Synthesis and potential use of 1,8-naphthalimide type \( ^1 \)O\(_2\) sensor molecules

Tamás Kálai, Éva Hideg, Ferhan Ayaydın and Kálmán Hideg*

New double (fluorescent and spin) sensor molecules (4 and 7) were synthesized and their responses to ROS \textit{in vitro} are reported with perspectives of plant physiology use \textit{in vivo}.

Please check this proof carefully. Our staff will not read it in detail after you have returned it.

Translation errors between word-processor files and typesetting systems can occur so the whole proof needs to be read. Please pay particular attention to: tabulated material; equations; numerical data; figures and graphics; and references. If you have not already indicated the corresponding author(s) please mark their name(s) with an asterisk. Please e-mail a list of corrections or the PDF with electronic notes attached – do not change the text within the PDF file or send a revised manuscript.

Please bear in mind that minor layout improvements, e.g. in line breaking, table widths and graphic placement, are routinely applied to the final version.

We will publish articles on the web as soon as possible after receiving your corrections; no late corrections will be made.

Please return your final corrections, where possible within 48 hours of receipt, by e-mail to: pps@rsc.org

Reprints—Electronic (PDF) reprints will be provided free of charge to the corresponding author. Enquiries about purchasing paper reprints should be addressed via: http://www.rsc.org/publishing/journals/guidelines/paperreprints/. Costs for reprints are below:

<table>
<thead>
<tr>
<th>No of pages</th>
<th>Cost (per 50 copies)</th>
<th>Each additional</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–4</td>
<td>£225</td>
<td>£125</td>
</tr>
<tr>
<td>5–8</td>
<td>£350</td>
<td>£240</td>
</tr>
<tr>
<td>9–20</td>
<td>£675</td>
<td>£550</td>
</tr>
<tr>
<td>21–40</td>
<td>£1250</td>
<td>£975</td>
</tr>
<tr>
<td>&gt;40</td>
<td>£1850</td>
<td>£1550</td>
</tr>
</tbody>
</table>

Cost for including cover of journal issue: £55 per 50 copies
Dear Author,

Paper No. c2pp25253h

Please check the proofs of your paper carefully, paying particular attention to the numerical data, tables, figures and references.

When answering the queries below please ensure that any changes required are clearly marked on the proof. There is no need to e-mail your answers to the queries separately from the rest of your proof corrections.

Editor’s queries are marked like this [Q1, Q2, ...], and for your convenience line numbers are indicated like this [5, 10, 15, ...].

Many thanks for your assistance.

<table>
<thead>
<tr>
<th>Query</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>For your information: You can cite this article before you receive notification of the page numbers by using the following format: (authors), Photochem. Photobiol. Sci., (year), DOI: 10.1039/c2pp25253h.</td>
</tr>
<tr>
<td>Q2</td>
<td>Please carefully check the spelling of all author names. This is important for the correct indexing and future citation of your article. No late corrections can be made.</td>
</tr>
<tr>
<td>Q3</td>
<td>Ref. 25: Please indicate where ref. 25 should be cited in the text.</td>
</tr>
<tr>
<td>Q4</td>
<td>Ref. 29: Can this reference be updated yet?</td>
</tr>
</tbody>
</table>
Synthesis and potential use of 1,8-naphthalimide type $^{1}\text{O}_2$ sensor molecules†

Tamás Kálati,a Éva Hideg,b,c Ferhan Ayaydin d and Kálmán Hideg*a

New double (fluorescent and spin) sensor molecules containing 4-amino substituted 1,8-naphthalimide as a fluorophore and a sterically hindered amine (pre-nitroxide) or pyrrole nitroxide as a quencher and radical capturing moiety were synthesized. All sensors were substituted with a diethylaminoethoxy side-chain to increase the water solubility. Steady state fluorescence properties of these compounds and their responses to ROS in vitro are reported with perspectives of plant physiology use in vivo.

