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

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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

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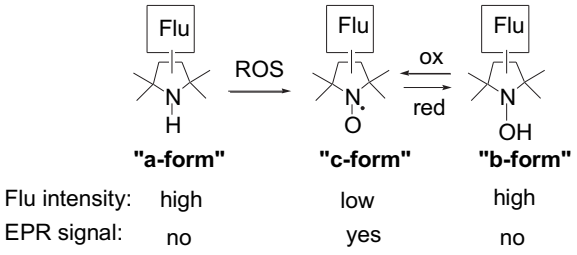



**Fig. 1.** The fluorescence intensity and EPR signal change depending on nitrogen oxidative status in nitroxide-fluorophore adducts.

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 ($\varepsilon$) 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 $\Phi'$ 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. $I$, $A$, $n$, $\Phi$ 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.

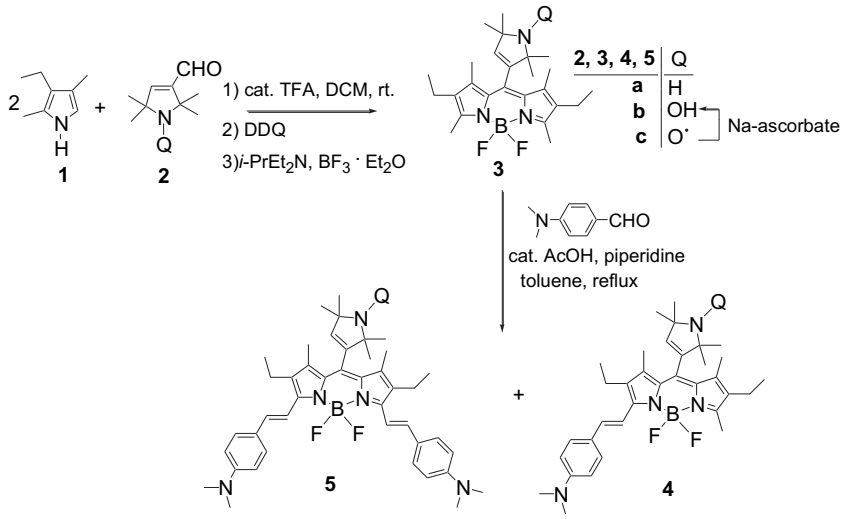
*2.3.1.2. 2,2,5,5-Tetramethyl-3-[4,4-difluoro-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-2,5-dihydro-1H-pyrrole (**3a**).* Yield: 750 mg (35%), mp 135–137 °C, $R_f$: 0.30 ($CHCl_3$:$Et_2O$: MeOH, 8:3:1). MS: *m/z* (%): 427 ($M^+$, 51), 412 (9), 370 (100), 355 (66). $^1$H NMR ($CDCl_3$): $\delta$: 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.




**Scheme 1.** Synthesis of BODIPY-based redox sensitive dyes **4** and **5**.

*2.3.2.3. 2,2,5,5-Tetramethyl-3-[3-(4-dimethylaminostyryl)-4,4-difluoro-1,5,7-trimethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-2,5-dihydro-1H-pyrrole (**4a**).* Yield: 84 mg (15%), mp 137–139 °C, $R_f$ 0.29 ($CHCl_3$/$Et_2O$/MeOH, 8:3:1). MS ESI: 559 $[M+H]^+$. $^1H$ NMR ($CDCl_3$): $\delta$: 7.72–7.65 (m, 4H), 7.64 (d, $J = 5.5$ Hz, 2H), 5.80 (s, 1H), 3.18 (s, 6H), 2.67–2.59 (q, $J = 7.8$ Hz, 2H), 2.56 (s, 3H), 2.52–2.48 (m, 2H), 2.33 (s, 3H), 2.30 (s, 3H), 1.92 (s, 6H), 1.71 (d, $J = 6.3$ Hz, 6H), 1.17 (t, $J = 7.5$ Hz, 3H), 1.03 (t, $J = 7.7$ Hz, 3H). Anal. Calcd. for $C_{34}H_{45}BF_2N_4$: C 73.11; H 8.12; N 10.03. Found: C 73.15; H 8.25; N 10.01.

