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A new dual probe for hydrogen abstraction

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ABSTRACT

A new dual (fluorescent and spin) probe is described, where a *N*-aryl-2,4,6-triphenylpyridinium fluorophore is attached to a TEMPO fragment through an amide link. The resulting sensor **4** was evaluated as a hydrogen-abstracting agent in acetonitrile and in an aqueous solution of reduced Triton-X 100, being as resistant to hydrolysis as quinoline-TEMPO **1**, but more hydrophobic than this probe.

1. Introduction

The use of fluorescence as a method to sense different processes at molecular level is well established.[1–6] Recently, pre-fluorescent probes obtained by attaching a nitroxide moiety to a fluorophore were successfully employed to monitor hydrogen-transfer reactions.[6]

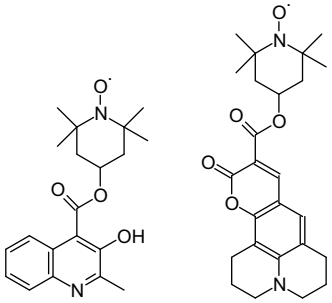
The mechanism of action of these pre-fluorescent probes is well described.[7–9] The paramagnetic nitroxide fragment attached to the quenched fluorophore selectively abstracts a hydrogen atom from a good hydrogen donor[6] or efficiently traps carbon-centered radicals.[10,11] The resulting diamagnetic hydroxylamine or alkoxyamine thereby restores the fluorescence of the chromophore providing experimental evidence for the reaction and an easily measurable way of following its kinetics.

Pre-fluorescent probes are very sensitive and appear less prone to suffer from the problems of electron-deficient probes such as DPPH or of mechanisms of complex stoichiometry, as when ABTS radicals are used.[12–14]

Another remarkable advantage of these sensors is their high selectivity. Hydrogens in polyphenols are abstracted exclusively from the most reactive hydroxyl group, leading to clean and easily identifiable processes.[6,15]

In previous reports, we have employed quinoline-TEMPO **1** and coumarin-TEMPO **2** as pre-fluorescent probes to specifically evaluate the hydrogen-transfer reaction rates of antioxidants.[6,15–17]

As pointed out by us in a recent study[18] when measuring antioxidant activities in micro-heterogeneous media, the lipophilicity of the assessing probe is a key factor to be considered. For this reason, we have been interested in developing new easily available pre-fluorescent probes with different lipophilicities.



Substituted pyridinium salts seemed to us an interesting family to be explored, because of their synthetic versatility and accessibility. Their preparation starts from substituted pyrylium salts, available with a variety of substituents.[19–21]

Their fluorescence is well documented.[22–27] Although there is one report in the literature of a pyridinium derivative incorporating a TEMPO fragment[28] the resulting probe was not pre-fluorescent, a limitation that restricts its use to measurements employing electron paramagnetic resonance spectroscopy.

In the present report we describe the preparation of the dual pre-fluorescent probes **3** and **4**, and evaluate them as hydrogen-abstractors, comparing their stability and lipophilicity with that of quinoline-TEMPO **1**.

2. Results and discussion

The pyridinium TEMPO probe **3** was prepared in two steps from the 2,4,6-triphenylpyrylium perchlorate, which was converted into the *N*-(4-carboxyphenyl)pyridinium salt,[29] and esterified with 4-hydroxyTEMPO. The fluoroborate salt **4** was prepared by reaction of the *N*-(4-carboxyphenyl)pyridinium salt with 4-amino-TEMPO.[30]

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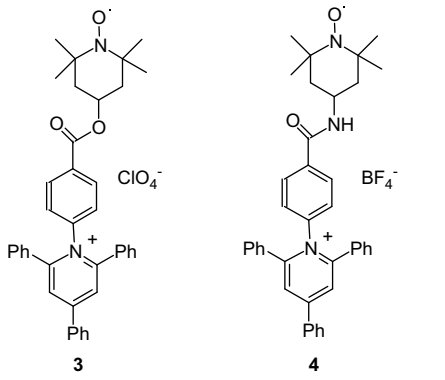

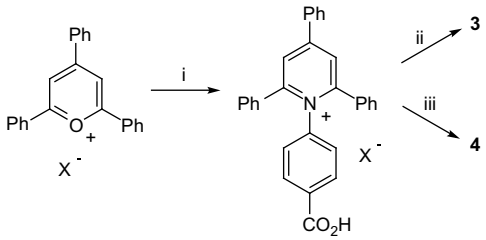

X = ClO_4 or BF_4

(i) 4-$HO_2CC_6H_4NH_2$, EtOH, reflux 48 h;
(ii) 4-hydroxy-TEMPO, DMAP, EDAC;
(iii) 4-amino-TEMPO, DMAP, DCC.

