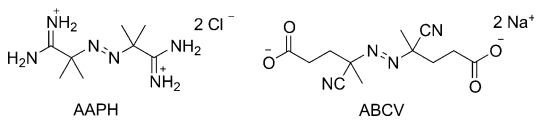


Figure 2. Initiation rate (R_i) as a function of the concentration of the azo initiators AAPH and ABCV in buffered water at 30 °C with THF (25% v/v). Lines represent the linear regression for AAPH at pH 7.4 and ABCV at pH 11.



tor than AAPH, so that concentrations six-fold larger are needed to achieve the same R_i. This made its use less practical and AAPH was preferred in our studies.

Does the autoxidation of THF in water follow the kinetics of a radical chain?

The oxidisability ($k_p/(2k_t)^{1/2}$) of a "well-behaved" oxidisable substrate is expected to be independent of the experimental conditions at a given temperature. Therefore, we determined this value, according to Equation (7), for THF in buffered water at pH 2.1, 7.4 and 12.0 at 30 °C under a variety of settings. The results displayed in Figure 3 show that $k_p/(2k_t)^{1/2}$ is indeed constant (within experimental error) both when changing R_i (Figure 3A) and when changing the concentration of THF (Figure 3B). This demonstrates that THF autoxidation in buffered water is a chain mechanism that follows the same kinetic behaviour that is well established for other oxidisable materials in organic solvents. The recorded chain length was in the range of 15 to 60 under the tested settings. As expected, the oxidisability remains constant when changing the pH (see Table 1). The values recorded in water (average $(5.9\pm0.6)\times10^{-4}$ M$^{-1/2}$ s$^{-1/2}$) are slightly lower than the value reported for the autoxidation of THF in neat form, which, however, is identical to the value we measured in organic solution (PhCl) for comparison ($(7.9\pm0.4)\times10^{-4}$ M$^{-1/2}$ s$^{-1/2}$).

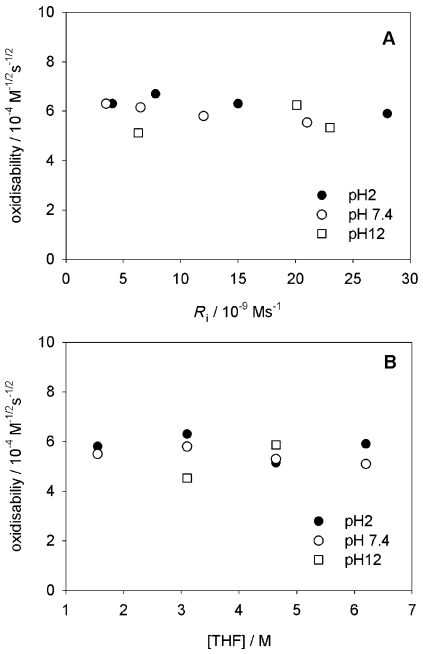


Figure 3. A) Plot of the oxidisability of THF against R_i ([THF] = 3.1 M). B) Plot of the oxidisability of THF against its concentration, with [AAPH] = 0.05 M. Measurements in buffered water at 30 °C.

Table 1. Kinetic parameters for the autoxidation of THF at 30 °C.[a,b]

Solvent	k_p [M^{-1} s^{-1}]	$2k_t$ [10^7 M^{-1} s^{-1}]	$k_p/\sqrt{2k_t}$ [10^{-4} M$^{-1/2}$ s$^{-1/2}$]
water pH 2.1			6.0 ± 0.6
water pH 7.4			5.9 ± 0.5
water pH 12			5.8 ± 0.5
av. (pH 2.1–12)	4.8 ± 0.6	6.6	5.9 ± 0.6
chlorobenzene			7.9 ± 0.4
neat THF[c]	4.3	3.1	7.8

[a] Oxidisability (± SD) of THF measured in water at various pH, in chlorobenzene or in the neat form along with the corresponding rate constants for chain propagation (k_p) and termination ($2k_t$). [b] Unless otherwise noted, the reaction mixture contained 3:1 (v/v) solvent/THF. [c] Data in the absence of solvent from ref. [19].

Determination of k_p and $2k_t$ for THF in water

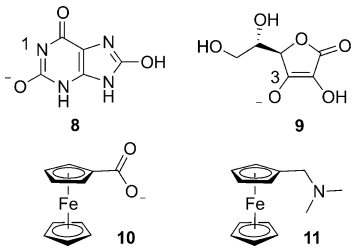
Because the recorded small difference in oxidisability could, in principle, be due to the variation of k_p, $2k_t$ or both, we independently determined the value of k_p for THF by measuring the oxygen consumption in the presence of a reference inhibitor for which the rate constant for trapping peroxyl radicals ($k_{inh,}$) is known. The kinetic data were processed by using Equation (11), which is the integrated form of the well-known Equation (12),[15] in which AH is the inhibitor.

$$\Delta[O_2] = -\left(k_p[THF]/k_{inh}\right)\ln(1 - t/\tau) \quad (11)$$

$$-\frac{d[O_2]}{dt} = \frac{k_p[THF]R_i}{nk_{inh}[AH]} \quad (12)$$

From the limited suitable kinetic data available in water, we selected as reference the reactions of urate at pH 7.3 and of ascorbate at pH 7.0, both with MeOO$^\bullet$ radicals, measured by pulse radiolysis, respectively, by Alfassi et al.[23] and Jovanovic et al.[24]

