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ORIGINAL PAPER

TEMPO-Attached Pre-fluorescent Probes Based on Pyridinium Fluorophores

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Abstract The photophysical behavior of three pyridinium-derived fluorophores, the N-aryl-2,4,6-triphenylpyridinium, the N-aryl-5,6-dihydro-2,4-diphenylbenzo[*h*]quinolinium and the N-aryl-5,6,8,9-tetrahydro-7-phenyldibenzo[*c,h*]acridinium perchlorates, was investigated. Comparison of their fluorescence quantum yields led to the preparation of a novel, more sensitive pyridinium-based, TEMPO-attached prefluorescent probe for H-abstraction processes, the N-{4-[4-(N-oxyl-2,2,6,6-tetramethylpiperidinyl)carbonylamino]phenyl}-5,6,8,9-tetrahydro-7-phenyldibenzo[*c,h*]-acridinium perchlorate.

Keywords Pre-fluorescent probe · Pyridinium · Pyrylium · Fluorescence · Nitroxide · EPR

Introduction

Pre-fluorescent probes with an attached TEMPO (2,2,6,6-tetramethylpyperidin-1-oxyl) fragment have been employed for sensing H-donor molecules and carbon-centered radicals [1]. Their interaction with these molecules or radicals may be followed by EPR or fluorescence spectroscopy [2]. While the attached TEMPO fragment secures their EPR response, their sensitivity to fluorescence measurements relies on the fluorophore that it is linked to the spin moiety. The presence of the TEMPO radical quenches the fluorophore emission in the molecule. Conversion, by hydrogen abstraction, of the nitroxyl radical into a diamagnetic hydroxylamine restores this emission, by eliminating the spin-exchange process responsible for the fluorescence quenching [3].

At the same time, the TEMPO triplet observed in the EPR spectrum of the radical probe also disappears. Since both processes occur simultaneously, the hydrogen abstraction may be followed by either an increase of the fluorescence of the reaction, or the decrease of the triplet intensity in its EPR spectrum [4, 5].

In looking for new, versatile fluorophores, capable of giving rise to new pre-fluorescent probes, we have described the preparation and properties of a TEMPO fragment attached to a triphenylpyridinium fluorophore. The resulting probe, the 4-{4-[(2,4,6-triphenylpyridinio)benzamido]}-2,2,6,6-tetramethylpiperidine-1-oxyl tetrafluoroborate successfully monitored the hydrogen abstraction of the H-donor TROLOX in homo- and microheterogeneous environments, both by EPR and fluorescence measurements [4].

However, its poor fluorescence quantum yield prompted the investigation of other related, more strongly fluorescent, pyridinium ring systems. By modification of the 2,4,6-triphenylpyridinium ring, through the introduction of one or two ethylene bridges, we hoped to obtain analogs of that probe with improved photophysical properties.

In the present report, we compare the three fluorophores **1b**, **2b** and **3b**, prepared from the corresponding pyrylium perchlorates **1a**, **2a** and **3a**, respectively (Scheme 1).

The result of the photophysical comparison parameter such as fluorescence quantum yield suggested to us the preparation of probe **3d**, as the most promising of the three ring-systems (Scheme 2).

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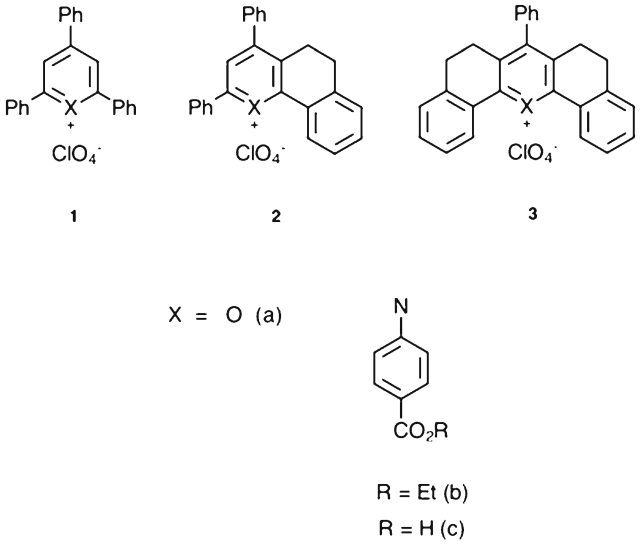