In order to test probes **3** and **4** as dual sensors by using fluorescence and EPR techniques, we evaluated their ability to monitor the hydrogen-transfer reaction from a well-known hydrogen donor such as TROLOX.

2.1. Hydrogen abstraction in a pure solvent

Abstraction of a hydrogen atom from TROLOX by the pre-fluorescent probes **3** or **4** led to the formation of the corresponding hydroxylamines. Several examples of such a conversion for a variety of TEMPO derivatives are found in the literature.[6,31–33] Indirect evidence for the formation of these fluorescent hydroxylamines was obtained by comparison of the emission spectrum of the product of the reaction of **3** and TROLOX with the fluorescence spectrum of the *N*-(4-carboxyphenyl)-2,4,6-triphenylpyridinium fluoroborate. This was based on the fact that the restored emission of the fluorophore in these nitroxide-fluorophore adducts is unaffected by the nitroxide substituent.[34] Both spectra exhibited the same emission at 466 nm, when excited at 355 nm, indicating that the formation of a hydroxylamine from **3** had restored the emission of the carboxyphenylpyridinium fluorophore.

The reactions of probes **3** or **4** with TROLOX in acetonitrile were followed by fluorescence and EPR. Both compounds showed a similar kinetic behavior.

A plot of the fluorescence intensity I/I_0 vs time for the reactions of probe **3** with TROLOX is shown in curve A of Figure 1. An analogous plot for probe **4** is shown in Figure 3, curve A. The calculated rate constants in MeCN were 0.24±0.01 $M^{-1}\,s^{-1}$ for probe **3** and 0.17±0.01 $M^{-1}\,s^{-1}$ for probe **4**. Figure 2, curve A, and Figure 3, curve B, represent the course of the same reaction, as followed by EPR. Addition of 10 mM of TROLOX to a 20 μM solution of the probe **3** or **4** in acetonitrile led to a gradual decrease of the radical triplet signal with time. The calculated rate for hydrogen abstraction in both cases was 0.19±0.01 $M^{-1}\,s^{-1}$.

In order to confirm that the mechanism involved a hydrogen transfer from the hydroxyl group of TROLOX to the TEMPO

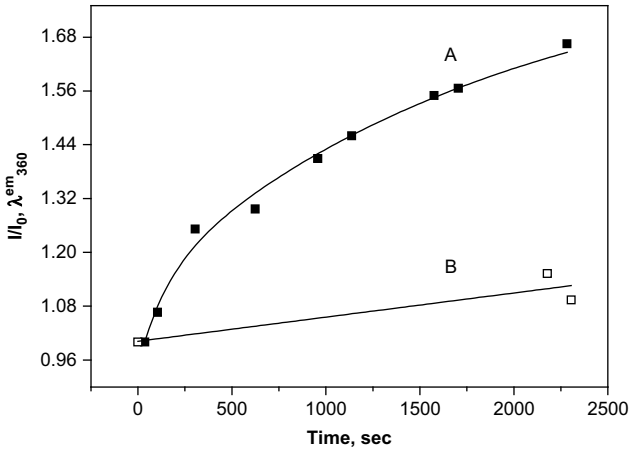

Figure 1. (A) Variation of the fluorescence of **3** (10 μM), evaluated at 466 nm, after addition of TROLOX (10 mM) in acetonitrile (excitation wavelength λ_{exc}=355 nm) (■); (B) control experiment for probe **3** in acetonitrile, in the absence of TROLOX (□).

derivative **3**, we compared the obtained rate value in acetonitrile with the rate constant obtained in methanol-d_6. In Figure 2 the exponential decay curves for the two pseudo-first order reactions are compared. It is seen that the reaction in methanol-d_6 is much slower than in acetonitrile. In contrast with the reaction in acetonitrile, practically finished after 1500 s, in deuterated methanol, only about 20% of the TEMPO derivative had been consumed. Calculation of the second-order constants from these plots led to a deuterium isotope effect of 8.9, arising from the fully deuterated phenolic group. The observed value of 0.019 $M^{-1}\,s^{-1}$ in the deuterated solvent (Fig. 2, curve B) reproduced the value obtained for the reaction of probe **1** with a phenolic compound in methanol-d_6.[35] This kinetic result was another indication that, for both TEMPO derivatives **1** and **3**, the same hydrogen/deuterium abstraction from the hydroxyl group of the phenol to the TEMPO derivative was operating, with the formation of analogous hydroxylamine derivatives. Further evidence that no hydrogen abstraction by probe **4** took place in MeCN in the absence of TROLOX was obtained from a blank experiment, by measuring the fluorescence of a solution of derivative **4** in acetonitrile (Fig. 3, curve C). Under these circumstances, no variation of the fluorescence intensity of the sample was observed after 30 min.