Uric acid has $pK_{a1} = 5.4$ and $pK_{a2} = 9.8$ in water, therefore at pH 7.3 it exists predominantly in the monoanionic (urate) form **8** due to deprotonation at the 1-position.[25] Similarly, ascorbic acid has $pK_{a1} = 4.1$ and $pK_{a2} = 11.4$ in water, and therefore at pH 7.0 it exists predominantly in the monoanionic (ascorbate) form **9** due to deprotonation of the OH at the 3-position.[8,26]



Reference values of k_{inh} at 30 °C were determined for **8** and **9** from the reported Arrhenius data[23,24] to be 1.6×10^5 and 2.0×10^6 M^{-1} s^{-1}, respectively, thereby falling in the optimal operative range for our kinetic measurements (10^3–10^6 M^{-1} s^{-1}). Two additional compounds, the ferrocene derivatives **10** and **11**, have k_{inh} values in the suitable range;[24] however, they were not used as primary standards because, due to the modest absorbance of the transient reaction products, their

rate constants were measured by pulse radiolysis in an indirect way.[24] Because they are expected to react with peroxyl radicals by pure ET, they were investigated to challenge our calibration using a different system.

The k_p value for THF thus obtained is reported in Table 1, together with the $2k_t$ value calculated from the average oxidisability at pH 2.1–12. As expected, k_p in water is very close to the value in neat THF, whereas $2k_t$ is slightly larger, conceivably accounting for the interference of solvent in the complex self-reaction chemistry of secondary peroxyl radicals.[27]

Representative oxygen uptake plots in the presence of the four reference inhibitors are shown in Figure 4, and the kinetic parameters obtained by using our calibration are collected in Table 2. The agreement with literature data obtained by pulse radiolysis is excellent not only for the two reference compounds used for calibration (**8** and **9**), but also for the two ferrocenes (**10** and **11**), despite the different chain-breaking chemistry, highlighted by the different stoichiometric factors.

Indeed, both ascorbate and urate trap two peroxyl radicals, as expected from their antioxidant chemistry in organic solution. Both of them have "transferable" hydrogen atoms, and therefore their reactions with peroxyl radicals can be described as a formal hydrogen-atom transfer followed (or preceded) by ET to another peroxyl radical (see the Supporting Information).

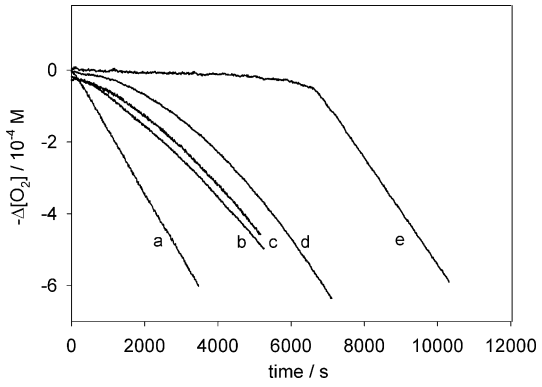


Figure 4. Oxygen consumption measured during the autoxidation of THF (3.1 M) initiated by 30 mM AAPH at 30 °C a) in the absence of antioxidants and with b) 2.0×10^{-5} M ferrocenecarboxylic acid (**10**, pH 8.0), c) 2.0×10^{-5} M (dimethylaminomethyl)ferrocene (**11**, pH 8.0), d) 2.0×10^{-5} M uric acid (**8**, pH 7.3) and e) 1.3×10^{-5} M ascorbic acid (**9**, pH 7.0), only in this case [AAPH] = 12.5 mM.

Table 2. Kinetic parameters for the reaction of reference compounds with peroxyl radicals in buffered water at 30 °C.[a]

Compound	Measured k_{inh} [$M^{-1}s^{-1}$]	n	Ref. k_{inh} [$M^{-1}s^{-1}$]
uric acid (**8**), pH 7.3	$(1.4\pm0.2)\times10^{5}$[b]	1.8 ± 0.2	1.6×10^{5}[c]
ascorbate (**9**), pH 7.0	$(2.2\pm0.3)\times10^{6}$[b]	2.0 ± 0.1	2.0×10^{6}[d]
ferrocenecarboxylic acid (**10**), pH 8	$(1.5\pm0.8)\times10^{5}$	1.0 ± 0.1	2.8×10^{5}[c,e]
[(dimethylamino)methyl]ferrocene (**11**), pH 8	$(1.6\pm0.3)\times10^{5}$	1.3 ± 0.3	2.0×10^{5}[c,e]

[a] Measured by using the calibration data for THF (k_p, $2k_t$) reported in Table 1. [b] The calibration was averaged between compounds **8** and **9**, hence neither of the two has k_{inh} coincident with the literature. [c] Data from ref. [24]. [d] Data from ref. [23]. [e] Value at 293 K.

Conversely, ferrocenes have no transferable hydrogen atoms and are found to break one oxidative chain, expectedly by ET to the peroxyl radical.

Measurement of k_{inh} for phenols in water: pH and solvent effects

Encouraged by the performance of our method, we set to apply it to an investigation of the antioxidant chemistry of representative phenols (see Figure 5 for typical plots) featuring the typical structural motives encountered in RTAs or molecules of biological interest.