Emerging biological applications demanded the synthesis of new fluorophore-nitroxide acceptor–donor molecules. New probes included various nitroxide moieties (nitronyl-, pyrrolidine-, piperidine-13) coupled to diverse fluorophores (acridine, cyanine dye, dansyl, fluorescamine, BODIPY, Nile-red, CdSe quantum dots and naphthalimides). Our aim was to fuse nitroxide to a fluorophore more suitable for plant biology applications than dansyls which need ultraviolet wavelengths for fluorescence excitation. Nitroxides bound to 4-substituted-1,8-naphthalimides were recently reported to quench fluorescence via triplet deactivation. Bojinov and co-workers found that 4-substituted-1,8-naphthalimides bound to pre-nitroxide had increased photostability, due to the presence of the sterically hindered amine ring. These compounds produced increased fluorescence in the presence of protons and metal ions (Cu$^{2+}$, Pb$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Co$^{2+}$). The advantage of 4-substituted-1,8-naphthalimide fluorophores is that substituents can be introduced easily into the imide nitrogen and into the naphthalene ring. In addition to the possibility of introducing new substituents into the naphthalimide ring selectively, longer (>400 nm) fluorescence excitation wavelengths of 4-aminonaphthalimide also appeared advantageous. The advantage of sensors excitable at longer wavelengths over ones needing ultraviolet excitation includes better penetration of fluorescence excitation into tissues and a smaller risk of damage by high energy irradiation. Our aim was to develop new 4-aminonaphthalimide based molecules using visible fluorescence excitation and having similar ROS selectivity as previously-described dansyl derivatives. This idea was supported by the fact that both dansyl and naphthalimide fluorophores contained a naphthalene chromophore.

In this paper we describe the conversion of 4-nitro-1,8-naphthalic-anhydride to imide and selective replacement of the 4-nitro group of the aromatic ring with a pyrrole nitroxide or a diethylaminoethylamine moiety respectively.

Introduction

Optical sensors for biomolecules and biochemical processes are widely used in biochemical and medical studies. Detection based upon fluorescence has received much attention and significant progress has been made both in fluorescence instrumentation and in the synthesis of novel fluorophores. Fluorophores combined with nitroxide free radicals or their precursors offer more advanced application than simple fluorophores. Quenching the singlet and triplet states of fluorophores by a nitroxide allows the detection of redox and free radical processes: when the nitroxide function is reduced to hydroxylamine, the fluorescence is quenched again. This principle was utilized in our earlier design of reactive oxygen species (ROS) sensitive reporter molecules in which a pre-nitroxide, a sterically hindered pre-cursor amine, was attached to a fluorophore. In these sensors oxidation of amine by ROS resulted in nitroxide formation as well as partial fluorescence quenching.

The function of our previously developed dansyl fluorophore-containing sensor is based on that only $\alpha,\alpha,\alpha',\alpha'$-tetra-substituted pyrrolines or piperidines can form a stable quencher (nitroxide) upon oxidation.

†Electronic supplementary information (ESI) available: Supplementary figure: Green and red fluorescence intensities over comparable regions in Fig. 4C and 4F to support the idea that compound 4 – unlike 7 – penetrates chloroplasts. See DOI: 10.1039/c2pp25253h

*Department of Organic and Medicinal Chemistry, University of Pécs, Pécs, Hungary.
E-mail: kalmant@kultek.hu; Fax: +36 72 503 663; Tel: +36 72 503 621
†Institute of Biology, University of Pécs, Pécs, Hungary.
E-mail: hideg@kultek.hu; Fax: +36 72 503 634; Tel: +36 72 503 621
‡Institute of Plant Biology, Biological Research Center, Szeged, Hungary.
Fax: +36 72 505 304; Tel: +36 72 505 417
§Cellular Imaging Laboratory, Biological Research Center, Szeged, Hungary.
E-mail: ferhan@brc.hu; Fax: +36 72 505 304; Tel: +36 72 505 417

This journal is © The Royal Society of Chemistry and Owner Societies 2012
The steady state fluorescence properties of these new compounds and their response to ROS in vitro and in vivo including the scope and limitation of their utilization in plant physiology are also presented.

