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Synthesis of redox sensitive dyes based on a combination of long wavelength emitting fluorophores and nitroxides

Balázs Bognár [a], József Jekő [b], Tamás Kálai [a], Kálmán Hideg [a,*]

[a] Institute of Organic and Medicinal Chemistry, University of Pécs, P.O. Box 99, H-7602 Pécs, Hungary
[b] Department of Chemistry, College of Nyíregyháza, H-4440 Nyíregyháza, Sóstói st. 31/B, Hungary

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ABSTRACT

New, nitroxide-fluorophore acceptor-donor compounds were synthesized based on long wavelength (570–790 nm) emitting 9-diethylamino-5*H*-benzo[*a*]phenoxazin-5-one, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene and metal-ligand complex fluorophores. The fluorophores and nitroxides were linked via a robust C=C bond. The steady-state spectral properties of the new donor-acceptor compounds and their diamagnetic (sterically hindered amine) derivatives were studied. Titration of nitroxides with ascorbic acid sodium salt to diamagnetic *N*-hydroxy compounds resulted in fluorescence enhancement. The Ru-complex modified with nitroxide exhibited fluorescence increase and electron paramagnetic resonance band broadening upon B-deoxyribonucleic acid addition providing evidence of binding with B-deoxyribonucleic acid.

1. 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 in both fluorescence instrumentation and in the synthesis of novel fluorophores [3,4]. The development of the EPR technique [5] inspired researchers to synthesize new spin labels and construct new double (EPR active and fluorescent) sensors [6,7].

Fluorophore-nitroxide donor-acceptor compounds have been utilized mainly for the detection of radicals or probing the redox reactions in biological and chemical systems, including the detection of hydroxyl [8] or glutathionyl radical [9], Fe^{2+} or ascorbic acid [10,11].

The fluorescence of nitroxide-fluorophore compounds is weak owing to electron transfer from the fluorophore to nitroxide radical or electron exchange between nitroxide and the excited singlet state of the fluorophore [7]. When the nitroxide ("**c**-form", Fig. 1) function is reduced to *N*-hydroxylamine ("**b**-form") the fluorescence intensity increases, while the intensity of the EPR signal of nitroxide decreases. In other words, the nitroxide redox status can be followed by both fluorescence and EPR spectroscopy. A further extension of this idea, when the sterically hindered precursor amine ("**a**-form") instead of the nitroxide is attached to fluorophore and its oxidation by reactive oxygen species (ROS) results in a decrease in fluorescence with nitroxide formation [12]. In the past decade a series of new donor-acceptor probes have been synthesized varying both the nitroxide (nitronyl- [13], pyrrolidine- [14], piperidine-nitroxide [15]) and the fluorophore (acridine [9], umbelliferone [11], naphthyl [7], cyanine dye [16], polyaromatics [14,17], naphthalimides [18], dansyl [6,15], fluorescamine [19] and BODIPY [13,20]) moiety. However, these fluorophores emit mainly below 600 nm and for biological and clinical application it is preferable to apply long wavelength excitation and emission. At longer wavelengths there is less sample absorbance, e.g. biological samples are more transparent to red light, less autofluorescence and the light sources are less expensive. In our laboratory the first red fluorophore (sulforhodamine B)-nitroxide adduct was synthesized for the purposes of studying the interaction of singlet molecular oxygen and a double sensor [21].

The continuation of this research was inspired by the fact that application of long wavelength emitting fluorophores has become widespread in the past decade [22,23], however to find an ideal fluorophore is not easy and always determined by the application. Water-solubility, chemical stability, sensitivity toward polarity of

* Corresponding author. Tel.: +36 72 536 221.
E-mail address: kalman.hideg@aok.pte.hu (K. Hideg).