Scheme 1 Structures of the pyrylium precursors and the pyridinium-derived fluorophores investigated in this work

The evaluation of probe **3d** as a dual, prefluorescent probe for hydrogen abstraction was successfully carried out with TROLOX, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analog of vitamin E widely used as hydrogen donor standard, [4]) thus confirming our expectations of its increased sensitivity as a fluorophore, coupled with an adequate response as a radical H-abstractor.

Materials and Methods

Melting points were obtained with a Kruss Optonic equipment, and were not corrected.

FTIR spectra were recorded in a Bruker IFS 66v and in a Perkin Elmer series 2000 equipments. The UV–vis spectra were recorded with a Cary 50 spectrometer. NMR spectra were obtained with a 400-MHz Bruker Avance equipment. EPR spectra were obtained with a Bruker EMX series 1572 equipment. Fluorescence spectra were recorded with a Perkin Elmer LS55 spectrofluorimeter. Fluorescence lifetimes (τ) were obtained with an EasyLife-X PTI system, using a 350 nm LED pulse excitation, and the lifetimes were calculated with the EasyLife integrated software.

Perchloric acid, acetophenone, benzaldehyde, α-tetralone, sodium hydroxide, 4-aminoTEMPO, ethyl 4-aminobenzoate, di-chlorohexylcabodiimide, N,N-dimethyl-4-aminopyridine, were purchased from Sigma-Aldrich and were used as received. All solvents were of spectroscopic grade.

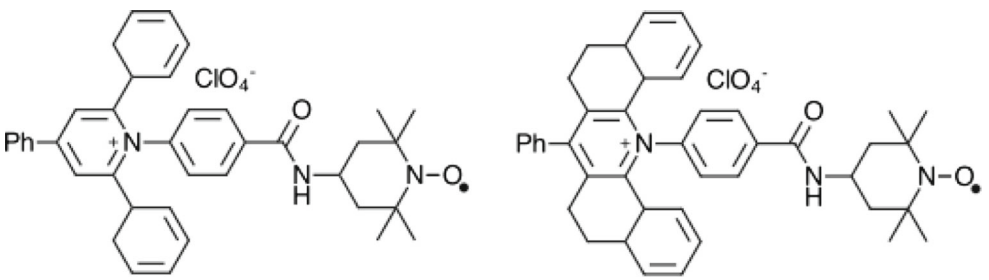
2,4,6-Triphenylpyrylium perchlorate (**1a**), 5,6-dihydro-2,4-diphenylbenzo[*h*]chromenylium perchlorate (**2a**) and 5,6,8,9-tetrahydro-7-phenyldibenzo[*c,h*]xanthylium perchlorate (**3a**) were prepared by methods reported in literature [6–10].

N-(4-ethoxycarbonylphenyl)-2,4,6-triphenylpyridinium perchlorate (1b): To a stirred solution of the corresponding pyrylium salt **1a** (0.49 g,1.20 mmol) dissolved in CH_3CN (50 mL) was added triethylamine (0.06 g, 0.60 mmol) at 40 °C. Subsequently ethyl 4-aminobenzoate (0.22 g, 1.32 mmol) was added to the reaction mixture, which was stirred under reflux for 6 h. After cooling down, the solvent was evaporated under vacuum to afford a brown crude oil. Addition of ethanol (15 mL) afforded the crude pyridinium salt, which separated as a solid, purified by column chromatography (*n*-hexane/ethyl acetate 4:1) to give the N-(4-ethoxycarbonylphenyl)-2,4,6-triphenylpyridinium perchlorate as a pale yellow solid (0.41 g, 62 % yield), m.p. 259–261 °C. HRMS (TOF MS ES+) m/z calcd. for $C_{32}H_{26}NO_2$ [M - ClO_4^-] 456.1958 found 456.1964.