Materials and methods

Chemistry

Treatment of 4-nitronaphthalene anhydride with diethylaminomethyl or paramagnetic allylic amine\(^\text{18}\) in EtOH at reflux temperature gave compounds 2 and 5, respectively. The reaction of compounds 2 and 5 with paramagnetic amine and diethylaminomethylamine in DMF at ambient temperature furnished compounds 3 and 6. Reduction of these paramagnetic compounds with iron powder in acetic acid\(^\text{19}\) yielded compounds 4 and 7 with a hydrophilic moiety – the diethylaminomethyl side chain – and a reactive oxygen species (ROS) trapping moiety, the 2,2,5,5-tetramethylpyrroline ring (Scheme 1). To access some information on ROS trapping of compounds 4 and 7 we synthesized a paramagnetic aromatic amine 10 as a model compound. The paramagnetic aromatic amine 9 was synthesized by reduction of the Schiff base of aniline and aldehyde\(^\text{20}\) with NaBH\(_3\)CN in acetonitrile in the presence of acetic acid. The paramagnetic aromatic amine was reduced to sterically hindered amine 10 by iron powder in acetic acid as described above (Scheme 2).

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on a Fisons EA 1110 CHNS elemental analyzer. Mass spectra were recorded on a Thermoquest Automass Multi. \(^1\)H NMR spectra were recorded with a VarianUNITYINova 400 WB spectrometer. Chemical shifts are referenced to Me\(_4\)Si. Measurements were run at a 298 K probe temperature in CDCl\(_3\) solution. ESR spectra were taken on a Miniscope MS 200 in 10\(^{-4}\) M CHCl\(_3\) solution and all mono-radicals gave a triplet line \(\Delta\nu = 14.4\) G. Flash column chromatography was performed on Merck Kieselgel 60 (0.040-0.063 mm). Qualitative TLC was carried out on commercially available plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel GF254. Compounds 1,\(^\text{17}\) 3-aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-1-ylxoyl,\(^\text{18}\) 8,\(^\text{20}\) were prepared according to published procedures and other reagents were purchased from Aldrich.

Synthesis of 4-nitro-N-(2-diethylamino)ethyl)-1,8-naphthalimide (2) and 4-nitro-N-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl)-1,8-naphthalimide radical (5): To a suspension of 4-nitro-1,8-naphthalenedicarboxylic acid anhydride 1 (1.22 g, 5.0 mmol) in ethanol (20 mL) a solution of diethylaminomethylamine (580 mg, 5.0 mmol) or compound 3-aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-1-ylxoyl (845 mg, 5.0 mmol) in ethanol (5 mL) was added dropwise at ambient temperature. The resulting mixture was stirred at 60 °C for 4 h and the mixture was allowed to settle overnight. The precipitated product was filtered and washed with cold ethanol (2 mL) to give compound 2 or 5 which were used in the next step without further purification.

Compound 2: brown shiny crystals 1.05 g (62%), mp 129 °C, Ms (EI) \(m/z\) (%): 341 (M\(^+\), 1), 269 (3), 86 (100). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.81 (d, 1H, \(J = 5.2\) Hz), 8.71 (d, 1H, \(J = 6.8\) Hz), 8.67 (d, 1H, \(J = 8\) Hz), 8.38 (d, 1H, \(J = 8\) Hz), 7.96 (t, 1H, \(J = 6.8\) Hz), 4.36 (2H, t), 2.94 (t, 2H), (2.82, q, 4H), 1.61 (t, 6H). Anal. Calcd for compound 3-aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-1-ylxoyl (1.0 equiv.), rt, 60 °C, EtOH 4 h, then rt 12 h (53%); (c) Fe (10 equiv.), AcOH, 80 °C, 30 min then K\(_2\)CO\(_3\), 39-49%.

Scheme 1  Reagents and conditions: (a) H\(_2\)N(CH\(_2\))\(_2\)N(CH\(_3\))\(_2\) (1 equiv.), rt, 0 °C → rt, 1 h, 79%; (b) PhNH\(_2\) (1 equiv.), NaBH\(_3\)CN (1.5 equiv.), acetonitrile, 15 min, rt, then AcOH pH = 7, 1 h, rt, then NaOH, 44%; (c) Fe (10 equiv.), AcOH, 80 °C, 30 min then K\(_2\)CO\(_3\), 49-64%.