*2.3.2.4. 2,2,5,5-Tetrametil-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 (**5a**).* Yield: 280 mg (40%), mp >360 °C, $R_f$ 0.28 ($CHCl_3$/$Et_2O$/MeOH, 8:3:1). MS ESI: 690 $[M+H]^+$. $^1H$ NMR ($CDCl_3$): $\delta$: 7.85–7.60 (m, 8H), 7.55–7.41 (m, 4H), 5.86 (s, 1H), 3.18 (s, 12H), 2.70–2.63 (m, 4H), 2.35 (s, 6H), 1.92, 1.70 (2s, 12H), 1.20 (t, $J = 6.9$ Hz, 6H). $^{13}C$ NMR ($CDCl_3$): 154.73, 149.12, 138.26, 135.63, 135.12, 134.43, 133.12, 131.22, 130.11, 127.72, 123.28, 117.32, 74.42, 69.33, 40.32, 27.12, 26.82, 17.22, 17.10, 15.21, 14.10, 12.65. Anal. Calcd. for $C_{43}H_{54}BF_2N_5$: C 74.88; H 7.89; N 10.15. Found: C 74.83; H 7.76; N 10.10.

*2.3.3. Synthesis of Nile Red derivatives (**8c** and **8a**)*

*2.3.3.1. 9-Diethylamino-2-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)-5H-benzo[a]phenoxazin-5-one radical (**8c**).* To a deoxygenated solution of compound **6** (466 mg, 1.0 mmol) in dioxane (10 mL) $Pd(PPh_3)_4$ (50 mg, 0.05 mmol) was added and the mixture was stirred at rt. for 10 min. Then compound **7** (184 mg, 1.0 mmol) and aq. 10% $Na_2CO_3$ (5 mL) was added and the mixture was stirred and refluxed for 10 h under $N_2$. After cooling the dioxane was evaporated off and the red residue was partitioned between brine (10 mL) and EtOAc (20 mL). The organic phase was separated, the aqueous phase was extracted with EtOAc (20 mL). The combined organic phase was dried ($MgSO_4$), filtered, evaporated and the residue was purified by flash column chromatography (hexane/EtOAc) to yield compound **8c** 173 mg (38%), red crystals, mp 240–242 °C, $R_f$ 0.50 ($CHCl_3$/$Et_2O$, 2:1). MS (EI): *m/z* (%): 441 ($M^+$-15, 14), 426 (100), 396 (12), 277 (12), 206 (36). Anal. Calcd. for $C_{28}H_{30}N_3O_3$: C 73.66; H 6.62; N 9.20. Found: C 73.48; H 6.56; N 9.15.

*2.3.3.2. 9-Diethylamino-2-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)-5H-benzo[a]phenoxazin-5-one (**8a**).* To a solution of compound **8c** (228 mg, 0.5 mmol) in glacial acetic acid (5 mL) Fe powder (140 mg, 2.5 mmol) was added and the mixture was warmed up to 70 °C until the reaction started and the mixture was stirred at rt. for 60 min. After diluting with water (20 mL) the solution was decanted from iron residue and in a 250 mL baker the solution was made alkaline (pH = 9) by solid $K_2CO_3$ (intense foaming!). The mixture was extracted with $CHCl_3$ (2 × 15 mL), the organic phase was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography ($CHCl_3$:MeOH) to yield the title compound 112 mg (51%), reddish–purple crystals, mp 233–235 °C, $R_f$: 0.31 ($CHCl_3$:MeOH, 9:1). MS (EI): *m/z* (%): 441 ($M^+$, 12), 426 (100), 396 (13), 309 (18), 206 (44). $^1H$ NMR ($CDCl_3$): $\delta$: 8.49 (s, 1H), 8.12 (d, $J = 7.2$ Hz, 1H), 7.73 (d, $J = 9.7$ Hz, 1H), 7.64 (d, $J = 9.1$ Hz, 1H), 6.85 (d, $J = 11.2$ Hz, 1H), 6.67 (s, 1H), 6.30 (s, 1H), 6.25 (s, 1H), 3.52–3.47 (q, $J = 6.9$ Hz, 4H), 1.70, 1.61 (2s, 12H), 1.15 (t, $J = 7$ Hz, 6H). $^{13}C$ NMR ($CDCl_3$): 182.69, 152.14, 150.78, 146.86, 144.57, 139.11, 135.51, 132.19, 131.89, 131.47, 131.01, 129.06, 126.18, 125.33, 123.07, 110.36, 105.74, 105.43, 72.04, 67.90, 45.27, 28.40, 27.64, 12.32. Anal. Calcd. for $C_{28}H_{31}N_3O_2$: C 76.16; H 7.08; N 9.52. Found: C 76.10; H 7.01; N 9.43.