^{1}H NMR ($CDCl_3$) δ=1.32 (t, 3H, J=7.1 Hz, CH_3), 4.28 (q, 2H, J=7.1 Hz, CH_2), 7.21–7.33 (m, 6H, Ar), 7.42 (m, 8H, Ar), 7.53 (m, 1H, Ar), 7.75 (m, 4H, Ar), 7.93 (s, 2H, Ar). ^{13}C NMR ($CDCl_3$) δ =14.3, 61.8, 126.7, 128.7, 129.0, 129.7, 129.9, 130.2, 130.4, 131.6, 132.2, 132.8, 134.8, 142.5, 156.3, 158.1. 165.0.

N-(4-ethoxycarbonylphenyl)- 5,6-dihydro-2,4-diphenylbenzo[*h*]quinolinium perchlorate (2b): Following the same procedure described for compound **1b**, the quinolinium perchlorate **2b** was prepared from the benzochromenylium salt **2a** in 22 % yield as a white solid, m.p. 285–288 °C. HRMS (TOF MS ES+) m/z calcd. for $C_{34}H_{28}NO_2$ [M - ClO_4^-] 482.2115 found 482.2120.

Scheme 2 Structure of the prefluorescent probes **1d** and **3d**



1d **3d**

^{1}H NMR ($CDCl_3$) δ=1.37 (t, 3H, J=7.1 Hz, CH_3), 3.02 (s, 4H, CH_2CH_2), 4.34 (q, 2H, J=7.1 Hz, CH_2), 6.72 (m, 1H, Ar), 6.82 (m, 1H, Ar), 7.17–7.32 (m, 7H, Ar), 7.41–7.54 (m, 5H, Ar), 7.58 (m, 2H, Ar), 7.75 (m, 1H, Ar), 7.87 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$) δ=14.3, 61.9, 126.4, 126.9, 128.3, 128.5, 129.0, 129.1, 129.5, 129.8, 129.96, 130.03, 130.3, 130.6, 131.6, 131.8, 133.0, 136.1, 138.9, 143.2, 143.9, 150.8, 153.8, 158.1, 165.0.

N-(4-ethoxycarbonylphenyl)-5,6,8,9-tetrahydro-7-phenyldibenzo[*c,h*]acridinium perchlorate (3b): A mixture of the 5,6,8,9-tetrahydro-7-phenyldibenzo[*c,h*]xanthylium perchlorate (**3a**) (0.230 g, 0.50 mmol), ethyl 4-aminobenzoate (0.091 g, 0.55 mmol) and Et_3N (0.036 g, 0.36 mmol) in absolute EtOH (5 mL) was placed into a MW-vial. The reaction mixture was heated to 180 °C and stirred at 700 rpm for 1 h. The mixture was concentrated under vacuum to give a brown oil, which was purified by column chromatography (*n*-hexane/ethyl acetate=4/1) to give the perchlorate **3b** as a white solid, m.p 284–286 °C, in 6 % yield. HRMS (TOF MS ES+) m/z calcd. for $C_{36}H_{30}NO_2$ [M - ClO_4^-] 508.2277 found 508.2277 ^{1}H NMR ($CDCl_3$) δ=1.43 (t, 3H, J=7.1 Hz CH_3), 2.73 (m, 4H, CH_2CH_2), 2.95 (m, 4H, CH_2CH_2), 4.42 (q, 2H, J=7.1 Hz, CH_2), 6.51 (d, 2H, J=8.1 Hz, Ar), 6.84 (m, 3H, Ar), 7.16–7.30 (m, 4H, Ar), 7.44 (m, 2H, Ar), 7.48–7.61 (m, 5H, Ar), 8.34 (d, 2H, J=8.1 Hz, Ar).

