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A versatile fluorescence approach to kinetic studies of hydrocarbon autoxidations and their inhibition by radical-trapping antioxidants†

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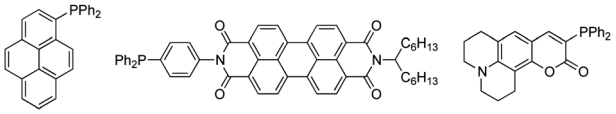
We report a simple coumarin–triarylphosphine conjugate that undergoes fluorescence enhancement upon reaction with hydroperoxides and demonstrate its use to follow autoxidations of 7-dehydrocholesterol (at 37 °C) and hexadecane (at 160 °C) and their inhibition by antioxidants.

Autoxidation is the radical chain process responsible for the oxidative degradation of all hydrocarbons (R–H, Scheme 1),[1] from polymers, lubricating oils and other petroleum-derived products to the lipids that comprise the biological membranes of the cells of living organisms. Given the importance of this process in understanding the fate of industrial and biological materials in an aerobic atmosphere, as well as its putative involvement in the pathogenesis of degenerative disease, much effort has been devoted to the detailed study of these reactions.

Autoxidations are followed either by consumption of O_2 or formation of the hydroperoxide product (ROOH). The former is generally preferred since it can be done in real time by differential pressure measurements,[2] and the latter, which are often carried out by either iodometric titration or HPLC/GC following reduction of ROOH to ROH with PPh_3,[3] are tedious and can be difficult to reproduce with good precision. Recently, several (pro-)fluorescent molecular probes have been developed to detect and/or image ROOH formation in real time in solution and/or cells. These compounds generally feature known fluorophores conjugated to phosphines and operate similarly: oxidation of the phosphine by ROOH precludes photoinduced electron transfer (PeT) from the phosphine to the fluorophore, leading to an enhancement in fluorescence. Two representative examples are diphenyl-1-pyrenylphosphine (DPPP, **1**)[4,5] and Spy-LHP (**2**).[6]



1 **2** **3**

We sought to use these molecules in a microplate assay to facilitate our studies of hydrocarbon autoxidations and their inhibition by antioxidants, but quickly learned that these compounds (and their synthetic precursors) suffer from very poor solubility and are generally very expensive.[7] Inspired by the work of Bertozzi and co-workers,[8] Xian *et al.*[9] developed the more accessible and soluble coumarin-based dye **3** for the determination of *S*-nitrosothiols, but unfortunately we found that reactions of **3** with ROOH are sluggish ($k < 0.1$ M^{-1} s^{-1}), and that background oxidation with O_2 can be problematic. Since triphenylphosphine reacts much more quickly with ROOH,[10] we opted to include a phenyl spacer for our investigations. Moreover, given the ease with which simpler 7-substituted coumarins can be synthesized (by Koneovenagel condensation or Wittig olefination and *in situ* lactonization of the cinnamate), we elected to focus on this less-electron rich fluorophore. We considered 4 substitutions (Scheme 2) in an attempt to optimize both PeT and rate of reaction with ROOH.

The *N*,*N*-diethylamino-substituted coumarins had relatively poor fluorescence quantum yields for both the phosphine (**4–5**) and phosphine oxide (**8–9**) conjugates ($\Phi_{f,ox}$ and $\Phi_{f,red}$, Table 1). The erosion in quantum yield compared to the oxidized form of **3** ($\Phi_{f,ox} = 0.79 \pm 0.04$)[9] presumably arises due to the increased conformational flexibility of the *N*,*N*-diethylamino substituent as well as the intervening phenyl linkage. Furthermore, the lack of any significant quenching of the coumarin's fluorescence by PeT from the phosphine moiety indicated a mismatching of the energies of the phosphine and coumarin HOMOs in this system. Substituting the *N*,*N*-diethylamino substituent for a methoxy group provided a sharp increase in quantum yield, and decreased the energy of the coumarin HOMO energy, leading to a slightly more effective quenching of the fluorescence. However, it was upon replacement of the

Initiation $\xrightarrow{R_i}$ R• (1)

Propagation R• + O_2 $\xrightarrow{\text{v. fast}}$ ROO• (2)

ROO• + R–H $\xrightarrow{k_p}$ R• + ROOH (3)

Termination ROO• + ROO• $\xrightarrow{k_t}$ Non-radical products (4)

Scheme 1 Radical chain mechanism of hydrocarbon autoxidation. Assuming steady state in [ROO•] the rate of autoxidation is given by: $d[ROOH]/dt = -d[O_2]/dt = R_p = k_p[R–H]R_i^{1/2}/2k_t^{1/2}$.