*2.3.4. Synthesis of paramagnetic ligand (**11**)*

*2.3.4.1. 2-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl) dipyrido[3,2-a:2′,3′-c]quinoxaline radical.* A solution of 5,6-diamino-1,10-phenantroline **10** (210 mg, 1.0 mmol) and compound **9**

**Table 1**
Optical properties of compound **3a**–**13c** synthesized.

| Compound | Solvent | $\lambda_{abs}$ nm | $\varepsilon$ $M^{-1}$ $cm^{-1}$ | $\lambda_{ex}$ nm | $\lambda_{em}$ nm | $\Phi$[a] |
|---|---|---|---|---|---|---|
| **3a** | MeOH | 537 | $3.59 \times 10^4$ | 541 | 560 | 0.315 |
| | Dioxane | 541 | | 540 | 559 | 0.417 |
| **3c** | MeOH | 536 | $4.79 \times 10^4$ | 535 | 558 | 0.194 |
| | Dioxane | 536 | | 541 | 561 | 0.308 |
| **4a** | MeOH | 600 | $2.82 \times 10^4$ | 600 | 697 | 0.002[b] |
| | Dioxane | 600 | | 615 | 676 | 0.052[b] |
| **4c** | MeOH | 629 | $2.81 \times 10^4$ | 629 | 695 | 0.005[b] |
| | Dioxane | 638 | | 626 | 678 | 0.025[b] |
| **5a** | MeOH | 648 | $1.49 \times 10^4$ | 642 | 788 | 0.001[b] |
| | Dioxane | 648 | | 648 | 772 | 0.039[b] |
| **5c** | MeOH | 730 | $1.42 \times 10^4$ | 730 | 785 | 0.001[b] |
| | Dioxane | 731 | | 733 | 766 | 0.011[b] |
| **8a** | MeOH | 564 | $3.32 \times 10^4$ | 573 | 640 | 0.220 |
| | Dioxane | 525 | | 529 | 578 | 0.795 |
| **8c** | MeOH | 564 | $1.55 \times 10^4$ | 567 | 638 | 0.038 |
| | Dioxane | 530 | | 530 | 584 | 0.185 |
| **13c** | MeOH | 448 | $1.36 \times 10^4$ | 453 | 600 | 0.053 |

[a] Referred to fluorescein in 0.1 M NaOH at 496 nm.
[b] Referred to Cresyl Violet in MeOH at 640 nm, $n = 3$, accuracy ±10%.

(196 mg, 1.0 mmol) in anhydr. EtOH (10 mL) was heated under condenser for 4 h. After evaporation of the solvent the residue was purified by flash column chromatography ($CHCl_3$/MeOH) to yield compound **11** 259 mg (70%), yellow solid, mp 230–232 °C, $R_f$ 0.29 ($CHCl_3$/MeOH, 9:1). MS (EI) *m/z* (%): 370 ($M^+$, 12), 356 (25), 340 (100), 325 (23). Anal. Calcd. for $C_{22}H_{20}N_5O$: C 71.33; H 5.44; N 18.91. Found: C 71.16, H 5.49, N 18.94.

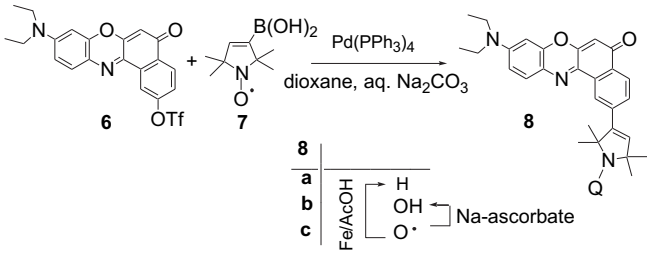
*2.3.5. Synthesis of Ru-complex (**13c**)*