Scheme 2  Reagents and conditions: (a) PhCOCl (1 equiv.), Et\(_3\)N (1 equiv.), 
CH\(_2\)Cl\(_2\), 0 °C → rt, 1 h, 79%; (b) PhNH\(_2\) (1 equiv.), NaBH\(_3\)CN (1.5 equiv.), acetonitrile, 15 min, rt, then AcOH pH = 7, 1 h, rt, then NaOH, 44%; (c) Fe (10 equiv.), AcOH, 80 °C, 30 min then K\(_2\)CO\(_3\), 49-64%.
for C_{18}H_{19}N_{3}O_{4}: C 63.33; H 5.61; N 12.31; found: C 63.21; H 5.63; N 12.25.

Compound 5: brown crystals 1.04 g (53%), mp 225–227 °C, Ms (El) m/z (%): 394 (M^+ 5), 364 (4), 243 (59), 107 (100). Anal Calcd for C_{24}H_{30}N_{3}O_{5}: C 69.35; H 7.12; N 12.06; found: C 69.34; H 7.08; N 12.11.

Synthesis of 4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylaminomethyl)-N-[2-(diethylamino)ethyl]-1,8-naphthalimidine radical (3) and 4-(2-(diethylamino)ethylamino)-N-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl)-1,8-naphthalimidine radical (6): To a stirred solution of compound 2 (1.705 g, 5.0 mmol) or compound 4 (1.97 g, 5.0 mmol) in MeOH (10 mL) or compound 5 (1.97 g, 5.0 mmol) in DMF (30 mL) or compound 5 (1.97 g, 5.0 mmol) in acetonitrile (20 mL) NaBH₃CN (472 mg, 7.5 mmol) was added in one portion then the mixture was stirred at room temperature for 15 min. Then NaBH₄ was added to adjust the pH to 7 and the mixture was stirred for a further 1 h. After filtration of the mixture, the solid was evaporated off, water (20 mL) was added and the mixture was basified with NaOH to pH = 12 and then extracted with CH₂Cl₂ (3 × 20 mL). The organic phase was dried (MgSO₄), filtered and evaporated and the residue was purified by flash column chromatography (CHCl₃/EtO and CHCl₃-MeOH) to afford compounds 3 and 6.

Compound 3: 1.62 g (70%), yellow solid, mp 88–90 °C, Ms (El) m/z (%): 463 (M^+ <1), 362 (1), 86 (100). Anal Calcd for C_{27}H_{35}N_{4}O₃: C 69.95; H 7.61; N 12.09; found: C 69.88; H 7.63; N 12.06.

Compound 4: 272 mg (39%), yellow solid, mp 75–77 °C, Ms (El) m/z (%): 448 (M^+ <1), 376 (1), 122 (37), 86 (100). 1H NMR (DMSO-d₆) δ 8.0 (d, 1H, J = 8.4 Hz), 8.44 (d, 1H, J = 7.2 Hz), 8.25 (d, 1H, J = 8.8 Hz), 8.16 (s, 1H), 7.69 (t, 1H, J = 8.6 Hz), 6.66 (d, 1H, J = 8.8 Hz), 5.32 (s, 1H), 4.07 (2H, m), 2.60 (t, 2H), 2.53 (q, 4H), 1.83 (s, 1H), 1.27 (s, 6H), 1.07 (s, 6H), 0.96 (t, 6H). Anal Calcd for C_{27}H_{36}N_{2}O₄: C 69.95; H 7.61; N 12.09; found: C 69.84; H 7.45; N 12.03.

Compound 10: 225 mg (49%), white crystals, mp 97–99 °C, Ms (El) m/z (%): 230 (M^+ 4), 215 (11), 106 (100). 1H NMR (CDCl₃) δ 7.18 (t, 2H), 6.72 (t, 1H), 6.63 (d, 2H, J = 7.6 Hz), 5.49 (s, 1H), 3.73 (s, 2H), 1.34 (s, 6H), 1.26 (s, 6H). Anal Calcd for C_{18}H_{18}N₂O₅: C 78.21; H 9.63; N 12.16; found: C 78.15; H 9.59; N 12.03.

