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ORIGINAL PAPER

Disabling Photoinduced Electron Transfer in 4,4-Difluoro-8 (-4′-hydroxyphenyl)-1,3,5,7-tetramethyl-4-bora-3a, 4a-diaza-*s*-indacene by Phosphorylation

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Abstract The synthesis and photophysical characterization of the phosphorylated Bodipy dye **5** are reported and compared to those of its hydroxyphenyl counterpart **1**. Conversion of the latter by three methods of phosphorylation yields the strongly fluorescent dye **5** which exhibits similar steady-state spectra like **1** but an approximately five times prolonged fluorescence lifetime τ_{Fl}. We attribute this distinct change from τ_{Fl}=0.7 ns for **1** to τ_{Fl}=3.7 ns for **5** to the suppression of photoinduced electron transfer in **5**. This photochemical reaction was previously held responsible for fluorescence quenching in **1**. Fluorescence correlation spectroscopy reveals that **5** can be detected by single-molecule methods and that uncaging of phosphate in **5** is a minor problem.

Keywords Phosphorylation · Fluorescence correlation spectroscopy · Bodipy · Photoinduced electron transfer · Single-molecule · Phenol

Introduction

Dyes with a 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene core, the so-called *Bordipy*rromethene (BODIPY) dyes, find widespread application as fluorescent labels in bio-analytics or as laser dyes [1, 2]. The fluorescence properties of the alkyl-substituted representatives are comparable to those of Xanthene-dyes like Fluorescein or Rhodamine dyes, but in contrast to these, Bodipy dyes are uncharged. Their synthesis is established and lead to the development of sensors for numerous ions [3–10]. Most often, the unit which binds the analyte is connected to the fluorescing core via the *meso*-position of the latter. Ion sensing then is achieved by suppression of fluorescence quenching mechanisms, notably photoinduced electron transfer [2, 3]. Excited dyes are redoxamphoteric and can act as electron-donor, i.e. reductant, or as electron-acceptor, i.e. oxidant. In the majority of the mentioned cases, the latter is realized (Scheme 1) [2]. With an oxidizable counterpart in close vicinity, i.e. the substituent in *meso*-position, electron transfer to the excited dye occurs faster than fluorescence emission. If ligand binding or a chemical transformation now increases the oxidation potential of the donor, then fluorescence quenching is suppressed. For enhancing the analytical ability, one can match their redox potential by fine-tuning the donor or the acceptor [3, 11].

In the present work, we demonstrate that the electron-donor properties of a hydroxyphenyl-moiety in *meso*-position can be removed by phosphorylation. Different methods for obtaining phosphomonoesters of a weakly fluorescent *p*-hydroxyphenyl-Bodipy dye are employed which lead to a highly water-soluble and strongly fluorescent product [12–14]. Infrared (IR), ultraviolet visible (UV/Vis), fluorescence emission, lifetime (time-correlated single photon counting (TCSPC)) and fluorescence correlation spectroscopy (FCS) are performed for characterization. We finally discuss the suitability of the product as substrate for studying the activity of phosphatases on the single-molecule level where both the substrate and its product should be detectable but distinguishable by their photophysical properties.

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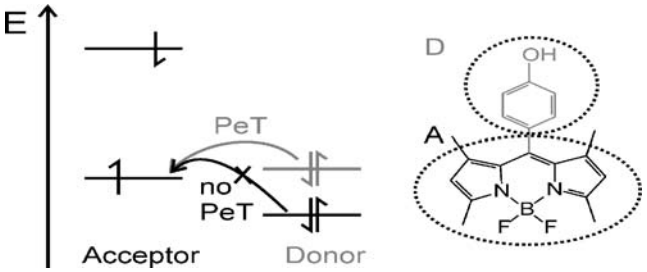


Scheme 1 Disabling PeT in **1**. Upon lowering the energy of the highest occupied molecular orbital in the donor moiety (*D*), i.e. raising the oxidation potential, electron transfer to the singly occupied molecular orbital of the acceptor (*A*) is disabled

Materials and methods

Reagents

4,4-Difluoro-8-(4′-hydroxyphenyl)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene 1 as educt was obtained following the literature [16]. The successful synthesis was verified by ^{1}H-NMR spectroscopy. Reagents for the syntheses were used at least in *purum* quality; solvents for spectroscopic measurements were in Chromasolv or comparable quality.