We next investigated the stability of our newly synthesized probes and the possible hydrolysis of the ester or amide functions

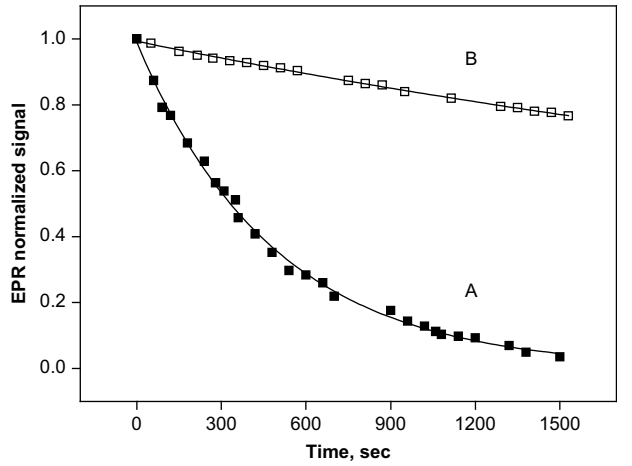

Figure 2. (A) Variation with time of the intensity of the first signal of the EPR triplet of **3** (20 μM) by reaction with excess TROLOX (10 mM) in MeCN at 25 °C (■); (B) decrease of the EPR signal of **3** (20 μM) in methanol-d_6 in the presence of TROLOX (11 mM) at 25 °C (□).

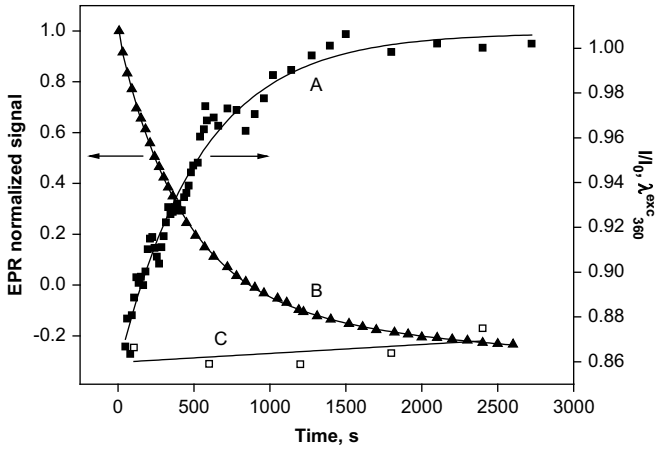

Figure 3. (A) Fluorescence of **4**, evaluated at 465 nm, after addition of TROLOX (10 mM) in acetonitrile (excitation wavelength λ_{exc}=355 nm) (■); (B) variation with time of the intensity of the first signal of the EPR triplet of **4** (10 μM) by reaction with excess TROLOX (10 mM) in acetonitrile at 25 °C; (C) variation of the fluorescence of **4** (10 μM) in acetonitrile, in the absence of TROLOX (□).

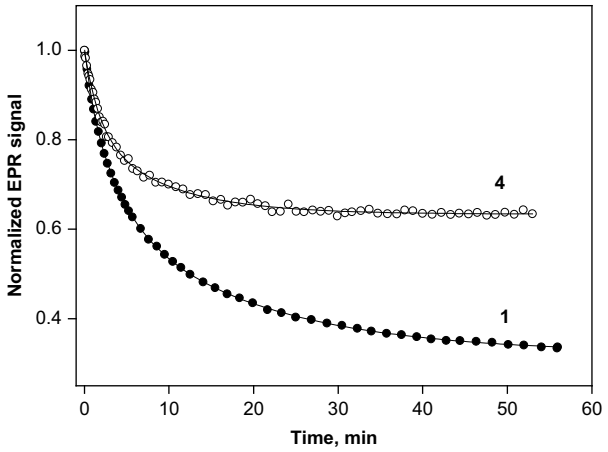

Figure 4. Variation with time of the intensity of the first signal of the EPR triplet of **1** or **4** (20 μM), by reaction with excess TROLOX (20 mM) in an aqueous micellar solution of reduced Triton-X 100 (20 mM).

