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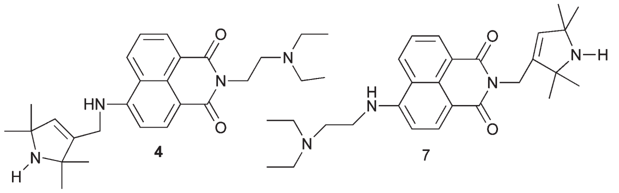
Synthesis and potential use of 1,8-naphthalimide type $^1O_2$ sensor molecules

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

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



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Synthesis and potential use of 1,8-naphthalimide type $^1O_2$ sensor molecules†

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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 pyrroline nitroxide as a quencher and radical capturing moiety were synthesized. All sensors were substituted with a diethylaminoethyl 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*.

Introduction

Optical sensors for biomolecules and biochemical processes are widely used in biochemical and medical studies.[1,2] 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.[3,4]

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 *N*-hydroxylamine the fluorescence intensity increases, when hydroxylamine is oxidized to nitroxide, the fluorescence is quenched again.[5] This principle was utilized in our earlier design of reactive oxygen species (ROS) sensitive reporter molecules in which a pre-nitroxide, a sterically hindered precursor amine, was attached to a fluorophore. In these sensors oxidation of amine by ROS resulted in nitroxide formation as well as partial fluorescence quenching.[6]

The function of our previously developed dansyl fluorophore-containing sensor is based on that only α,α,α′,α′-tetrasubstituted pyrrolines or piperidines can form a stable quencher (nitroxide) upon oxidation.[7]

Emerging biological applications demanded the synthesis of new fluorophore-nitroxide acceptor–donor molecules. New probes included various nitroxide moieties (nitronyl-,[8] pyrrolidine-,[9] piperidine-[5]) coupled to diverse fluorophores (acridine,[10] cyanine dye,[11] dansyl,[5,6] fluorescamine,[12] BODIPY,[8,13] Nile-red,[13] CdSe quantum dots[14] and naphthalimides[15,16]). 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.[15] 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+}).[15] 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,[17] 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.[5,6] 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 pyrroline nitroxide or a diethylaminoethylamine moiety respectively.

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† 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

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

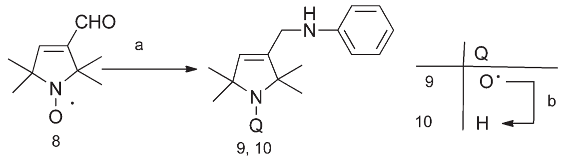
Chemistry

Treatment of 4-nitronaphthalic anhydride with diethylaminoethylamine or paramagnetic allylic amine[18] in EtOH at reflux temperature gave compounds **2** and **5**, respectively. The reaction of compounds **2** and **5** with paramagnetic amine and diethylaminoethylamine in DMF at ambient temperature furnished compounds **3** and **6**. Reduction of these paramagnetic compounds with iron powder in acetic acid[19] yielded compounds **4** and **7** with a hydrophilic moiety – the diethylaminoethyl 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 **8** aldehyde[20] with $NaBH_3CN$ 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).

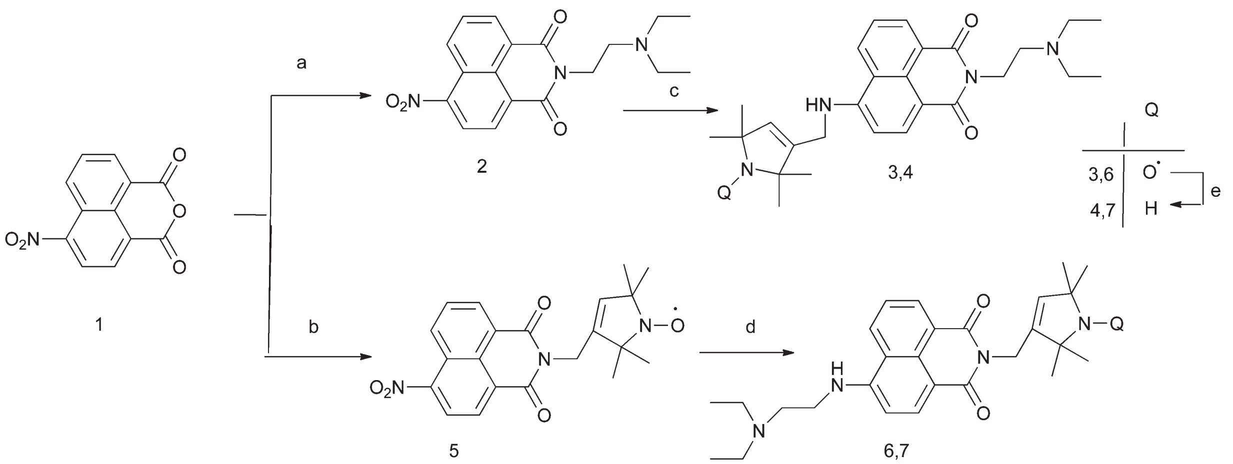
Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on a Fisons EA 1110 CHNS elemental analyzer. Mass spectra were recorded on a Thermoquest Automass Multi. ^{1}H NMR spectra were recorded with a Varian UNITYINOVA 400 WB spectrometer. Chemical shifts are referenced to Me_4Si. 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 monoradicals gave a triplet line a_N = 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**,[17] 3-aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl,[18] **8**[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-1*H*-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 diethylaminoethylamine (580 mg, 5.0 mmol) or compound 3-aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl (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 ($CDCl_3$) 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