Phosphorylation methods

a) Phosphoryl chloride ($POCl_3$) [12]

Twenty milligrams (0.06 mmol) **1** were dissolved in 15 ml CH_2Cl_2 as solvent and cooled to 0°C. 17 µl $POCl_3$ (0.18 mmol) and 21 µl NEt_3 (0.15 mmol) in 2 ml CH_2Cl_2 were consecutively added. After 2 h, the reaction was stopped by pouring the solution on ice. The strongly fluorescent organic phase was separated from the colorless aqueous phase and dried over $MgSO_4$. Thin-layer chromatography (TLC) indicated the coexistence of the educt and a more polar product species. Hydrolysis of the latter was achieved by mixing the fluorescent CH_2Cl_2 solution with an equal amount of a saturated K_2CO_3 solution. The immediately green fluorescent aqueous solution was separated from the organic phase and neutralized with diluted HCl (Scheme 2).

b) Dibenzylphosphite (DBP) [13]

One hundred eighty milligrams (0.53 mmol) **1** were dissolved in 20 ml MeCN and cooled to −10°C. Successively, 256 µl CCl_4 (2.65 mmol), 194 µl Diisopropylethylamin (1.11 mmol), 6.5 mg Dimethylaminopyridine (0.05 mmol) and finally 174 µl DBP (0.77 mmol) were added and stirred for 1 h under a N_2-atmosphere. Afterwards, a mixture of MeCN and 0.5 M KH_2PO_4-solution (1:3) was used for terminating the reaction. After eluting three times with 20 ml Ethylacetate, the combined organic phases were washed with water and saturated NaCl solution, and dried over $MgSO_4$. After evaporation of the solvent, the remainder was dissolved in CH_2Cl_2 and subjected to column chromatography. Two fractions, which were more polar than the educt, could be eluted by increasing solvent polarity (mixture CH_2Cl_2/Tetrahydrofurane ranging from 1:0 to 1:1; finally methanol). Both fractions could be converted to a water-soluble, highly fluorescent product by stirring with diluted NaOH (0.1 M). The aqueous solutions were neutralized with diluted HCl (Scheme 2).

c) Diethylchlorophosphonate on magnesia [14]

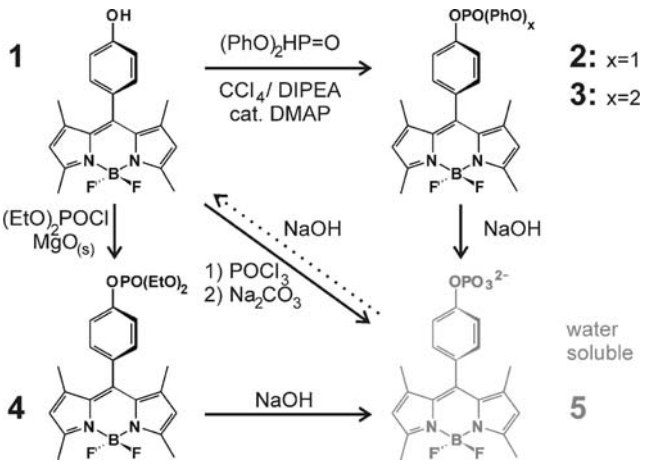
Eighty-five milligrams (0.25 mmol) **1** were mixed with 44 µl Diethylchlorophosphonate (0.3 mmol) and 15 mg MgO and heated for 1 h at 60°C. After cooling down, the remainder was eluted with CH_2Cl_2. Treatment with diluted NaOH (0.1 M) again yielded a yellowish, strongly fluorescent solution. The aqueous solutions were neutralized with diluted HCl (Scheme 2).