N-{4-[4-(N-oxyl-2,2,6,6-tetramethylpiperidinyl) carbonylamino]phenyl}-2,4,6-triphenylpyridinium perchlorate (1d): The N-(4-ethoxycarbonylphenyl)-2,4,6-triphenylpyridinium perchlorate (**1b**) (0.69 g, 1.25 mmol) was refluxed with NaOH (0.06 g, 1.5 mmol) in ethanol (100 mL) for 2 h. The resulting dark solution was neutralized with concentrated $HClO_4$, the solvent was rotary evaporated, water (20 mL) was added and the insoluble N-(4-carboxyphenyl)pyridinium perchlorate **1c** filtered and dried to give the N-(4-carboxyphenyl)-2,4,6-triphenylpyridinium perchlorate **1c** (0.57 g, 86 % yield), m.p. > 290 °C, ir (KBr) 3400–3000 (broad, OH), 1710 (C=O), 1610, 1600, 1290, 1050 (broad, ClO_4^-), 810, 760, 700 cm^{-1}. This crude carboxylic acid was employed without any further purification in the subsequent reaction.

A solution of the crude N-(4-carboxyphenyl)pyridinium perchlorate **1c** (0.53 g, 1 mmol), 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-aminoTEMPO, 0.26 g, 1.4 mmol), 4-dimethylaminopyridine (DMAP, 0.18 g, 2.2 mmol) and N,N-dicyclohexylcarbodiimide (DCC, 0.3 g, 1.5 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 added to the residue to precipitate the crude TEMPO-attached pyridinium perchlorate **1d**, purified by column chromatography (silica, MeOH/CH_2Cl_2 (1:4) as eluent) to give the N-{4-[4-(N-oxyl-2,2,6,6-tetramethylpiperidinyl)carbonylamino]phenyl}-2,4,6-triphenylpyridinium perchlorate **1d,** obtained as a pale yellow solid (0.57 g, 78 % yield), m.p. 222–226 °C. Ir (KBr) 1620 (CONH), 1598, 1095 (ClO_4^-) cm^{-1}.

Radical **1d** was further characterized by its epr spectrum in acetonitrile, which showed a triplet with an a_N value of 16.6 G. ^{1}H- and ^{13}C-NMR spectra of the radical could not be obtained, due to its paramagnetic nature.

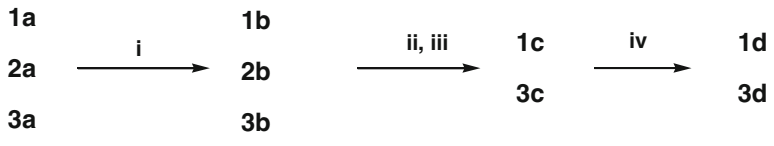
N-{4-[4-(N-oxyl-2,2,6,6-tetramethylpiperidinyl) carbonylamino]phenyl}-5,6,8,9-tetrahydro-7-phenyldibenzo[*c,h*]acridinium perchlorate (3d): The preparation of perchlorate **3d** was achieved following the same method described for perchlorate **1d**, by hydrolyzing the ester **3b** to give the N-(carboxyphenyl) derivative **3c**, which was employed without any further purification in the subsequent conversion to the amide **3d**.

The N-{4-[4-(N-oxyl-2,2,6,6-tetramethylpiperidinyl) carbonylamino]phenyl}-5,6,8,9-tetrahydro-7-phenyldibenzo[*c,h*]acridinium perchlorate (**3d**), m.p. 219–224 °C, was obtained in 70 % yield after purification of the crude product by column chromatography (SiO_2) with a MeOH/CH_2Cl_2 (1:4) as eluent. IR (KBr) 3326, 2920, 2850, 1626 (NC=O), 1574, 1087 (ClO_4^-) cm^{-1}.