Scheme 2 *Reagents and conditions*: (a) PhCOCl (1 equiv.), Et_3N (1 equiv.), CH_2Cl_2, 0 °C → rt, 1 h, 79%; (b) $PhNH_2$ (1 equiv.), $NaBH_3CN$ (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_2CO_3, 49–64%.



Scheme 1 *Reagents and conditions*: (a) $H_2N(CH_2)_2N(CH_3)_2$ (1 equiv.), rt → 60 °C, EtOH 4 h, then rt 12 h (62%); (b) 3-aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl (1.0 equiv.), rt → 60 °C, EtOH 4 h, then rt 12 h (53%); (c) **8** (1 equiv.), rt, DMF, 24 h (70%); (d) $H_2N(CH_2)_2N(CH_3)_2$ (1 equiv.), rt, DMF, 24 h, 55%; (e) Fe (10 equiv.), AcOH, 80 °C, 30 min then K_2CO_3, 39–49%.

for $C_{18}H_{19}N_3O_4$: 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 (EI) *m*/*z* (%): 394 (M^+, 5), 364 (4), 243 (59), 107 (100). Anal Calcd for $C_{21}H_{20}N_3O_5$: C 63.95; H 5.11; N 10.65; found: C 63.84; H 5.13; N 10.69.

Synthesis of 4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylaminomethyl)-*N*-(2-(diethylamino)ethyl)-1,8-naphthalimide radical (**3**) and 4-(2-(diethylaminoethyl)amino)-*N*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)-1,8-naphthalimide radical (**6**): To a stirred solution of compound **2** (1.705 g, 5.0 mmol) or compound **5** (1.97 g, 5.0 mmol) in DMF (30 mL) the appropriate amine (5.0 mmol) was added and the mixture was stirred at ambient temperature for 24 h. The resulting solution was poured into water (300 mL). After extraction with EtOAc (3 × 50 mL) the organic layer was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography ($CHCl_3$–Et_2O and $CHCl_3$–MeOH) to afford compounds **3** and **6**.

Compound **3**: 1.62 g (70%), yellow solid, mp 88–90 °C, Ms (EI) *m*/*z* (%): 463 (M^+, <1), 362 (1), 86 (100). Anal Calcd for $C_{27}H_{35}N_4O_3$: C 69.95; H 7.61; N 12.09; found: C 69.88; H 7.63; N 12.01.

Compound **6**: 1.27 g (55%), yellow solid, mp 142–144 °C, Ms (EI) *m*/*z* (%): 463 (M^+, 1), 433 (2), 122 (34), 86 (100). Anal Calcd for $C_{27}H_{35}N_4O_3$: C 69.95; H 7.61; N 12.09; found: C 69.91; H 7.55; N 11.92.

Synthesis of 4-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylaminomethyl)-*N*-(2-(diethylamino)ethyl)-1,8-naphthalimide (**4**) and 4-(2-(diethylaminoethyl)amino)-*N*-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3ylmethyl)-1,8-naphthalimide (**7**), *N*-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)aniline (**10**): To a solution of nitroxide **3** or **6** or **9** (2.0 mmol) in AcOH (10 mL) Fe powder (1.12 g, 20.0 mmol) was added and the mixture was warmed to 80 °C until the reaction started. The mixture was stirred at room temperature for 1 h, diluted with water (30 mL), decanted, and the decanted aq. solution made alkaline with solid K_2CO_3 with resultant intense foaming. The mixture was extracted with $CHCl_3$–MeOH (9 : 1) (3 × 15 mL), dried ($MgSO_4$), filtered, evaporated and chromatographic purification ($CHCl_3$–MeOH) gave compounds **4** or **7** or **10** (39–49%).