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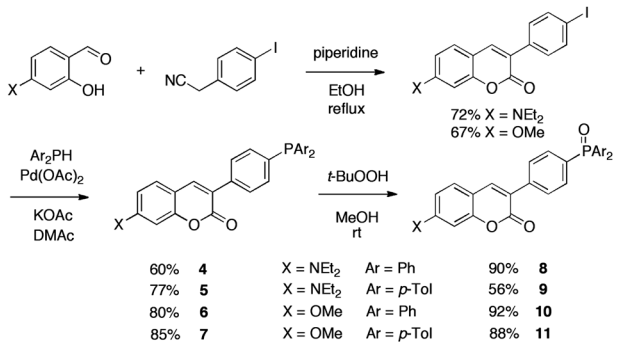

Scheme 2 Synthesis of coumarin–triarylphosphine conjugates.[17]

Table 1 Relevant spectral parameters of coumarin–phosphine conjugates **4–7** and corresponding phosphine oxides (**8–11**) at 298 K in MeOH

	λ_{ex} (nm)	λ_{em} (nm)	$\Phi_{f,red}$[a]	$\Phi_{f,ox}$[a]	$\Phi_{f,ox}/\Phi_{f,em}$
4, **8**	408	486	0.17	0.21	1.2
5, **9**	406	486	0.15	0.17	1.3
6, **10**	345	426	0.20	0.48	2.4
7, **11**	343	422	0.05	0.52	10.6

[a] Determined *versus* sodium difluorescein in methanol (Φ_f = 0.92).[11]

diphenylphosphine moiety with the more electron-rich di(*p*-tolyl)-phosphine that the fluorescence enhancement upon oxidation became useful ($\Phi_{f,ox}/\Phi_{f,red}$ = 10.6). Moreover, **7** was indefinitely stable in air at 25 °C in non-protic solvents, and underwent negligible oxidation in protic solvents over several hours.

It is obvious from the ample kinetic data in the literature that the rates of reactions of triarylphosphines and ROOH are too slow for accurate determinations of ROOH concentration based on a single 'end-point' fluorescence measurement (*e.g.* k = 1.3, 1.0 and 0.4 M^{-1} s^{-1} for reactions of *n*-, *sec*-, and *tert*-BuOOH, respectively, with triphenylphosphine in EtOH at 25 °C).[10,12] Therefore, in order to determine ROOH concentrations with greater accuracy and precision, a second order rate constant for the reaction of **7** and a model hydroperoxide was determined. Tetralin hydroperoxide (TetOOH) was chosen as a representative secondary hydroperoxide (mimicking lipids and other relevant autoxidation substrates) because it is stable and can be recrystallized to very high purity.[13] Fig. 1 shows the initial rates for the reactions of **7** (20 μM) with TetOOH, which were used to obtain k = 9.1 and 1.2 M^{-1} s^{-1} in MeOH at 37 °C and *t*-amyl alcohol (*t*-AmOH) at 25 °C, respectively (see ESI†). With these rate constants in hand, ROOH concentrations of unknowns can be determined from the initial rates of their reactions with **7**.

To demonstrate the quantitative utility of **7** to follow the progress of hydrocarbon autoxidations, we carried out experiments using both a representative biological lipid and industrial model compound. 7-Dehydrocholesterol (7-DHC) was recently reported by Porter and co-workers to be "one of the best chain-carrying molecules that has been evaluated in free radical oxidations" with a propagation rate constant of k_p = 2260 M^{-1} s^{-1}.[14] However, the experiments used to determine k_p were not actually chain oxidations of 7-DHC, and therefore did not allow for the determination of its oxidizability ($k_p/(2k_t)^{1/2}$), which requires knowledge of the rate of oxidation (R_p, see

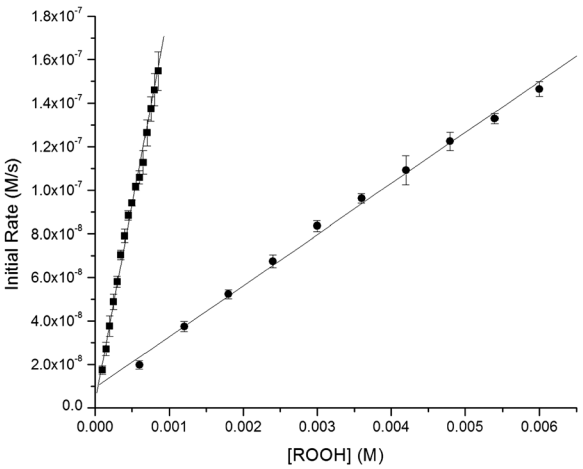

Fig. 1 Initial rates for the reaction of **7** (20 μM) with TetOOH in MeOH (■) at 37 °C and *t*-AmOH (●) at 25 °C.

Scheme 1 caption). The use of compound **7** enabled us to measure R_p in a 7-DHC autoxidation. Thus, azo-initiated[15] oxidations of 7-DHC were carried out in 1,2-dichlorobenzene in a 96-well microplate and the fluorescence of each well was recorded at 4 minute intervals following dilution with MeOH and addition of **7** (as a concentrated solution in CH_3CN) in order to determine [ROOH] (Fig. 2).[17] The rate of ROOH formation afforded a kinetic chain length ($\upsilon = R_p/R_i$) of ~400, which provided an oxidizability of 21.5 from which $2k_t$ could be calculated as 1.1×10^4 M^{-1} s^{-1}.[18,19] These results indicate that the high oxidizability of 7-DHC is therefore not only due to its very high k_p, but also its very low $2k_t$.