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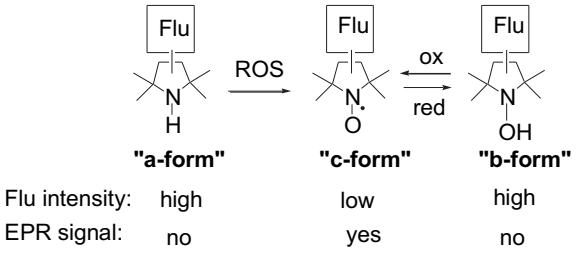

Fig. 1. The fluorescence intensity and EPR signal change depending on nitrogen oxidative status in nitroxide-fluorophore adducts.

microenvironment, intrinsic fluorescence of the environment, Stokes shift, quantum yield, fluorescence lifetime are the possible parameters for consideration. The objective of this work was to synthesize new double sensor compounds with different, long wavelength emitting fluorophores (Nile Red (C.I. Basic Blue 12), BODIPY and metal-ligand complex) attached by C=C bond to a nitroxide unit thereby achieving redox probes utilizable in biological systems.

2. Experimental

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on Carlo Erba EA 1110 CHNS elemental analyzer. Mass spectra were recorded on an Automass Multi or VG TRIO-2 instruments in the EI mode (70 eV, direct inlet). ESI-TOF MS measurements were performed with a BioTOF II instrument (Bruker Daltonics, Billerica, MA). ^{1}H NMR spectra were recorded with Varian ^{UNITY}*INOVA* 400 WB spectrometer. Chemical shifts are referenced to Me_4Si, the exchangeable NH signal was not observed. Measurements were run at 298 K probe temperature in $CDCl_3$ solution. ESR spectra were obtained from 10^{-5} molar solutions ($CHCl_3$), using a Magnettech MS200 spectrometer, and all monoradicals gave triplet signal $a_N = 14.5–14.7$ G.). Preparative 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 GF_{254}.

2.1. Materials

Calf thymus B-DNA sodium salt was purchased from Sigma and concentration was estimated spectrophotometrically ($\varepsilon_{260} = 6600\ M^{-1}\ cm^{-1}$). Compounds **2a** [24], **2c** [25], **6** [26], **7** [27], **9** [28], **10** [29], **12** [30] were prepared as published earlier and all other reagents and compounds were purchased from Aldrich or Fluka.

2.2. Spectroscopic measurements

The UV spectra were taken with a Specord 40 (Jena Analytic), the molar extinction coefficients (ε) at absorption maxima were obtained from slope of absorbance vs concentration using five solutions of different concentrations. Fluorescence spectra of compounds dissolved in dioxane or MeOH or NaCl/Tris buffer were measured with Perkin Elmer LS50B spectrofluorimeter, with 10 nm slits, with correction of instrumental factors by means of a rhodamine B quantum counter and correction files supplied by the manufacturer. Quantum yields were referred to Cresyl Violet dissolved in MeOH ($\lambda_{ex} = 640$ nm, $\Phi' = 0.54$) or fluorescein dissolved in 0.1 M NaOH ($\lambda_{ex} = 496$ nm, $\Phi' = 0.95$). The values were calculated on 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 reference sample. I, A, n, Φ are related to the sample with the same definitions applied to reference sample.

2.3. Dyes

2.3.1. Synthesis of BODIPY core *3a* and *3c*

To a deoxygenated solution of compound **2a** or **2c** (5.0 mmol) and compound **1** (10.0 mmol 1.23 g) in CH_2Cl_2 (30 mL) trifluoroacetic acid (57 mg, 0.5 mmol for compound **3c** and 627 mg, 5.5 mmol for compound **3a**) was added and the mixture was stirred at rt. overnight (10 h) in dark under nitrogen. Then DDQ (1.13 g, 5.0 mmol) was added and after 30 min *i*-Pr_2EtN (8.0 mL) and $BF_3{\cdot}Et_2O$ (8.0 mL) was added at 0 °C and the solution was stirred for 40 min at this temperature. The deep red solution was washed with sat. $NaHCO_3$ solution (20 mL), with brine (20 mL), the organic phase was separated, dried ($MgSO_4$). In the case of compound **3c** PbO_2 (478 mg, 2.0 mmol) was added and O_2 was bubbled through. The solutions were filtered, evaporated and the residue was purified by flash column chromatography (hexane/EtOAc or $CHCl_3/Et_2O$) to afford the BODIPY dyes in 10–35% yield as red–purple crystals.