*2.3.5.1. [Ru(phen)$_2$ 2-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)dipyrido[3,2-a:2′,3′-c]quinoxaline](PF$_6$)$_2$ salt radical.* A solution of compound **11** (185 mg, 0.5 mmol) and Ru-complex (**12**) (380 mg, 0.5 mmol) in anhydr. EtOH (75 mL) was heated under reflux condenser for 2 h. After cooling the solution was filtered and the filtrate was treated with $NH_4PF_6$ upon which the complex precipitated and the solution was allowed to stay in a refrigerator (−18 °C) overnight. The precipitate was filtered, washed with $Et_2O$ (20 mL) to give orange–brown solid 202 mg (36%), mp >360 °C, MS ESI: 977 $[M^{2+} + PF_6^-]^+$. Anal. Calcd. for $C_{46}H_{36}F_{12}N_9OP_2Ru$: C 49.25; H 3.23; N 11.24 Found: C 49.02, H 3.20, N 11.08.

## 3. Results and discussion

### 3.1. Synthesis and characterization of BODIPY-based sensors

For the synthesis of new BODIPY derivatives the mixture of paramagnetic aldehyde (**2c**) [25] and 2,4-dimethyl-3-ethylpyrrole (**1**) was treated with a catalytic amount of trifluoroacetic acid (TFA) in $CH_2Cl_2$ followed by treatment with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), *i*-$Pr_2$EtN and boron trifluoride diethyl etherate at ambient temperature to give compound **3c**. To synthesize the diamagnetic derivative (**3a**) aldehyde **2a** [24] and 1.1 equivalent TFA was used [20]. The emission wavelength of these derivatives (**3a** and **3c**) are in the shorter wavelength region (~560 nm), and Stokes shift are also small, 20 nm. Only a slight difference was noted in the excitation and emission spectra when recorded in polar (MeOH) and apolar (dioxane) solvents. We hoped that extension of conjugation would shift both excitation and emission toward longer wavelength. These BODIPY cores (**3a** and **3c**) were condensed with 4-(*N,N′*-dimethylamino)benzaldehyde in benzene in the presence of a catalytic amount of acetic acid and piperidine under azeotropic removal of water [31] to give mixture of the 3-monostyryl derivative **4c** and 3,5-distyryl **5c** and diamagnetic **4a** and **5a** compounds, respectively (Scheme 1). The 3,5-distyryl derivatives **4a** and **5a** exhibited long (788 nm) emissions (Table 1). The Stokes shift of these compounds in MeOH are 97 and 146 nm for **4a** and **5a** dyes, respectively and the quantum yields decreased because of strong charge transfer caused by amines [32].