that linked the TEMPO fragment with the fluorophore. Blank experiments with compound **3** or **4** in acetonitrile at 25 °C, in the absence of TROLOX, indicated that, under these conditions, probe **3** underwent some degree of hydrolysis by the water present in MeCN (Fig. 1, curve B). This concomitant hydrolysis of **3** would account for the larger value obtained by fluorescence measurements for the rate of hydrogen abstraction from TROLOX ($0.24\ M^{-1}\ s^{-1}$), when compared with the rate for probe **4** ($0.17\ M^{-1}\ s^{-1}$) and the value reported for **1** in MeOH ($0.19\ M^{-1}\ s^{-1}$).[6] Under the same conditions, the amide **4** was stable (Fig. 3, curve C). The greater stability of probe **4** was further confirmed by running blank experiments in water, at pH 7 and 4. In both cases, the amide **4** showed no signs of hydrolysis, proving to be as stable as quinoline-TEMPO **1**. The agreement between the results from the two techniques when probe **4** was employed was also an indication that the rise in fluorescence and decay in EPR are due solely to the hydrogen abstraction from TROLOX. These results made us concentrate our interest in probe **4**.

2.2. Hydrogen abstraction in a micellar environment

Previous work had shown that compound **1** does not undergo any self-quenching by the presence of a phenolic hydroxyl group in the molecule.[6] This might be attributed to the conditions under which kinetics of hydrogen abstraction are normally performed. In the presence of a 1000-fold excess of a phenol, the competing intermolecular abstraction of a hydrogen atom from the phenolic hydroxyl group of **1** should not be significant. A second, reasonable explanation for the absence of self-quenching might be that compound **1** exists as a zwitterion, and its OH group is deprotonated to form a quinolinium cation. The resulting zwitterionic form of the quinoline-TEMPO **1** should then be more hydrophilic than its uncharged isomer.

The relative hydrophobicities of probes **1** and **4** were evaluated in terms of their partitioning in an aqueous neutral micellar solution of reduced Triton-X 100. Their reactions with TROLOX in this medium were followed by EPR and are shown in Figure 4.

Hydrogen abstraction from the hydrophilic TROLOX by either probe can be assumed to take place in the aqueous phase of the micellar system. The rates of hydrogen abstraction do not vary with the nature of the TEMPO derivative, as confirmed by the first-order exponential decay curves for both probes, which yielded very similar rate constants. However, their different plateau values at the end of the reactions point to different final concentrations of **1** and **4** in the aqueous phase. The intensity of the normalized EPR signal of **1** decayed by nearly 65% at the end of the reaction. By contrast, in the case of compound **4**, the observed decay was only 35% (Fig. 4). Assuming that the hydrophilic TROLOX, responsible for quenching radicals **1** and **4**, was overwhelmingly present in the aqueous phase, we conclude that 65% of probe **1** and 35% of probe **4** were present in water. Thus, this simple method allowed us to estimate partitioning ratios between the hydrophobic micelle and water of 0.35/0.65–0.5 and 0.35/0.65–1.9 for compounds **1** and **4**, respectively, indicative of the greater hydrophobic character of compound **4** when compared with **1**.

3. Conclusion

In summary, attachment of a TEMPO fragment to a tetraphenylpyridinium skeleton leads to two new, dual (fluorescent and spin) probes for hydrogen abstraction processes. Like quinoline-TEMPO probe **1**, in the pyridinium system **3**, the TEMPO fragment is attached to the chromophore by an ester function. Blank experiments indicated that this ester functionality was more resistant to hydrolysis in the case of probe **1** than of probe **3**. Replacement of the ester in **3** by an amide function led to the more stable probe **4**, which was not hydrolyzed under the same experimental conditions nor in water at pH 7 or 4. Compound **4** was also more hydrophobic than probe **1**, as shown by estimates of relative partitioning in an aqueous micellar solution of reduced Triton-X 100. Thus, the stable pyridinium salt **4** should complement the quinoline-TEMPO derivative **1** as a more hydrophobic hydrogen-abstracting probe, in those cases where the antioxidant activity of hydrophobic substrates in micro-heterogeneous systems is evaluated.

Development of new dual probes with variously substituted pyridinium fluorophores is presently under way in our laboratories.