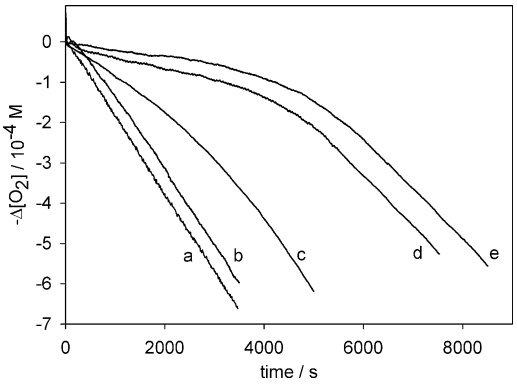


Figure 5. Oxygen consumption measured during the autoxidation of THF (3.1 M) initiated by AAPH at 30 °C and pH 7.4 a) in the absence of antioxidants and in the presence of the following antioxidants: b) 4.0×10^{-5} M **6**, c) 4.0×10^{-5} M **4** (both with [AAPH] = 50 mM), d) 1.0×10^{-5} M **1**, and e) 1.0×10^{-5} M **3** (both with [AAPH] = 12.5 mM).

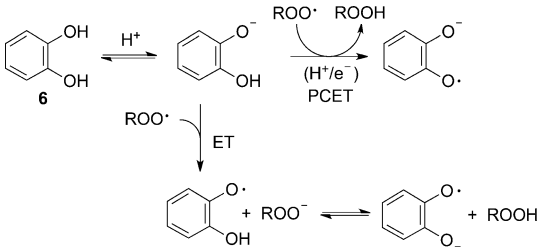
Compounds **1**–**3** share the 6-hydroxychromane active moiety of α-tocopherol, but the phytyl chain is replaced by less lipophilic groups. The reactivity of pentamethylchromanol (PMHC) **1** in buffered water at pH 7.4 ($k_{inh} = (2.0\pm0.3)\times10^{5}\,M^{-1}s^{-1}$, see Table 3) is marginally lower than that recorded in unbuffered water ($k_{inh} = (2.6\pm0.3)\times10^{5}\,M^{-1}s^{-1}$), which shows that the buffer itself has a modest effect on the kinetics. Additionally, the rate constant did not change on decreasing the pH to 2.1, which suggests that acid catalysis, previously observed in acetonitrile,[11] is not relevant in water under our current settings. The same finding applies to 2,6-di-*tert*-butyl-4-methoxyphenol (**4**), which has also previously shown acid catalysis in acetonitrile.[11]

Conversely, the rate constants for phenols **2**, **3**, **5** and **6** are up to four times larger at pH 7.4 compared with at pH 2.1. The enhanced reactivity of catechol (**6**) at pH 7.4 (two-fold increase) can be attributed to partial deprotonation ($pK_{a1} = 9.3$[8]) to yield the more electron-rich phenoxide, which can more rapidly undergo EPT (CPET) to the peroxyl radical (Scheme 4) due to the lower bond dissociation enthalpy (BDE) of the (second) O–H in the phenoxide. This results from the much larger electron-donating (ED) ability of O^- (e.g., $\sigma_p^+ = -2.3$[28]) compared with OH ($\sigma_p^+ = -0.92$[28]). Indeed, when pH was raised to

Table 3. Kinetic parameters for the reactions of phenols with peroxyl radicals in water at 30 °C.[a]

	Water, pH 2.1[b]		Water, pH 7.4[b]		Water, pH 12[b]		PhCl	MeCN
	k_{inh} [10^5 M^{-1} s^{-1}]	n	k_{inh} [10^5 M^{-1} s^{-1}]	n	k_{inh} [10^5 M^{-1} s^{-1}]	n	k_{inh} [10^5 M^{-1} s^{-1}]	k_{inh} [10^5 M^{-1} s^{-1}]
1[c]	1.9 ± 0.3	1.8 ± 0.1	2.0 ± 0.3	1.8 ± 0.1	10 ± 1	2.1 ± 0.1	32[d]	6.8[e]
2	1.4 ± 0.3	2[f]	4.1 ± 0.7	2[f]	15 ± 4	2[f]	11[g]	
3	1.5 ± 0.2	1.9 ± 0.1	3.2 ± 0.4	1.9 ± 0.1	–	–	19[g]	
4	0.33 ± 0.04	1.7 ± 0.2	0.32 ± 0.04	1.8 ± 0.2	–	–	1.1[f]	0.25[e]
5	0.03 ± 0.01	2[h]	0.12 ± 0.04	2[h]	1.5 ± 0.5	2.0 ± 0.1	2.2 ± 0.3[i]	0.05[e]
6	0.03 ± 0.01	2[h]	0.07 ± 0.02	2[h]	7.0 ± 2.1[j]	2.2 ± 0.2[j]	5.5[k]	0.25[d]
7	–	–	0.17 ± 0.05	2[h]	–	–	11[l]	0.20[e]

[a] Values in chlorobenzene and acetonitrile at 30 °C from the literature are reported for comparison. [b] The reaction mixture was 3:1 water/THF containing 0.1 M phosphate buffer. [c] The rate constants measured for the reaction of **1** with ROO• in unbuffered water/THF (3:1) and PhCl/THF (3:1) at 30 °C were (2.6 ± 0.3) × 10^5 and (2.8 ± 0.5) × 10^5 M^{-1} s^{-1}, respectively. [d] From ref. [38]. [e] From ref. [11]. [f] Reference value. [g] From ref. [15]. [h] No distinct inhibited period was observed in the oxygen uptake plots, therefore the value of n was assumed as 2 based on the known behaviour in other solvents. [i] Measured in this work from the inhibited autoxidation of styrene. [j] Measured at pH 10.0. [k] From ref. [39]. [l] From ref. [40].