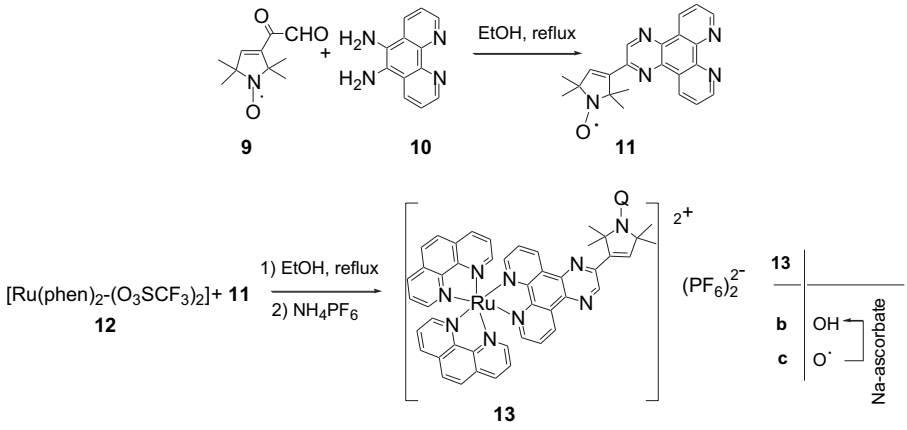

**Scheme 2.** Synthesis of Nile Red redox sensitive dye **8**.

### 3.2. Synthesis and characterization of Nile Red based redox probes

Nile Red is a phenoxazine dye, which fluoresces intensely and has been used for histochemical detection of lipids [33]. Very recently Nile Red linked with chemiluminescent donor has been used in the construction of energy-transfer cassettes [26] and in fluorescent probe molecules in a silicate matrix [34]. In our approach the 2-triflate of Nile Red **6** was coupled with paramagnetic boronic acid **7** [27] using a Suzuki coupling in aq. dioxane in the presence of $Na_2CO_3$ and $Pd(PPh_3)_4$ to yield compound **8c**. Subsequent reduction with Fe powder in AcOH [24] afforded compound **8a** (Scheme 2). These dyes emit at 640 nm in MeOH and at a shorter wavelength 584 nm in dioxane. The fluorescence quantum yield is increased fourfold for the diamagnetic derivative **8a** over the paramagnetic derivative **8c**. These dyes seem to be an ideal redox sensor emitting at longer wavelengths.

### 3.3. Synthesis and characterization of paramagnetically modified Ru(II) complex

The metal-ligand complexes have been recognized as long-lifetime luminescent probes and as new diagnostic and therapeutic agents [31,35]. Polypyridyl complexes of Ru(II) are intensely colored owing to well-characterized, localized metal-to-ligand charge transfer transitions. The nitroxide moiety can be attached only to a polypyridyl ligand via a C=C bond to a modified 1,10-phenanthroline, e.g. dipyrido[3,2-*a*:2′,3′-*c*]quinoxaline ligand (**11**) which was obtained by condensing 1,2-dicarbonyl compound (**9**) [28] with 5,6-diamino-1,10-phenanthroline (**10**) [29]. Treatment of [Ru(phen)$_2$-($O_3SCF_3$)$_2$] complex (**12**) [30] with compound **11** in




**Scheme 3.** Synthesis of Ru-complex-based redox sensitive dye **13**.

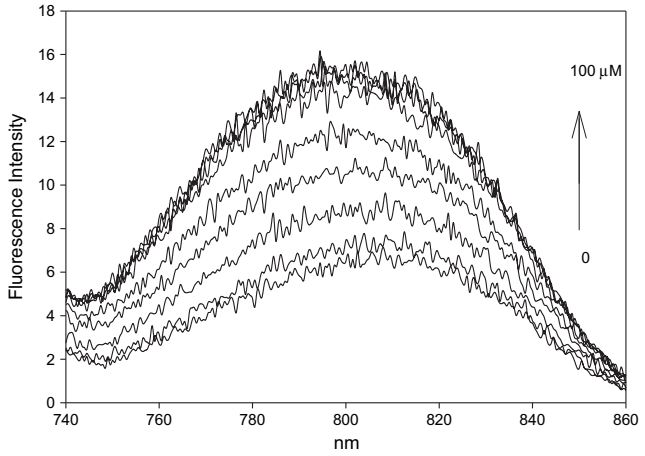



**Fig. 2.** Fluorescence emission spectra of compound **5c** (50 μM) in MeOH with sodium ascorbate (dissolved in MilliQ water) 0, 5, 10, 15.5, 25, 40, 50 and 100 μM (final concentration). $\lambda_{ex}$: 730 nm.

**Table 2**
Quantum yield increase upon reduction of **5c**, **8c**, **13c** nitroxides dissolved in 10% $H_2O$ (V) containing MeOH.

| Compound | Φ[a] | Compound | Φ[a] |
|---|---|---|---|
| **5c** | 0.0018[b] | **13c** | 0.050 |
| **5b** | 0.0041[b] | **13b** | 0.065 |
| **8c** | 0.024 | **13c** + B-DNA | 0.134 |
| **8b** | 0.031 | | |

[a] Referred to fluorescein in 0.1 M NaOH at 496 nm.
[b] Referred to Cresyl Violet in MeOH at 640 nm, $n = 3$, accuracy ±10%.

refluxing ethanol followed by precipitation with $NH_4PF_6$ yielded compound **13c**, which exhibits emission at 600 nm with low ($\Phi = 0.05$) quantum yield (Scheme 3).