Synthesis of N-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-ylmethyl)aniline radical (9): To a stirred solution of 8 aldehyde (840 mg, 5.0 mmol) and aniline (465 mg, 5.0 mmol) in acetone (20 mL) NaBH₃CN (472 mg, 7.5 mmol) was added in one portion then the mixture was stirred at room temperature for 15 min. Then NaBH₄ was added to adjust the pH to 7 and the mixture was stirred for a further 1 h. After filtration of the mixture, the solid was evaporated off, water (20 mL) was added and the mixture was basified with NaOH to pH = 12 and then extracted with CH₂Cl₂ (3 × 20 mL). The organic phase was dried (MgSO₄), filtered and evaporated and the residue was purified by flash column chromatography to give the title compound 539 mg (44%) as a yellow solid, mp 147–149 °C, Ms (El) m/z (%): 245 (M^+ 17), 215 (9), 106 (100), 77 (61). Anal Calcd for C_{18}H_{17}N₂O₅: C 73.43; H 8.36; N 11.42; found: C 73.29; H 8.51; N 11.38.

Absorption and fluorescence spectroscopy

The UV spectra were taken with a Spectord 40 (Jena Analytic) to measure the optical density. The values were set OD < 0.05. The molar extinction coefficients (ε) at absorption maxima were obtained from the slope of absorbance vs. concentration using five solutions of different concentrations. Fluorescence spectra of compounds dissolved in EtOH were measured with a Perkin Elmer LS50B spectrofluorimeter using 3 nm (ex) and 5 nm (em) slits. Corrections for instrumental factors were made by a rhodamine B quantum counter and correction files supplied by the manufacturer. Quantum yields were referred to Rhodamine 101 dissolved in EtOH (λ_{ex} 450 nm, Φ = 1.0). The values were calculated by the equation Φ = ([I′]/[I]) [A/I] [n/z] [Φ], where I′, I, and Φ′ are the integrated emission, absorbance (at the excitation wavelength), and quantum yield of the reference sample, respectively. n′ is the refractive index of the solvent used for the reference sample. I, A, n, Φ are related to the sample with the same definitions applied to the reference sample. Results are listed in Table 1.

Response to ROS in vitro

Reactivity to singlet oxygen was measured using illuminated Rose Bengal as the O₂ source and the ESR signal of TEMPO from the TEMP + O₂ → TEMPO reaction as the O₂ detector. The reaction mixture contained 25 µM Rose Bengal, 1 mM TEMP. In the absence of light, there was no EPR signal detected from the above reaction. EPR triplet signals of TEMPO were detected after 5 min illumination with green TEMPO reaction as the O₂ detector.
towards singlet oxygen was characterized by monitoring the decrease in the ESR TEMPO signal due to the competition between the probes for the reaction with singlet oxygen.

The interaction between fluorescent sensors 4/HCl and 7/HCl and \( {^1}O_2 \) was also evaluated by measuring changes in their relative fluorescence emission (% quenching) in response to \( {^1}O_2 \) that was generated from illuminated Rose Bengal as above. To test whether fluorescence quenching was brought about by \( {^1}O_2 \) and not for other artifacts, experiments using Rose Bengal as the photosensitizer were repeated in the presence of the 5 mM NaN3, which is a \( {^1}O_2 \) quencher.23

Sensitivity to the hydroxyl radical was measured as fluorescence change of 4/HCl or 7/HCl upon incubation with \( \cdot OH \) from a Fenton reaction (10 µM EDTA, 10 µM FeSO4, 100 µM H2O2 and 100 µM ascorbate24) in 50 mM potassium phosphate buffer at pH 7.0. Reactivities to H2O2 or to \( \cdot OH \) were tested by incubating the compounds with these ROS for 15 min. In a separate experiment, sensors were tested with 100 µM H2O2 only. The xanthine/xanthine-oxidase system (75 µM/0.05 U mL\(^{-1}\)) was used to generate superoxide radicals and effects of 4/HCl or 7/HCl on the superoxide inducible absorption increase of nitro blue-tetrazolium were measured at 560 nm24 after 15 min.