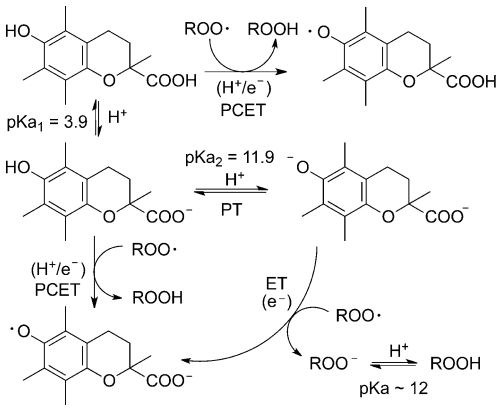
Scheme 4. Mechanistic possibilities for the enhanced reactivity of catechol on increasing the pH.

10, at which catechol is largely deprotonated, k_{inh} increased to 7 × 10^5 M^{-1} s^{-1}, over 200-fold larger than at pH 2.1.

However, an alternative explanation cannot be excluded on the basis of the results of Litwinienko and co-workers from the reactions of flavonoids with the DPPH• radical:[29] at higher pH catechol might react by a SPLET-like mechanism that would be suppressed in acidic medium. This second possibility implies that the deprotonation of the catechol is followed by stepwise ET from the phenoxide and proton exchange (Scheme 4). Although it is difficult to distinguish between the two possibilities for catechol, the scenario becomes clearer with monophenolic compounds.

Trolox (**2**) increases its reactivity three-fold upon increasing the pH from 2.1 to 7.4, which can be attributed to the dissociation of the carboxylic group in **2** (pK_{a1} = 3.9);[30] the dissociation causes the carboxylic group to shift from being inductively electron-withdrawing (EW, σ_I = 0.30)[31] to electron-donating (ED, σ_I = −0.19),[31] thereby decreasing the BDE_{OH} of the phenolic group and increasing its reactivity (Scheme 5).[15,17b] Similar but less pronounced behaviour (two-fold increase in the rate constant) is shown by the analogous hydroxytetramethylchromanacetic acid **3**, in which the carboxy group is partially electronically insulated by the methylene group.[17b]

Upon further increasing the pH from 7.4 to 12 the reactivity of Trolox (**2**) further increases about four-fold. This could conceivably be due to a SPLET-like mechanism consisting of deprotonation of the phenolic OH group (pK_{a2} = 12[8]) followed by



Scheme 5. Mechanistic possibilities for the reaction of **2** (Trolox) with peroxyl radicals in water: concerted PCET versus stepwise PT-ET (SPLET).

ET from the electron-rich phenoxide. To test this hypothesis we extended the investigation on PMHC (**1**) to pH 12. Because PMHC has no acidic function other than the phenolic group (which has a pK_a similar to that of Trolox), the enhanced reactivity at pH 12 can be attributed to SPLET. Similarly to Trolox, the reactivity of PMHC increases five-fold from pH 7.4 to pH 12.

This is possibly the first direct evidence that the SPLET mechanism is relevant in enhancing the reactivity of RTAs with peroxyl radicals (besides DPPH•[32]) during inhibited autoxidations in homogeneous systems; however, interestingly, a minor enhancement (+30%) in the antioxidant performance of PMHC upon increasing the pH from 7 to 10 was recently reported in the autoxidation of a heterogeneous lipid system.[33] Indeed, we were surprised by the modest magnitude of the "SPLET effect". In our system, UV/Vis spectroscopy confirmed that around 60% of Trolox exists in the phenoxide form at pH 12 (see Figure 6), but nonetheless the increase in the rate constant recorded under our settings is modest compared with kinetic solvent effects of up to orders of magnitude reported in ionising organic solvents (e.g., alcohols) for acidic phenols reacting with DPPH•.[34–36] One possible explanation is

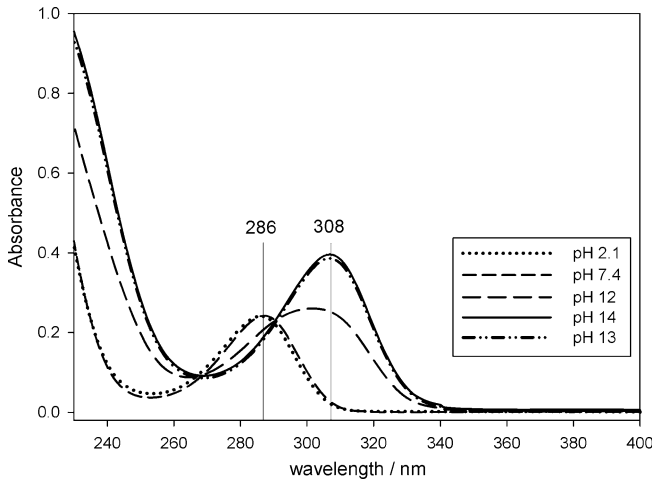


Figure 6. UV/Vis spectra of Trolox (**2**) in phosphate buffer at different pH (2.1–12) and in aqueous NaOH at pH 13 and 14.

that, in the case of autoxidations in water solution, although the redox potential of the phenol decreases upon increasing the pH due to progressive dissociation, which increases its reactivity, the peroxyl radical becomes progressively less oxidising, thereby partly compensating the effect. Indeed, the redox potential of $CH_3OO^{\bullet}$ decreases by about 0.6 V upon passing from pH 7 to pH 12,[23] which is a result of the deprotonation of the hydroperoxide product. The pK_a values of several organic hydroperoxides in water fall in the range 11.5–12.5[37] and we found that *tert*-butyl hydroperoxide is partly dissociated in phosphate buffer at pH 12 (see the Supporting Information). Indeed, when we turned our investigation to the more acidic 4-methoxyphenol (**5**, pK_a = 10.1[8]), the overall rate enhancement recorded upon passing from pH 2.1 to pH 12 was significantly larger (50-fold). UV/Vis spectroscopy confirmed that the dissociated fraction at pH 7.4 was $<1\%$, which accounts for the limited increase in k_{inh}, whereas it was nearly completely dissociated at pH 12 (see the Supporting Information).