### 3.4. Characterization of fluorescent dyes in the presence of ascorbate

Solutions of compounds **5c**, **8c** and **13c** (50 μM) in MeOH were titrated with sodium ascorbate dissolved in MilliQ water. The fluorescence intensity increased in all cases, from compound **5c** the *N*-hydroxylamine (5b) was formed with a 132% quantum yield

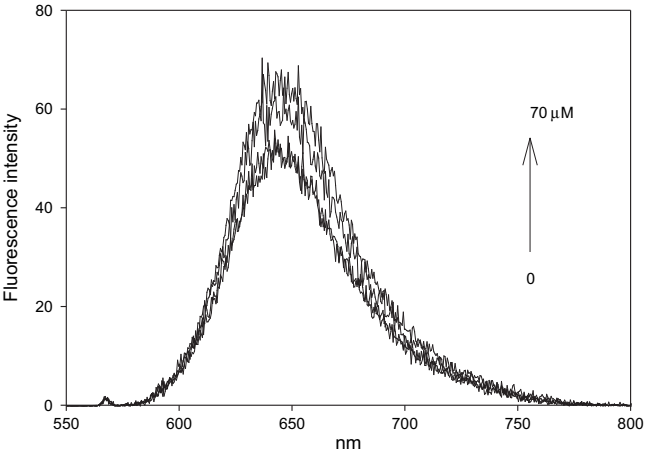



**Fig. 3.** Fluorescence emission spectra of compound **8c** (50 μM) in MeOH with sodium ascorbate (dissolved in MilliQ water) 0, 15.5, 50 and 70 μM (final concentration). $\lambda_{ex}$: 567 nm.

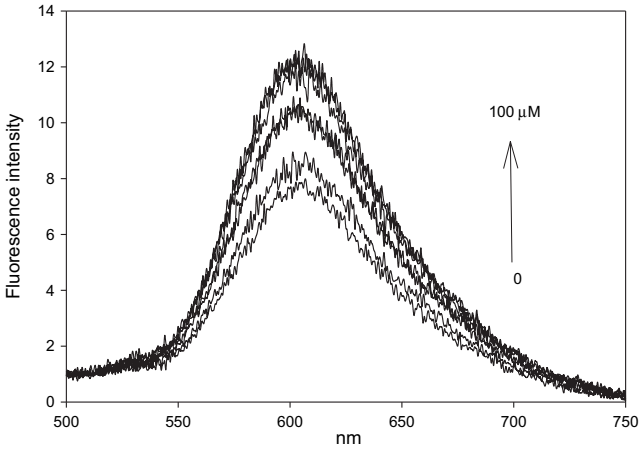



**Fig. 4.** Fluorescence emission spectra of compound **13c** (50 μM) in MeOH with sodium ascorbate (dissolved in MilliQ water) 0, 10, 25, 50 and 100 μM (final concentration), $\lambda_{ex}$: 453 nm.

increase (Fig. 2 and Table 2), from the paramagnetic Nile Red derivative (**8c**) compound **8b** was formed with 29% fluorescence quantum yield raise (Fig. 3) and from **13c** compound **13b** formed with a 31% fluorescence quantum yield enhancement (Fig. 4). In all cases the nitroxide was reduced to *N*-hydroxylamine with approximately one equivalent amount of ascorbate.

### 3.5. Characterization of fluorescent dye *13c* in the presence of B-DNA

Ru-complexes were reported to bind B-DNA resulting in a fluorescence increase, and for paramagnetically modified Ru-complexes EPR line broadening also was described. This broadening is attributed to surface bound complexes and intercalatively bound complexes [36]. Adding a solution of Calf thymus B-DNA (~270 μM) in 50 mM NaCl and 5 mM Tris buffer to a solution of **13c** caused a 169% quantum yield increase in fluorescence (Table 2, Fig. 5). This can not be attributed exclusively to reduction of **13c** to hydroxylamine (**13b**) because the treatment the solution of **13c** with sodium ascorbate produced minimal fluorescence increase in Tris buffer (Fig. 5). Although the intensity of the EPR lines decreased, some broadening of the EPR bands and the appearance of a high field peak (arrow in Figs. 6 and 7) also confirms the binding of compound **13c** with B-DNA.