Microlocalization of fluorescent sensors in plant leaves

Five mM solutions of 4, 7 (in 5 : 95 vol : vol ethanol : water) 4/HCl or 7/HCl (in water) were infiltrated into chlorophyll containing mesophyll cells of tobacco leaves as described earlier.26 Leaf segments were placed on microscope slides and were visualized using a 20× objective of an Olympus FV1000 laser scanning confocal microscope (LSM, Olympus Life Science). Chloroplasts and thylakoids were identified on the basis of chlorophyll fluorescence (argon laser excitation: 488 nm; detection: 650–750 nm) as described earlier,27 and localizations of 4, 7, 4/HCl and 7/HCl were detected by their fluorescence (argon laser excitation: 488 nm; detection: 500–600 nm).

Physiological responses of leaves to sensors

Responses of leaf tissue to the presence of sensors were evaluated by measuring the quantum yield of photosynthetic electron transport and activation of non-associative energy dissipating processes at various light intensities. Electron transport values were calculated from chlorophyll fluorescence yields using the MAXI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). Leaves infiltrated with

\[
\text{Table 1} \quad \text{Fluorescence characteristics of compounds} \; 3, 4, 6, 7
\]

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \lambda_{\text{abs}} ) (nm)</th>
<th>( \epsilon ) ( (1 \text{ mol}^{-1} \text{ cm}^{-1}) )</th>
<th>( \lambda_{\text{ex}} ) (nm)</th>
<th>( \lambda_{\text{em}} ) (nm)</th>
<th>( \Phi^e )</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>433</td>
<td>( 1.252 \times 10^4 )</td>
<td>449</td>
<td>517</td>
<td>0.03</td>
</tr>
<tr>
<td>4</td>
<td>438</td>
<td>( 1.564 \times 10^4 )</td>
<td>448</td>
<td>520</td>
<td>0.53</td>
</tr>
<tr>
<td>6</td>
<td>440</td>
<td>( 1.233 \times 10^4 )</td>
<td>448</td>
<td>526</td>
<td>0.03</td>
</tr>
<tr>
<td>7</td>
<td>439</td>
<td>( 1.489 \times 10^4 )</td>
<td>450</td>
<td>520</td>
<td>0.06</td>
</tr>
</tbody>
</table>

\( ^e \) Referred to Rhodamine 101 at 450 nm in EtOH, \( n = 3 \), accuracy ±10%.

Results and discussion

The fluorescence excitation and emission maxima of diamagnetic (4, 7) and paramagnetic (3, 6) derivatives are very similar, e.g. nitroxide does not cause any wavelength shift. However, the fluorescence is efficiently quenched by a nitroxide stable free radical in compounds 3 and 6 via triplet route deactivation (Fig. 1, Table 1).15 The quantum yields of diamagnetic
derivatives [4, 6] are higher, but upon protonation in aq. EtOH solution the fluorescence increases. It is in good accordance with the findings of Bojinov et al., proposing that in 4-amino-naphthalimides photoinduced electron transfer (PET) takes place decreasing the fluorescence intensity. Upon protonation the side chain amine oxidation potential increases, which prevents the electron transfer and hence the fluorescence increases. Zheng et al. recently reported that internal charge transfer (ICT) also has an influence on the fluorescence emission intensity, especially when the 4-dialkylamino group is part of a small ring.29 We found that upon protonation compound 4 exhibits 74% and compound 7 exhibits 760% fluorescence intensity increase, respectively (Fig. 1A and 1B). This observation is a cautionary sign, considering that the plant tissues and the growth media used in experiments are often acidic.

Studies on oxidative stress responses require ROS selective sensors. Compounds 4/HCl and 7/HCl reacted with singlet oxygen only (Fig. 2) and not with other ROS, such as superoxide radicals, hydroxyl radicals or hydrogen-peroxide (data not shown). However, reactions with singlet oxygen were not proportional to quenching of their fluorescence by $^{1}$O$_{2}$.