Compared with known kinetics in organic solution, such as chlorobenzene, the rate constants measured in water at acidic to neutral pH are generally smaller, the difference being more marked for phenols with greater hydrogen-bond-donating (HBD) ability. Indeed, the kinetic solvent effect (KSE) $k^{PhCl}/k^{H2O(2.1)}$ is, respectively, 183, 20 and 3 for phenols **6**, **1** and **4**, characterised by Abraham's α_2^H values of 0.73,[41] 0.37[42] and 0.18,[43] respectively. This KSE is typical for the EPT (or HAT) reaction X–H + Y$^{\bullet}$ → X$^{\bullet}$ + Y–H, in which X is an electronegative heteroatom (e.g., O, N) and Y$^{\bullet}$ is any radical species, and arises from hydrogen bonding of X–H to the solvent, which hampers its reactivity.[44] This KSE implies that the transfer of the hydrogen atom (or proton) is rate-determining and it is described quantitatively by Ingold's equation (Eq. 13),[42] in which k^S and k^0 are the rate constants, respectively, in the solvent of interest and in a non-HBA solvent (e.g., CCl_4), and α_2^H and β_2^H are Abraham's solvatochromic parameters (range 0–1) describing the HBD ability of the reactant X–H and the hydrogen-bond-accepting (HBA) ability of the solvent, respectively.[45]

$$\log(k^S/\text{M}^{-1}\,\text{s}^{-1}) = \log(k^0/\text{M}^{-1}\,\text{s}^{-1}) - 8.3\alpha_2^H\beta_2^H \quad (13)$$

On the basis of Equation (13), our rate constants in acidic water should be close to the values recorded in acetonitrile, which has an almost identical HBA ability (i.e., $\beta_2^H = 0.38$ for water[45] versus $\beta_2^H = 0.39$ for MeCN[38]). However, inspection of the data in Table 3 shows that they are generally lower. Clearly the relevant concentration of THF ($\beta_2^H = 0.51$[45]) in the reaction mixture (25% v/v; 3.1 M) contributes to the HBA ability of the medium. To confirm this point, we studied the reaction of phenol **1** in a 3:1 PhCl/THF solvent mixture and obtained $k_{inh} = 2.8\times10^5\,\text{M}^{-1}\,\text{s}^{-1}$, that is, significantly smaller than the value in PhCl and MeCN and similar to the value recorded in water/THF. Taken together, these data suggest that, at least under acidic conditions, in which the SPLET mechanism is suppressed, the reaction in water is subjected to an identical solvent effect as recorded in organic solution, hence it conceivably occurs by the same mechanism, one in which the formal transfer of hydrogen is rate-determining.

Indeed, as predicted by Equation (13), a semi-logarithmic plot of the ratio of the rate constants measured for each phenol in PhCl and in water versus α_2^H gave a perfect straight line ($r^2 = 0.995$, see the Supporting Information). From this correlation it is possible to estimate the HBA ability of our reaction medium (3:1 water/THF containing 0.1 M phosphate buffer) by means of Equation (14), which is derived from Equation (13).[46]

$$\log(k^{S1}/k^{S2}) = 8.3\alpha_2^H(\beta_2^{H\ S2} - \beta_2^{H\ S1}) \quad (14)$$

The resulting β_2^H value is 0.49,[47] sensibly higher than the value for neat water, but slightly lower than that for THF. For comparison, the β_2^H values obtained by using Equation (14) (using the data of phenol **1**) for unbuffered water/THF (3:1) and PhCl/THF (3:1) mixtures are, respectively, 0.44 and 0.43. Therefore, the presence of phosphate buffer makes a minor contribution to the HBA ability of the medium.

H(D) kinetic isotope effect

To gain a deeper insight into the mechanisms underlying the reactions with peroxyl radicals in water, we measured the H(D) kinetic isotope effect (KIE) by performing matching autoxidation studies in H_2O and D_2O as solvent, exploiting the dynamic OH → OD exchange in acidic H(D) atom donors.[48,49]

The results collected in Table 4 show that both PMHC (**1**) and Trolox (**2**) give the H(D) KIE as $k_H/k_D \approx 2$ at pH 2.1, at which they exist in the neutral form. Although this value is lower than typical values in organic solvents (k_H/k_D was reported to be 4.0 and 5.1 for α-tocopherol and PMHC at 30 °C in styrene[49]), it is still consistent with the proton (or hydrogen atom) being transferred in the rate-determining step. At pH 7.4, the H(D) KIE for Trolox decreases ($k_H/k_D = 1.2$), which suggests a change in mechanism, such as a stepwise PT–ET, in which the ET is rate-determining. However, it should be noted that the value measured at pH 7.4 is not significantly different from that recorded at pH 2.1 when the experimental errors in

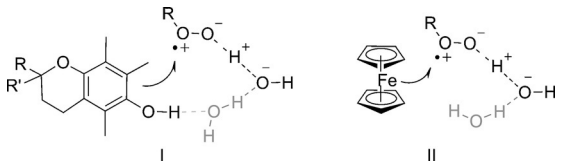
Table 4. Rate constants measured in D_2O at 30 °C and H(D) kinetic isotope effect.