Radical **3d** was further characterized by its epr spectrum in acetonitrile, which showed a triplet with an a_N value of 16.6 G and a *g*-factor of 2.001095. ^{1}H- and ^{13}C-NMR spectra of the radical could not be obtained, due to its paramagnetic nature.

Photophysical Measurements

The photophysical characterization of the N-(ethoxycarbonylphenyl)pyridinium perchlorates **2a**, **2b** and **2c** was carried out by UV-visible and static fluorescence spectroscopies. The fluorescence quantum yields Φ were determined by the comparative methodology [11, 12] using quinine sulfate as the reference fluorophore.



Scheme 3 Synthetic route for the preparation of the TEMPO-attached probes **1d** and **3d**

Results and Discussion

Preparation of probes **1d** and **3d** started from pyrylium salts **1a** and **3a**, respectively, which were converted into the corresponding N-(ethoxycarbonylphenyl) derivatives **1b** and **3b** by treatment with ethyl 4-aminobenzoate. Hydrolysis of esters **1b** and **3b** led to the corresponding carboxylic acids **1c** and **3c**, which were finally converted into the TEMPO-attached amides **1d** and **3d**, respectively (Scheme 3).

In order to compare the efficiency of the three pyridinium-based fluorophores (the 2,4,6-triphenylpyridinium, the 5,6-dihydro-2,4-diphenylbenzo[*h*]quinolinium and the 5,6,8,9-tetrahydro-7-phenyldibenzo[*c,h*]acridinium ring systems), the perchlorates **1b**, **2b** and **3b** were prepared and their fluorescence quantum yields and lifetimes were determined. Since a direct determination of the quantum yields of the TEMPO-attached probes could not be carried out, due to the fluorescence-quenching effect of the attached radical, their quantum yields were determined indirectly, by assuming them to be the same of the corresponding esters **1b**, **2b** and **3b.** The obtained fluorescence quantum yields and lifetimes (see below) indicated that the most sensitive TEMPO-attached prefluorescent probe would be the acridinium derivative **3d**. For this reason, we concentrated our efforts in the preparation of only the least (**1d**) and the most sensitive probe (**3d**) of the series.

Conversion of the starting pyrylium perchlorate into the corresponding N-(4-ethoxycarbonylphenyl)pyridinium salts **1b**, **2b** and **3b**, by reaction with ethyl 4-aminobenzoate had yields that depended heavily on the pyrylium-based system. Yields decreased with the increased steric hindrance of these systems: for compound **1b**, reflux in acetonitrile for 6 h, catalysed by the addition of triethylamine, sufficed to give the product in 62 % yield; for compound **2b**, the yield under the same conditions decreased to 22 %; finally for salt **3b**, the reaction required heating all reagents in ethanol in a microwave reactor for 1 h at 180 °C, and the yield after chromatographic purification was only 6 %.

The ethyl esters **1b**, **2b** and **3b** were hydrolysed and the obtained carboxylic acids were converted into the TEMPO-attached probes by reaction with 4-aminoTEMPO, in the presence of 4-(N,N-dimethylamino)pyridine (DMAP) and dicyclohexylcarbodiimide (DCC).

Table 1 Spectral and photophysical properties of pyridiniums **1b, 2b and 3b**

Compound	**1b**	**2b**	**3b**
Φ	0.06	0.16	0.23
τ (ns)	<0.1	0.1	0.4
λ_{max}^{abs} (nm)	310	350	380
λ_{max}^{em} (nm)	440	440	433

Spectral and Photophysical Measurements

The absorption and emission spectra of the pyridinium-based esters **1b**, **2b** and **3b** in acetonitrile are reproduced in Fig. 1.

As can be seen, there is a small bathochromic shift of the longest-wavelength band of these salts, as the ring-system rigidity increases **1b** (310 nm)<**2b** (350 nm)<**3b** (380 nm). The emission wavelengths do not differ appreciably. By contrast, the form and intensity of the emission bands are rather different for the three fluorophores. As the rigidity increases, so does the intensity of the emission band, which also becomes narrower in the more rigid dibenzoacridinium system **3b**, than in the less rigid triphenylpyridinium **3a**.