Compound **4**: 272 mg (39%), yellow solid, mp 75–77 °C, Ms (EI) *m*/*z* (%): 448 (M^+, <1), 376 (1), 122 (37), 86 (100). ^{1}H NMR (DMSO-d_6) 8.70 (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 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 $C_{27}H_{36}N_2O_4$: C 69.95; H 7.61; N 12.09; found: C 69.91; H 7.55; N 11.92.

Compound **7**: 412 mg (46%), mp 155–158 °C, yellow solid, Ms (EI) *m*/*z* (%): 433 (M^+ − 15, 2), 122 (64), 86 (100). ^{1}H NMR ($CDCl_3$) 8.59 (d, 1H, *J* = 7.2 Hz), 8.46 (d, 1H, *J* = 8.0 Hz), 8.38 (d, 1H, *J* = 8 Hz), 7.66 (t, 1H, *J* = 8 Hz), 6.66 (d, 1H, *J* = 8.8 Hz), 5.27 (s, 1H), 3.58 (m, 2H), 3.07 (d, 2H), 2.87 (q, 4H), 1.69 (s, 6H), 1.46 (s, 6H) 1.21 (t, 6H). Anal Calcd for $C_{27}H_{36}N_2O_4$: 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 (EI) *m*/*z* (%): 230 (M^+, 4), 215 (11), 106 (100). ^{1}H NMR ($CDCl_3$) 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 $C_{15}H_{22}N_2$: 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-1*H*-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 acetonitrile (20 mL) $NaBH_3CN$ (472 mg, 7.5 mmol) was added in one portion then the mixture was stirred at room temperature for 15 min. Then AcOH was added to adjust the pH to 7 and the mixture was stirred for a further 1 h. After filtration of the mixture, the solvent was evaporated off, water (20 mL) was added and the mixture was basified with NaOH to pH = 12 and then extracted with CH_2Cl_2 (3 × 20 mL). The organic phase was dried ($MgSO_4$), 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 (EI) *m*/*z* (%): 245 (M^+, 17), 215 (9), 106 (100), 77 (61). Anal Calcd for $C_{15}H_{21}N_2O$: 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 Specord 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, $\Phi = 1.0$).[21] The values were calculated by the equation $\Phi = (I/I')(A'/A)(n/n')\Phi'$, where I', A', 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 $^1O_2$ source and the ESR signal of TEMPO from the TEMP + $^1O_2$ → TEMPO reaction[22] as the $^1O_2$ 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 (520–560 nm, 75 μmol m^{-2} s^{-1}) light either in the absence or in the presence of one of the studied sensors (**4/HCl**, **7/HCl**, **10**) at the indicated concentrations. Reactivity of the sensor

Table 1 Fluorescence characteristics of compounds **3**, **4**, **6**, **7**

Compound	λ_{abs} (nm)	ε (1 mol^{-1} cm^{-1})	λ_{ex} (nm)	λ_{em} (nm)	Φ^a
3	433	1.252×10^4	449	517	0.03
4	438	1.564×10^4	448	520	0.53
6	440	1.233×10^4	448	526	0.03
7	439	1.489×10^4	450	520	0.06

[a] Referred to Rhodamine 101 at 450 nm in EtOH, $n = 3$, accuracy ±10%.

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 $^1O_2$ was also evaluated by measuring changes in their relative fluorescence emission (% quenching) in response to $^1O_2$ that was generated from illuminated Rose Bengal as above. To test whether fluorescence quenching was brought about by $^1O_2$ and not for other artifacts, experiments using Rose Bengal as the photosensitizer were repeated in the presence of the 5 mM NaN_3, which is a $^1O_2$ 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 $FeSO_4$, 100 μM H_2O_2 and 100 μM ascorbate[24]) in 50 mM potassium phosphate buffer at pH 7.0. Reactivities to H_2O_2 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 H_2O_2 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 nm[24] 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-assimilative 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 either one of the sensors (**4**, **7**) or their solvent only (5 : 95 vol : vol ethanol : water) were first kept in the dark for 20 min, then at various increasing intensities of photosynthetically active blue light, 5 min at each light level. Electron transport was calculated from quantum yields of chlorophyll fluorescence measured before and after a saturating pulse given either to dark adapted (Fo, Fm) or to blue light illuminated leaves (F and F′) as described by Schreiber *et al.*[28]