Compound (pH, pD)	k_{inh} [10^4 M^{-1} s^{-1}] in D_2O[a]	k_H/k_D
1 (2.1)	10 ± 3	1.9
2 (2.1)	6.1 ± 1.0	2.3
2 (7.4)	34 ± 7	1.2
5 (2.1)	0.024 ± 0.004	12.5
5 (7.4)	0.13 ± 0.05	9.2
5 (12)	5.8 ± 1.6	2.6
10 (8)	5.7 ± 0.5	2.6
11 (8)	6.3 ± 0.3	2.5

[a] From the kinetics of the inhibited autoxidation of THF/D_2O (3:1) containing 0.1 M phosphate buffer.

the relative rate constants are taken into account. Interestingly, upon extending the investigation to ferrocenecarboxylic acid (**10**) and [(dimethylamino)methyl]ferrocene (**11**) (both at pH 8), which have no transferable hydrogen and are known to react with peroxyl radicals purely by ET, we observed a similar H(D) KIE (2.5–2.6, see Table 4).

Although counterintuitive, this finding is not unprecedented. On studying the H(D) KIE for the ET reaction of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) with methylperoxyl and α-dioxanylperoxyl radicals in water, Neta et al. reported an average k_H/k_D of 2.2 (at 20 °C) and found that other one-electron reducing agents (including phenolate anions) react with $CCl_3OO^{\bullet}$ radicals with a similar H(D) KIE of about 2.[50] They explained the reaction as occurring by ET from the reducing agent to the peroxyl radical concerted with a PT from the solvent to the incipient hydroperoxide anion via a hydrogen-bonded transition state (TS) that includes water. In keeping with their proposal, the reactions of electron-rich phenols (PMHC and Trolox) or ET reducing agents (ferrocenes) with the peroxyl radical in water can be described as occurring by a multi-site EPT[7] (or separated CPET[8]) to the peroxyl radical, in which the electron moves from the reductant and the proton moves from water and/or the phosphate buffer. The process is tentatively illustrated in structures I and II, in which water molecules could be replaced by the phosphate buffer.

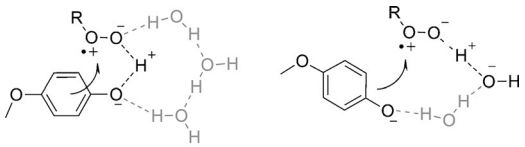


I II

In the case of phenols (or other acidic reducing agents), clearly the reaction is favoured by deprotonation to form the more electron-rich phenoxide anion,[8] in keeping with the observed increase in the rate of reaction upon increasing the pH (see Table 3)

At variance with the more electron-rich phenols, an isotope effect k_H/k_D of 12.5 was recorded for the electron-poorer and less reactive 4-MeOPhOH (**5**) at pH 2.1. This is larger than typical values recorded in organic solution for phenolic antioxidants (4–6) and is also larger than the limiting value of around 7 predicted by the semi-classical transition-state theory based on the difference in zero-point energies.[51] H(D) isotope effects exceeding such values have often been interpreted as indicative of quantomechanical tunnelling in the hydrogen (or proton) transfer.[52] Although the limiting value of 7 applies to C–H hydrogen atom transfer, the limiting value for the transfer of a phenolic O–H can be estimated from IR data to be $0.5(\nu_H - \nu_D) = 460$ cm^{-1}, which corresponds to 1.3 kcal mol^{-1}, and would yield a k_H/k_D value of 9, similar to the calculated value for hydroxylamines.[53] Therefore, it is unclear if tunnelling plays a role in the reactions of less electron-rich phenols with peroxyl radicals in water. Tunnelling has been identified as a key feature in enzymatic radical reactions in which the reactant centre and the abstracting radical are constrained by an enzyme active site.[54] Much less is known of the corresponding reactions occurring in solution,[52a] particularly in water.[55] Might water provide a sufficiently organised reaction environment? Our current data call for further investigation in this regard.

The H(D) KIE for 4-MeOPhOH decreases to 9.2 at pH 7.4 and drops to 2.6 at pH 12, at which the phenol is completely dissociated to the corresponding phenoxide. This clearly indicates a change in reaction mechanism. We suggest that, in water, less electron-rich phenols like 4-MeOPhOH (**5**) react with peroxyl radicals at low pH by EPT (CPET) in which both the electron and the proton are transferred from the undissociated phenol, that is, similarly to the mechanism accepted in aprotic organic solvents,[5] starting from a hydrogen-bonded pre-reaction complex possibly clustered in an organised pattern of water molecules as exemplified in structure III. Conversely, at higher pH (depending on the phenol's pK_a), below the pK_a of the hydroperoxide, the more electron-rich phenoxide anion reacts by a multi-site EPT (separated CPET), in which the electron moves from the phenoxide to the peroxyl radical and the proton moves from the hydrogen-bonded solvent to the incipient hydroperoxide anion, as depicted in structure IV, similarly to the mechanism proposed for Trolox and ferrocenes. It is worth stressing that the simplified structures III and IV are meant only to illustrate the concept with no aim to identify the actual TS, which might involve the phosphate buffer in addition/replacement of water.