It is important to point out that the oxidizability of 7-DHC is 1100-fold higher than the polyunsaturated lipid (methyl) linoleate and 3400-fold and 13 000-fold those of styrene and cumene, respectively,[20] the latter two of which are commonly used as substrates in inhibited autoxidations to determine the rate constant for the reaction of a radical-trapping antioxidant with a peroxyl radical (k_{inh}).[2] Indeed, the autoxidation of 7-DHC could be inhibited by 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMHC, a truncated version of α-tocopherol, Nature's

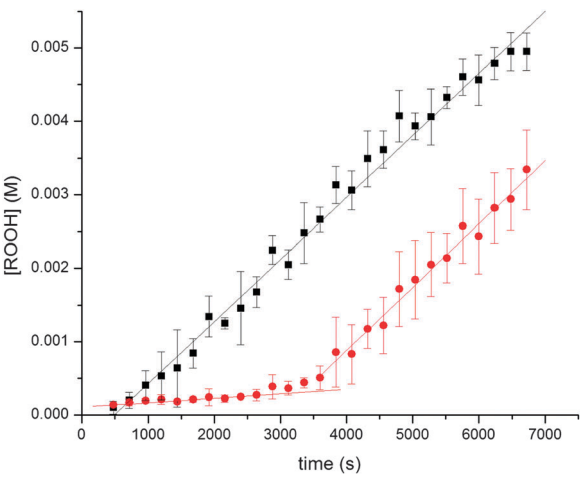

Fig. 2 Autoxidation of 7-dehydrocholesterol (78 mM) in 1,2-dichlorobenzene initiated with 52 μM of MeOAMVN (■), and its inhibition with 4 μM of PMHC (●) at 37 °C.

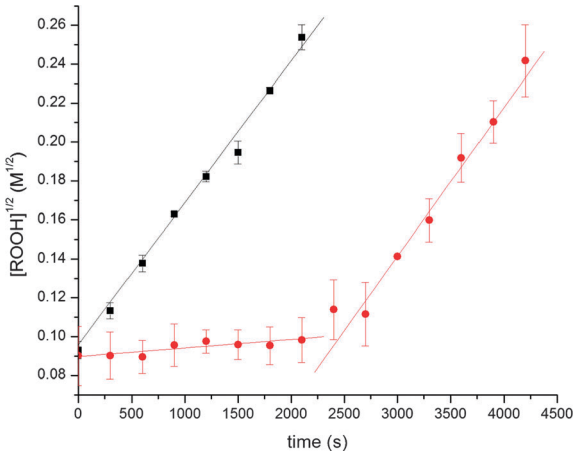

Fig. 3 Autoxidation of neat hexadecane initiated with 8 mM TetOOH (■), and its inhibition with 1 mM of BHT (●) at 160 °C.

premier lipid-soluble radical-trapping antioxidant), and the rate of the inhibited autoxidation could be used with the standard formulae[21] to determine $k_{inh} = 2.0 \times 10^6$ M^{-1} s^{-1}, in excellent agreement with the literature value of 2.1×10^6 M^{-1} s^{-1}.[2] The high oxidizability of 7-DHC suggests that it is an excellent substrate for kinetic studies of inhibited autoxidations, particularly for very effective antioxidants,[22,23] which are difficult with styrene.[24]

Although the fluorimetric determination of ROOH by reaction with **7** is very useful for the rapid determination of autoxidation/inhibition kinetics at ambient temperatures, its versatility is best demonstrated by its application to follow autoxidations carried out at elevated temperatures. These are impossible to study using the conventional O_2 uptake approach since the solutions must be continuously oxygenated in a stirred flow apparatus to prevent mass transfer of O_2 from being rate-limiting[3] and are therefore conventionally monitored by iodometry or lengthy GC analyses of reaction products.[3,25] Enabled by **7** we were able to monitor the TetOOH-initiated oxidation of hexadecane and its inhibition by the common industrial antioxidant additive BHT (2,6-di-*tert*-butyl-4-methylphenol) simply by removing aliquots from a stirred flow reactor, cooling them to 25 °C, diluting them with *t*-AmOH, loading them into a 96-well microplate and determining [ROOH] in each aliquot from the increase in fluorescence intensity due to reaction of the ROOH with **7** (added as a concentrated solution in CH_3CN).[17] The results are shown in Fig. 3.

Using this procedure, the measured rate of hexadecane autoxidation (7.3×10^{-5} $M^{1/2}$ s^{-1}) is in satisfactory agreement with that reported under similar conditions by Igarashi *et al.* (15×10^{-5} $M^{1/2}$ s^{-1}),[25,26] as is the length of the inhibited period observed with added BHT (~2500 s for 1 mM BHT *vs.* ~2600 s for 1.2 mM BHT).[25] The capability to determine [ROOH] at elevated temperatures is particularly useful as it simulates conditions encountered by lubricants and polymers under engine operating conditions or extrusion conditions, respectively. We are in the throes of applying this approach to study the high-temperature reactivities of our recently reported diarylamine radical-trapping antioxidants, which are up to 200-fold more reactive at ambient temperatures than current industry standards.[23]

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