Table 1 compares the quantum yields, lifetimes, and absorption and emission maxima of the three fluorophores.

Quantum yields and lifetimes increased with the rigidity of the pyridinium system, attaining the largest values in the case of fluorophore **3b**. This observation directed our efforts

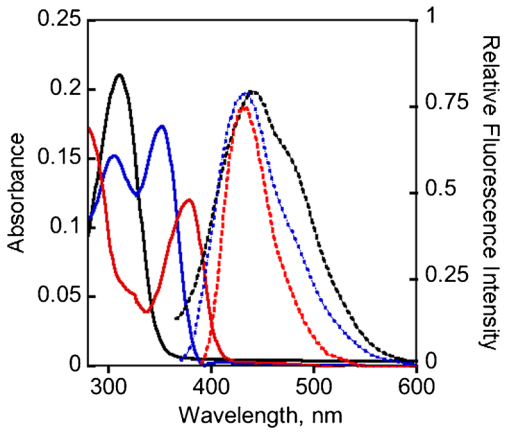


Fig. 1 Absorption and emission spectra of esters **1b** (——), **2b** (——) and **3b** (——) in acetonitrile. Emission spectra of compounds **1b** (- -), **2b** (– –) and **3b** (– –) were obtained by excitation at 310, 350 and 380 nm, respectively

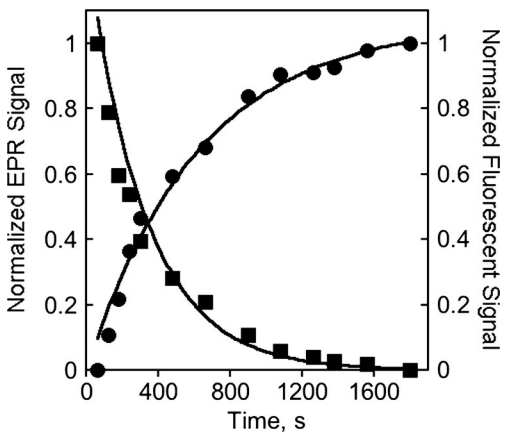


Fig. 2 Kinetics of the reaction of probe **3d** (10 μM) with TROLOX (10 mM) in acetonitrile at 25 °C, followed by fluorescence (●) (λ_{exc}= 380 nm, λ_{em}=430 nm) and epr (■)

toward the preparation of the TEMPO-attached probe **3d**, since it should be the most sensitive fluorescent probe of the series.

The evaluation of compound **3d** as a dual probe was carried out by following its reaction with TROLOX in acetonitrile. This hydrogen-abstraction process could be followed both by the restoration of the fluorescence of the probe, and/or by the disappearance of the TEMPO triplet in its epr spectrum. Figure 2 depicts both kinetic processes. The obtained symmetrical curves are an evidence of the dual behavior of the probe in H-abstraction processes.

Conclusion

In conclusion, the previous description of a prefluorescent probe for hydrogen-abstraction processes based on a TEMPO fragment attached to a pyridinium fluorophore [4] was further developed in the present communication.

A simple modification of the substituted pyridinium moiety, led to a considerable improvement of its sensitivity as a fluorophore. The reason for this increased performance may be ascribed to the greater rigidity of the resulting N-arylpyridinium systems, when their 2,6-diphenyl substituents have their rotation restricted by ethylene bridges. This led to moderate bathochromic shifts of the longest-wavelength absorption band of the probe fluorophore. More important, however, the same effect led to almost four-fold increase in the quantum yield of fluorescence in the most rigid ring-system **3b**. The corresponding prefluorescent probe **3d** proved as effective as its analog **1d**, as a hydrogen-abstractor with a dual spectroscopic response, with the advantage of being a much more sensitive fluorophore.

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Conflict of Interest The authors declare that they have no conflict of interest

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