III IV

Conceivably a combination of the two mechanisms would be operating at intermediate pH values, at which, depending of phenol's pK_a, variable amounts of the neutral and deprotonated forms coexist in solution. It is interesting to note that the apparent H(D) KIE measured at pH 11 for 4-MeOPhOH is 6.2. This high value, which suggests hydrogen (atom or proton)

transfer as the rate-determining step, appeared surprising given that 4-MeOPhOH has a pK_a value of 10.1 in water and should be largely deprotonated at pH 11. Indeed, we found it to originate from the combination of two different isotope effects: one on the kinetics of the reaction with peroxyl radicals and one on the equilibrium of the phenol's acid dissociation. The UV/Vis spectra of 4-MeOPhOH show that the phenoxide anion form accounts for about 91% in H_2O at pH 11, whereas it accounts only for around 36% in D_2O at pD 11 (see the Supporting Information).

The mechanism proposed for the reaction of electron-rich phenols, which claims the role of water as proton relay (cf. structure I), implies that more than one proton is transferred "simultaneously" in the rate-determining step. To gather support for this mechanism we performed a proton inventory experiment[56] for the reaction of Trolox at pH 2.1. The rate constant was determined in a series of matching autoxidation reactions in H_2O/D_2O mixtures with a variable deuterium atom fraction: as dictated by the Gross-Butler equation,[57] the observed isotope effect is related to the deuterium atom fraction in solution by a function the order of which is equal to the number of sites contributing to the isotope effect, that is, the number of protons "in flight" in the rate-determining step.[56]

For a typical hydrogen-atom transfer or PCET, in which only one hydrogen is transferred in the rate-determining step, it is expected that the rate constant measured in H_2O/D_2O mixtures (k_{Dmix}, hence the ratio k_{Dmix}/k_H) decreases linearly as the deuterium atom fraction of the mixture increases, as shown in Figure 7A (dotted line).

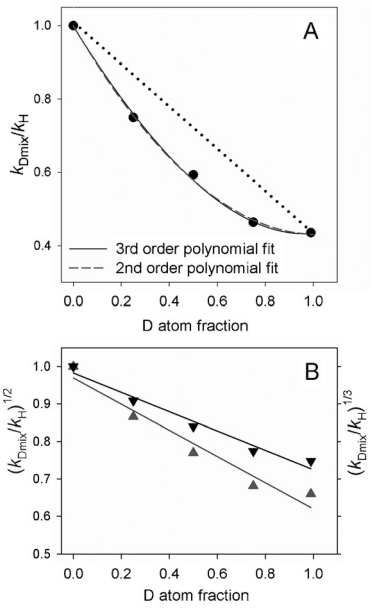


Figure 7. Proton inventory for the reaction of Trolox with peroxyl radicals in H_2O/D_2O mixtures at pL (pH/D) 2.1. A) A plot of the rate constant ratio k_{Dmix}/k_H as a function of deuterium atom fraction in solution; the dotted line represents the linear trend expected for the exchange of only one proton in the rate-determining step. (B) Plots of the square (▲) and cubic (▼) roots of the rate constant ratio k_{Dmix}/k_H as a function of deuterium atom fraction with the regression lines having r^2 of 0.952 and 0.971, respectively.

However, when we built this plot for Trolox it became apparent that the correlation was non-linear and had an order >1. To determine the number of protons being transferred in the rate-determining step, we fitted the experimental data to polynomial functions (of the type $y=a+bx+cx^2+dx^3+\ldots$) of growing order and found that both second- and third-order functions reproduced well the observed trend (Figure 7A). Matching was marginally better for the third-order function ($r^2=0.998$, $P=0.05$ vs $r^2=0.997$, $P=0.05$), whereas higher-order functions did not improve the quality of the fitting. To confirm the correlation order, the square and cubic roots of k_{Dmix}/k_H were plotted against deuterium atom fraction,[56] which afforded good linear correlations (Figure 7B). Again, linearity was slightly better for the cubic root ($r^2=0.971$) than for the square root ($r^2=0.952$); however, the experimental error does not allow it to be established unambiguously whether two or three protons are transferred in the rate-determining step. Both cases are compatible with the proposed mechanism, depending on the arrangement of hydrogen-bonded water or phosphate in the TS.

Conclusion

We have described a reliable and versatile method for investigating the kinetics of alkylperoxyl radical reactions in water solution, based on the inhibited autoxidation of water/THF mixtures. For its range of applications, this technique is the ideal complement to pulse radiolysis, against which it was calibrated. We have applied its power to dive into the complex mechanisms of reaction of typical radical-trapping antioxidants and other models of phenols of biological interest. We have shown for the first time that a SPLET-like mechanism, which consists of the acidic dissociation of phenols prior to their reaction with peroxyl radicals, plays a significant role in the trapping of peroxyl radicals by phenols during inhibited autoxidations in water. Concerning the actual rate-determining step of the reactions of phenols with peroxyl radicals, our results show the involvement of water as proton relay, evoking the chemistry previously found key to the functioning of some radical enzymes, such as those involving the oxidation of tyrosine.[13] Indeed, sufficiently electron-rich species like chromanol derivatives or the phenoxide anion of 4-methoxyphenol react with peroxyl radicals by MS-EPT (separated CPET) in which water acts as proton acceptor–donor and the electron is transferred to the peroxyl radical. Less electron-rich species, like undissociated 4-methoxyphenol, instead react by EPT (CPET) to the peroxyl radical, similarly to the mechanism established in organic solvents, but in which water might play a role by providing a favourable solvent cage.