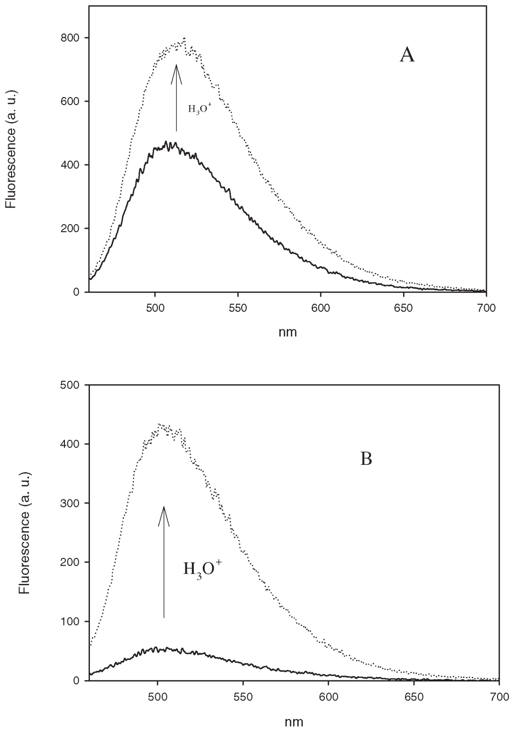


Fig. 1 (A) Fluorescence spectra of compound **4** (5.0 μM) in EtOH–H_2O (4 : 1) (solid line) and in EtOH–0.1 M aq. H_2SO_4 (4 : 1) (dotted line). (B) Fluorescence spectra of compound **7** (4.3 μM) in EtOH–H_2O (4 : 1) (solid line) and in EtOH–0.1 M aq. H_2SO_4 (4 : 1) (dotted line).

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

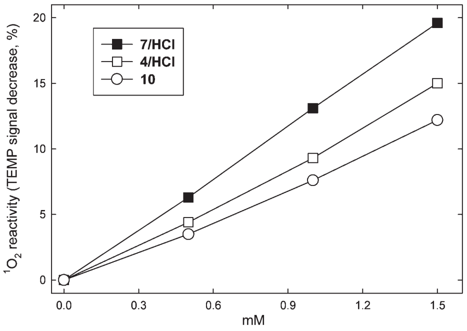


Fig. 2 Singlet oxygen quenching measured as percentage decrease in TEMPO production in the TEMP + $^1O_2$ → TEMPO reaction in the presence of compounds **4/HCl**, **7/HCl** or **10**.

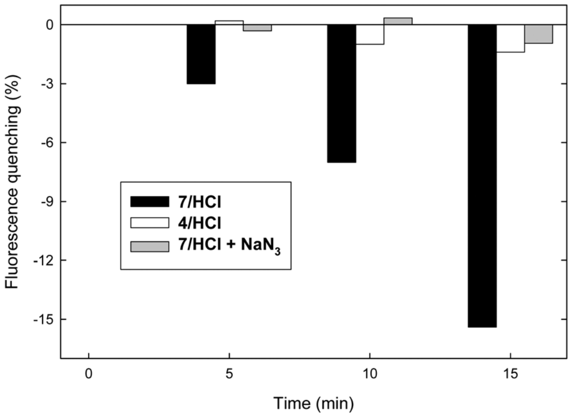


Fig. 3 Decrease in compound **4/HCl** and **7/HCl** fluorescence by singlet oxygen produced from illuminated Rose Bengal as described in Materials and methods.

Table 2 Fluorescence quenching[a] caused by ROS other than $^1O_2$ after 15 min incubation

ROS	H_2O_2	$O_2^{-\bullet}$	$^\bullet OH$
4HCl	1.2 ± 2.5%	−2.3 ± 3.5%	3.3 ± 2.5%
7HCl	−1.6 ± 2.7%	2.6 ± 3.1%	−3.8 ± 2.8%

[a] Relative quenching as % of fluorescence emission decrease (n = 3).

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.*[16] 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 $^1O_2$.