4. Experimental section

The melting points of compounds **3** and **4** were recorded with a capillary Electrothermal apparatus and were not corrected. Steady-state fluorescence measurements were performed in a Spex Fluorolog 1681 spectrofluorimeter. EPR experiments were carried out with a Bruker EMX 1572 spectrometer that operates in the X-band (9.2–9.9 GHz) equipped with a thermostatized cavity. UV–visible spectra were collected with a HP-8453 diode array spectrometer.

Chemicals were purchased from Sigma–Aldrich and used as received.

The *N*-(4-carboxyphenyl)-2,4,6-triphenylpyridinium perchlorate was prepared by refluxing equimolar amounts of

2,4,6-triphenylpyrylium perchlorate and 4-carboxyaniline in ethanol for 48 h.[25] The fluoroborate pyridinium salt was prepared following the same procedure. Excitation of this salt (10 μM) in MeCN at 355 nm led to an emission band at 466 nm.

4.1. 4-{4-[(2,4,6-Triphenylpyridinio)benzoyloxy]}-2,2,6,6-tetramethylpiperidine-1-oxyl perchlorate 3

A solution of *N*-(4-carboxyphenyl)-2,4,6-triphenylpyridinium perchlorate (400 mg, 0.76 mmol), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (217 mg, 1.26 mmol), 4-dimethylaminopyridine (60 mg, 0.49 mmol), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (160 mg, 0.83 mmol) in acetonitrile (20 mL) was warmed to 50 °C for 2 h. The solvent was then rotary evaporated, to the residue was added diethyl ether (30 mL) and the precipitate was filtered to give 410 mg (79% yield) of the product, purified by flash chromatography (silica 60H, acetone as eluent). The light yellow product **3** melted at 239–240 °C. Anal. Found: C, 68.13; H, 5.72; N, 3.81%; required for $C_{39}H_{38}ClN_2O_7$: C, 68.66; H, 5.61; N, 4.11%. IR (KBr) ν_{max} 1719 (CO), 1610, 1600, 1280, 1250, 1090 (broad, ClO_4) cm^{-1}.

4.2. 4-{4-[(2,4,6-Triphenylpyridinio)benzamido]}-2,2,6,6-tetramethylpiperidine-1-oxyl tetrafluoroborate 4

A solution of *N*-(4-carboxyphenyl)-2,4,6-triphenylpyridinium fluoroborate (644 mg, 1.25 mmol), 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (258 mg, 1.42 mmol), 4-dimethylaminopyridine (178 mg, 2.15 mmol), and *N*,*N*′-dicyclohexycarbodiimide (298 mg, 1.45 mmol) in $CHCl_3$ (50 mL) was stirred at 25 °C for 24 h. The separated solid was filtered off, the filtrate was rotary evaporated, and diethyl ether was added to the residue to precipitate the light yellow product that was filtered to give 636 mg (76% yield) of crude **4**, purified by column chromatography (silica gel, 60H, acetone), mp 180–183 °C. IR (KBr) ν_{max} 1656 (CO), 1620, 1598, 1551, 1497, 1279, 1240, 1100 (broad, BF_4) cm^{-1}. Anal. Found: C, 69.97; H, 5.98; N, 6.98%; required for $C_{39}H_{39}BF_4N_3O_2$: C, 70.06; H, 5.88; N, 6.29%.

4.3. Kinetic measurements

The kinetics of the reactions between probes **3** or **4** and a 100-fold excess TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was followed under pseudo-first-order conditions, by monitoring the increase of the fluorescence band of the probe at 466 nm after excitation at 355 nm in acetonitrile. The product band was identical to the emission of the *N*-(4-carboxyphenyl)-2,4,6-triphenylpyridinium fluoroborate, after excitation at 355 nm in acetonitrile. The observed rate constant was obtained by applying Eq. 1,

$$\ln\left(\frac{I^{\infty}-I^{0}}{I^{\infty}-I^{t}}\right) = kt \quad (1)$$

where I^{∞}, I^{0}, and I^{t} represent the fluorescence intensities in the plateau region, initially, and at time *t*, respectively.

The reactions between probes **3** or **4** and TROLOX in acetonitrile were followed by EPR under pseudo-first-order conditions at 25 °C, by monitoring the decrease with time of the first signal of the nitroxyl radical triplet. In an analogous way, the kinetics of hydrogen abstraction from TROLOX of probes **1** or **4** in an aqueous solution of reduced Triton-X 100 was followed by EPR.

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