We believe that our current data offer new insights into the fascinating redox chemistry of phenols in water, but possibly lead to even more questions that certainly we, and hopefully others, will enjoy investigating in the future.

Experimental Section

Materials: All chemicals and solvents were of the highest purity commercially available. 2,2,5,7,8-Pentamethyl-6-chromanol (**1**), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (**2**), uric acid (**8**), ascorbic acid (**9**), ferrocenecarboxylic acid (**10**) and [(dimethylamino)methyl] ferrocene (**11**) were used as received. 2,6-Di-*tert*-butyl-4-methoxyphenol (**4**), 4-methoxyphenol (**5**) and 3,5-di-*tert*-butylcatechol (**7**) were recrystallised from hexane. Catechol (**6**) was recrystallised from ethyl acetate/hexane. (*S*)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-acetic acid (**3**) was available from previous studies (purity 99% by HPLC on a 150×4.6 mm, 3 μm particle size C-18 column eluted at 1.0 mL min^{-1} with EtOH/H_2O (92:8, v/v) with detection at 295 nm).[17b] THF was distilled and stored under argon at 5 °C; the content in hydroperoxides was determined periodically by spectrophotometry at 262 nm in isopropanol upon reaction with triphenylphosphine, and found ≤50 ppm (μg g^{-1}). Styrene was purified by double percolation through silica and activated alumina columns.

Buffer preparation

General: Buffers were freshly prepared with bidistilled water or deuterium oxide and were stored in a refrigerator. The pH was adjusted with HCl or NaOH and checked by a glass-electrode pH-meter (±0.05). For measurements in deuterium oxide, the pD value was corrected with respect to the instrumental reading according to Equation (15).[57]

$$pD = pH_{read} + 0.4 \quad (15)$$

Buffered solutions in H_2O/D_2O solvent mixtures were prepared by mixing (up to the desired deuterium atom fraction) two buffered solutions in H_2O and D_2O, each previously adjusted to the desired pH or pD.

Buffer pH 2.1: NaH_2PO_4 $2H_2O$ (0.39 g, 0.05 M) and H_3PO_4 85% (0.17 mL, 0.05 M) were dissolved in water (50 mL).

Buffer pH 7.4: Na_2HPO_4 (0.595 g, 0.096 M) and NaH_2PO_4 $2H_2O$ (0.125 g, 0.016 M) were dissolved in water (50 mL).

Buffer pH 8.0: Buffers at pH 8.0 were obtained by adjusting the buffer at pH 7.4 by the addition of NaOH.

Buffer pH 12: Na_2HPO_4 (0.71 g, 0.025 M) and NaOH 0.1 M (53.8 mL, 0.027 M) were dissolved in water (200 mL).

Buffers at pH 11 and 13: Buffers at pH 11 and 13were obtained by adjusting the buffer at pH 12 with HCl or NaOH, respectively.

Buffer pH 14: Solutions at pH 14 were obtained as 0.97 M aqueous NaOH.

Buffer solutions were mixed with the desired amount of THF (typically 3:1 by volume) after having adjusted the pH or pD to the desired value.

Autoxidation experiments: Autoxidation experiments were performed in a two-channel oxygen-uptake apparatus based on a Validyne DP 15 differential pressure transducer built in our laboratory and described previously.[58] Azo initiators were prepared in concentrated stock solution that were injected into the reaction mixture to the desired final concentrations (typically 12.5–75 mM). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was freshly prepared every 4 h and stored at 5 °C to avoid excessive hydrolysis. 4,4′-Azobis(4-cyanovaleric acid) (ABCV) was dissolved in distilled water and titrated with 0.1 M NaOH to obtain the disodium salt. In a typical experiment, an air-saturated solution of THF/water in a variable ratio (1:1 to 1:7, v/v) containing the desired buffer (0.1 M) and AAPH (or ABCV) as initiator was equilibrated with an identical reference solution containing an excess of 2,2,5,7,8-pentamethyl-6-chromanol (**1**). After equilibration, and when a constant O_2 consumption was reached, a concentrated solution of the antioxidant was injected into the sample flask. The oxygen consumption of the sample was measured, after calibration of the apparatus, from the differential pressure recorded with time between the two channels. Initiation rates, R_i, were determined for each set of conditions by matching autoxidation experiments, using Trolox (**2**) as reference antioxidant, by means of Equation (10). Oxidisability values ($k_p/(2k_t)^{1/2}$) were determined in uninhibited autoxidations by means of Equation (7). Absolute k_{inh} values were determined, after independent assessment of R_i, from 4–10 inhibited autoxidation experiments with antioxidant concentration in the range 10–50 μM by means of Equation (11) or (12) and values of n were determined from the same experiments by Equation (10).[15,26,58]

UV/Vis Spectroscopy: UV/Vis absorption spectra were recorded at room temperature by using a Jasco V550 double-beam UV/Vis spectrometer with baseline correction. The solutions were placed in quartz absorption cuvettes with a pathlength of 10 mm and a chamber volume of 3.5 mL. Solutions were freshly prepared and the cuvettes were sealed with a rubber septum and purged with nitrogen to avoid air-oxidation of the phenoxide anions.

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Keywords: antioxidants · kinetics · proton inventory · radical reactions · reaction mechanisms

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