Although both fluorescent sensors (**4/HCl**, **7/HCl**) and their non-fluorescent version (**10**) reacted with $^1O_2$ and effectively competed with TEMP in the EPR active TEMPO forming reaction (Fig. 2), this was only accompanied by a decrease in fluorescence 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 $^1O_2$ (Fig. 3). Neither **4/HCl** nor **7/HCl** showed significant fluorescence quenching in the presence of ROS other than $^1O_2$ (Table 2). From these observations we concluded that a sterically hindered moiety (pre-nitroxide) has a role in $^1O_2$ 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 $^1O_2$ 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 $^1O_2$ 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 $^1O_2$ trapping are rather limited (Fig. 1A and 3). This suggests that the ROS sensor part (the pyrroline ring) should be bound to the imide and the diethylaminoethyl side-chain should be bound to the aromatic ring.

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).

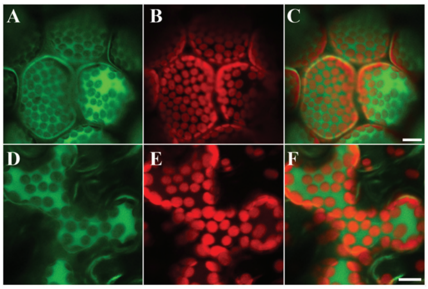


Fig. 4 Subcellular localization of compounds **4** (upper panel) and **7** (lower panel). Merged images of compound (A, D) and chlorophyll fluorescence (B, E) are shown in panels C and F. Scale bars: 10 μm.

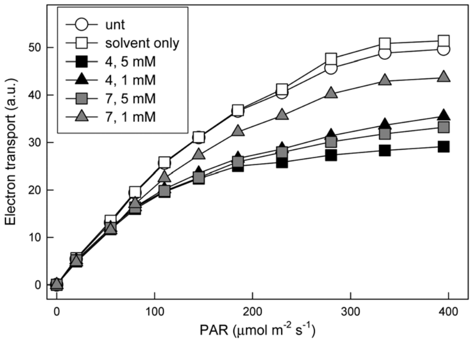


Fig. 5 Photosynthetic electron transport in tobacco leaves under various levels of photosynthetically active irradiation (PAR). Leaves were untreated (unt), infiltrated with either compound **4** or **7** dissolved in ethanol : water (solvent only) at the indicated concentrations. Symbols correspond to mean values calculated from three measurements. Standard deviations from the mean were smaller than 5% for all data points and are not shown.

For putative plant stress physiology applications, it is important that the presence of ROS sensors should not affect basic physiological functions. Since singlet oxygen has been shown to be produced in the photosynthetic apparatus both in isolated membranes and in leaves under special stress conditions[33,34] it is plausible to use the sensors' effects on non-stressed photosynthetic electron transport as a probe of their toxicity.

Chloroplast electron transport in tobacco leaves pre-treated with compounds **4** or **7** is shown in Fig. 5. In the absence of sensors electron transport increases with light (quantified as photosynthetically active radiation, PAR) and reaches a saturation corresponding to approximately double of PAR which was used to grow the plants. This trend was not affected by infiltrating 5% ethanol into the leaves, which was used as the solvent for compounds **4** and **7**. Electron transport, however, was strongly limited by the presence of compound **4**. Compound **7** was less toxic, with concentrations limiting photosynthesis to a small extent, so that for example 10–15% were tolerable in stress experiments. The difference between the effects of compounds **4** and **7** on photosynthesis corresponds to their cellular localization: plastid penetrating compound **4** had a greater effect than **7**, which was mainly found in the cytosol (Fig. 5).

Conclusions

In summary, 4-amino-1,8-naphthalimide-based singlet oxygen sensors were synthesized. Among the synthesized compounds **7** was the best $^1O_2$ quencher with the diethylaminoethyl chain bound to the aromatic ring. Compound **4** has excellent penetrating properties, however the fluorescence change upon $^1O_2$ 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 $^1O_2$ is chlorophyll-sensitized photo-production in chloroplasts. Singlet oxygen is capable of activating nuclear genes[35,36] 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 $^1O_2$ itself rather than the molecules oxidized by this ROS. Theoretically, it is unlikely that highly reactive $^1O_2$ would be able to leave the chloroplasts,[37] yet $^1O_2$ photo-generated in photosystem II of the alga *Chlamydomonas* was also traced in the cytoplasm.[38] 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 $^1O_2$ production in the chloroplasts.[34] Fluorescence quenching of compound **7** under these conditions would have proven the hypothesis, that some plastid-derived $^1O_2$ may leave this organelle, but there was no marked quenching (data not shown). This negative result however can not be regarded as positive proof against the above hypothesis because it can be caused by the relative insensitivity of the probe (too little $^1O_2$ 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.

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