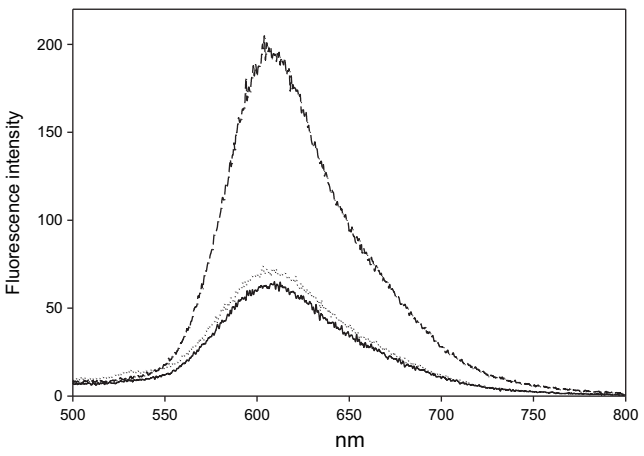



**Fig. 5.** Fluorescence emission spectra of 50 μM **13c** (—) in a buffer (50 mM NaCl and 5 mM Tris), 50 μM **13c** and 1.0 mM sodium ascorbate (· · · ·) in a buffer, 50 μM **13c** and 270 μM Calf thymus B-DNA (- - - -) in a buffer, $\lambda_{ex}$: 453 nm.

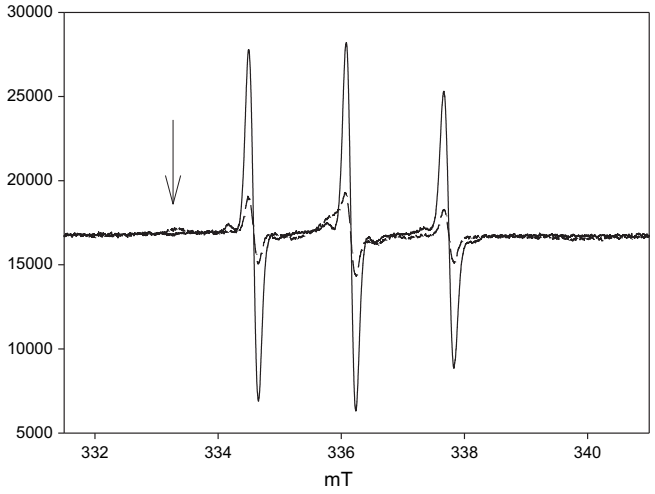



**Fig. 6.** EPR spectra of 50 μM **13c** (—) in a buffer (50 mM NaCl and 5 mM Tris), 50 μM **13c** and 250 μM Calf thymus B-DNA (- - - -) in a buffer.

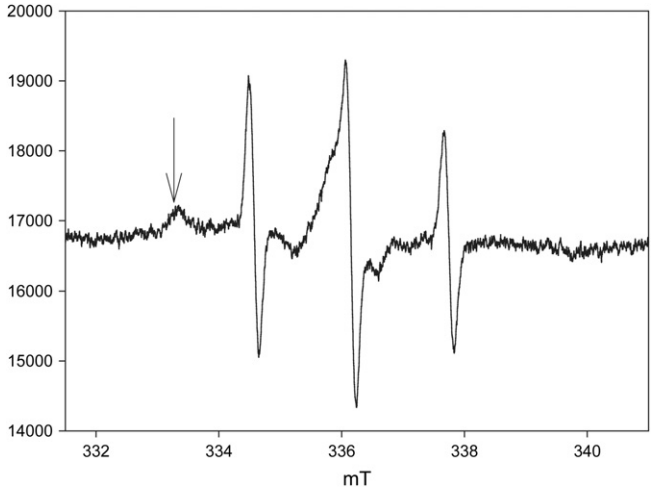



**Fig. 7.** EPR spectra of 50 μM **13c** and 250 μM Calf thymus B-DNA in a buffer (50 mM NaCl and 5 mM Tris).

## 4. Conclusions

In conclusion, we have reported the synthesis of a new series of double (spin and fluorescence) sensors emitting between 610 and 800 nm containing three different fluorophores: BODIPY dye, Nile Red and Ru-complex. Among the fluorophore-nitroxide adducts synthesized **5c** exhibited the highest sensitivity based on the measurements of quantum yields of nitroxide (**c**-form) and *N*-hydroxilamine (**b**-form) pairs (Table 2). The sensitivity of compound **8c** is rather limited, while compound **13c** is utilizable not only as a redox sensor but as an EPR and photophysical probe for monitoring the interaction with B-DNA. Further biological application and optimalization of nitroxide-fluorophore adducts as redox sensors or ROS scavengers such as **5a** and **8a**, emitting in the long wavelength region, is in progress.

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