Although both fluorescent sensors (4/HCl, 7/HCl) and their non-fluorescent version (10) reacted with $^{1}$O$_{2}$ and effectively competed with TEMP in the EPR active TEMPO forming reaction (Fig. 2), this was only accompanied by a decrease in fluorescence quenching in 7/HCl but not in 4/HCl (Fig. 3). Fluorescence quenching of 7/HCl was markedly decreased by the presence of NaN$_{3}$ indicating that it was caused by $^{1}$O$_{2}$ (Fig. 3). Neither 4/HCl nor 7/HCl showed significant fluorescence quenching in the presence of ROS other than $^{1}$O$_{2}$ (Table 2). From these observations we concluded that a sterically hindered moiety (pre-nitroxide) has a role in $^{1}$O$_{2}$ quenching, as a common structural building block in all three compounds (4, 7, 10). However, the N,N-diethylamino-N-ethylaniline moiety in compound 7 exhibits a notable contribution to physical quenching of $^{1}$O$_{2}$ probably with formation of an exciplex intermediate of charge transfer character, as proposed for the reaction of tertiary amines with singlet oxygen. A similar effect was observed in the case of antimalarial drugs and polyamines.30,31 The importance of tertiary amines is well supported by the fact that the absence of a tertiary amine moiety from compound 10 results in the weakest $^{1}$O$_{2}$ quenching among the three compounds (Fig. 2). Our data also show that when a precursor of nitroxide is bound to the aromatic ring directly as in compound 4, the changes in fluorescence upon protonation or $^{1}$O$_{2}$ trapping are rather limited (Fig. 1A and 3). This suggests that the ROS sensor part (the pyrroline ring) should be bound to the inner side of the chloroplasts (marked by red chlorophyll autofluorescence) as well as inside the cells (Fig. 4A–C).

Confocal laser scanning microscopy allows fluorescence detection of chlorophyll-containing mesophyll cells of leaves without mechanical injury. A 35 µm thick optical slice made from the upper surface of a tobacco leaf infiltrated with compound 4 shows the presence of the sensor in chloroplasts (marked by red chlorophyll autofluorescence) as well as inside the cells (Fig. 4A–C).

The ratio of plastid and non-plastid localization cannot be determined from relative fluorescence intensities, because fluorescence yields may be different in different biological environments, e.g. in the water rich cytosol and in the vicinity of biological membranes.32

Interestingly, compound 7, which is very similar to 4 in structure, penetrated the cells but not the chloroplasts (Fig. 4D–F, also see ESI figure† for comprehensive intensity plots of red and green fluorescence). Compounds 4/HCl and 7/HCl did not penetrate leaf mesophyll cells at all (data not shown).
Conclusions

In summary, 4-amino-1,8-naphthalimide-based singlet oxygen sensors were synthesized. Among the synthesized compounds 7 was the best 1O2 quencher with the diethylaminoethyl chain bound to the aromatic ring. Compound 4 has excellent penetrating properties, however the fluorescence change upon 1O2 quenching was quite small even in response to relatively high ROS fluxes in vitro and is not expected to be responsive to small amounts in vivo. Compound 7, on the other hand, would prove useful in experiments aimed at studying whether singlet oxygen can stimulate a response farther from its production site. This question is of special interest because in photosynthetic organisms the main source of 1O2 is chlorophyll-sensitized photo-production in chloroplasts. Singlet oxygen is capable of activating nuclear genes but its role in this chloroplast to nucleus (a.k.a. retrograde) signaling has not been fully explored so far. One of the open issues is to what extent the signaling is caused by 1O2 itself rather than the molecules oxidized by this ROS. Theoretically, it is unlikely that highly reactive 1O2 would be able to leave the chloroplasts, yet 1O2 photo-generated in photosystem II of the alga Chlamydomonas was also traced in the cytoplasm. To study whether a similar phenomenon was observable in higher plants, tobacco leaves were infiltrated with compound 7 and exposed to high intensity irradiation which was shown to trigger 1O2 production in the chloroplasts. Fluorescence quenching of compound 7 under these conditions would have proven the hypothesis, that some plastid-derived 1O2 may leave this organelle, but there was no marked quenching (data not shown). This negative result however cannot be regarded as positive proof against the above hypothesis because it can be caused by the relative insensitivity of the probe (too little 1O2 causing too small fluorescence quenching) as well as by other factors, for example possible modification of photosynthetic events by the probe. Experiments to overcome such and similar difficulties are in progress.

Acknowledgements

This work was supported by grants from the Hungarian National Research Fund to T. K. (OTKA-NKTH K67597, K104956), K. H. (OTKA K81123) and É. H. (OTKA NN-85349). The authors wish to thank Viola H. Csokona for elemental assistance.

References