*2.3.1.1. 1-Oxyl-2,2,5,5-tetramethyl-3-[4,4-difluoro-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-2,5-dihydro-1H-pyrrole radical (**3c**).* Yield: 220 mg (10%), mp 150–152 °C, R_f 0.47 (hexane/EtOAc, 2:1). MS: *m/z* (%): 442 (M^+, 25), 427 (62), 412 (50) 370 (100), 355 (86). Anal. Calcd. for $C_{25}H_{35}BF_2N_3O$: C 67.88; H 7.97; N 9.50. Found: C 67.87; H 7.86; N 9.53.

*2.3.1.2. 2,2,5,5-Tetramethyl-3-[4,4-difluoro-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-2,5-dihydro-1H-pyrrole (**3a**).* Yield: 750 mg (35%), mp 135–137 °C, R_f: 0.30 ($CHCl_3$:Et_2O: MeOH, 8:3:1). MS: *m/z* (%): 427 (M^+, 51), 412 (9), 370 (100), 355 (66). ^{1}H NMR ($CDCl_3$): δ: 5.75 (s, 1H), 2.50 (s, 6H), 2.26 (s, 6H), 2.33 (m, 4H), 1.81 (s, 6H), 1.69 (s, 6H), 1.02–0.98 (m, 6H). ^{13}C NMR ($CDCl_3$): 154.82, 137.19, 136.79, 134.40, 133.08, 131.22, 130.89, 74.47, 69.28, 26.99, 26.74, 17.19, 17.07, 15.21, 14.31, 14.05, 12.63. Anal. Calcd. for $C_{25}H_{36}BF_2N_3$: C 70.26; H 8.49; N 9.83. Found: C 70.20; H 8.46; N 9.75.

2.3.2. General procedure for dyes (*4a*, *4c*, *5a*, *5c*)

A solution of compound **3a** or **3c** (1.0 mmol) and 4-(*N*,*N*-dimethylamino)benzaldehyde (596 mg, 4.0 mmol), piperidine (0.6 mL) and AcOH (0.5 mL) in toluene (50 mL) was heated under reflux in a Dean and Stark apparatus for 24 h. Crude product was then concentrated under vacuum and purified by flash column chromatography (hexane/EtOAc or $CHCl_3/Et_2O$) to give the green or blue colored fractions in 10–45% yield.

*2.3.2.1. 1-Oxyl-2,2,5,5-tetramethyl-3-[3-(4-dimethylaminostyryl)-4,4-difluoro-1,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-2,5-dihydro-1H-pyrrole radical (**4c**).* Yield: 57 mg (10%), mp 200–202 °C, R_f 0.29 (hexane/EtOAc, 2:1). MS ESI: 573 $[M + H]^+$. Anal. Calcd. for $C_{34}H_{44}BF_2N_4O$: C 71.20; H 7.73; N 9.77. Found: C 71.13; H 7.73; N 9.75.

*2.3.2.2. 1-Oxyl-2,2,5,5-tetramethyl-3-[3,5-bis(4-dimethylaminostyryl)-4,4-difluoro-1,7-dimethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-2,5-dihydro-1H-pyrrole radical (**5c**).* Yield: 320 mg (45%), mp 222–223 °C, R_f 0.22 (hexane/EtOAc, 2:1). MS ESI: 704 $[M]^+$. Anal. Calcd. for $C_{43}H_{53}BF_2N_5O$: C 73.29; H 7.58; N 9.94. Found: C 73.18; H 7.53; N 9.90.