UV/Vis, Fluorescence and IR spectroscopy

Absorption and fluorescence emission spectra were recorded with a double-beam spectrometer (Lambda 5, Perkin Elmer) and a fibre coupled array spectrometer (SD 2000, Ocean Optics), respectively. IR spectra were recorded in attenuated total reflection (ATR) geometry (Spectrum 1000, Perkin Elmer). For that purpose, the dye solutions were put on the ZnSe prism and evaporated under N_2-atmosphere.

Lifetime measurements

We performed lifetime measurements of **1** and **5** in water. For that purpose, **1** was diluted in MeCN to ~10^{-5} M and



Scheme 2 Three syntheses of **5** starting from **1** using $POCl_3$ [12], (DBP, $(PhO)_2HP{=}O$) [13] or Diethylchlorophosphonate $(EtO)_2POCl$ on *MgO* [14]. See text for details

then added to water. The content of MeCN during the measurement was below 1%. Aqueous solutions of **5** were directly diluted to the appropriate concentration range (OD_{max}<0.1).

Fluorescence lifetimes were determined by a home-built TCSPC system in right-angle geometry. We used a picosecond-diode laser (LDH-P-C-470B, Picoquant) with a pulse width of ~70 ps at 40 MHz as excitation source, and detected the fluorescence photons under the magic angle and after filtering (HQ 500/35, AHF Analysentechnik) by a single-photon counting module (PD1CTC, Molecular Photon Device). The overall instrumental response function was about 200 ps (full width at half maximum). Data registration and analysis was performed using commercial software (SymPhoTime, Picoquant).

Fluorescence correlation spectroscopy

Experiments were performed with a home-built confocal setup based on an inverted microscope (Axiovert 200, Zeiss). A frequency-doubled diode laser (Picarro, Soliton), operating at λ_{exc}=488 nm, with a diameter of 0.7 mm was used as excitation source. The laser beam was directed to the microscope body and there deflected by a dichroic mirror (495 DRLP, Omega). The laser was focused by a water immersion objective lens (63×NA 1.2 WI, Zeiss) into an aqueous solution of the dyes **1** and **5** at a concentration of ~10^{-8} M. Fluorescence was collected by the same objective and was focussed by the tube lens to a pinhole with a diameter of 50 μm. After additional filtering (HQ 525/50, AHF Analysentechnik), fluorescence was split by a semitransparent mirror and detected by two avalanche photodiode modules (SPCM-14-AQR, PerkinElmer Optoelectronics). The electronic outputs of these devices were cross-correlated by a hardware correlator (FLEX 02 D, Correlator.com). The autocorrelation curves $g(\tau)$ were fitted using Eq. 1 by means of commercial software (Origin 7.5, Wolfram Scientific).

$$g(\tau) = 1 + \frac{1}{N}\cdot\frac{1}{1+\frac{\tau}{\tau_{diff}}}\cdot(1 + C\ \exp(-k\tau)) = 1 + \frac{1}{N}\cdot\frac{1}{1+\frac{\tau}{\tau_{diff}}}\cdot\left(1 + \frac{k_{23}^{eff}}{k_{31}}\exp\left(-\left(k_{23}^{eff} + k_{31}\right)\tau\right)\right) \quad (1)$$

N is the number of molecules in the detection volume, τ_{diff} is the diffusional time, C is the amplitude for the flickering dynamics, which is characterized by the overall rate constant $k.C$ and k are given by the reciprocal of the triplet lifetime, k_{31}, and the effective intersystem rate constant k_{23}^{eff}. The latter is determined by the intersystem rate constant k_{23}, the intensity I, the reciprocal of the excited state lifetime, k_{21}, and the absorption cross section σ (Eq. 2)

$$k_{23}^{eff} = k_{23}\frac{\sigma\cdot I}{\sigma\cdot I + k_{21}\cdot h\nu}. \quad (2)$$

From a saturation plot, i.e. plot of k_{23}^{eff} vs. I, k_{23} can be computed as the limit for high intensities without explicit need of the absorption coefficient σ [15].

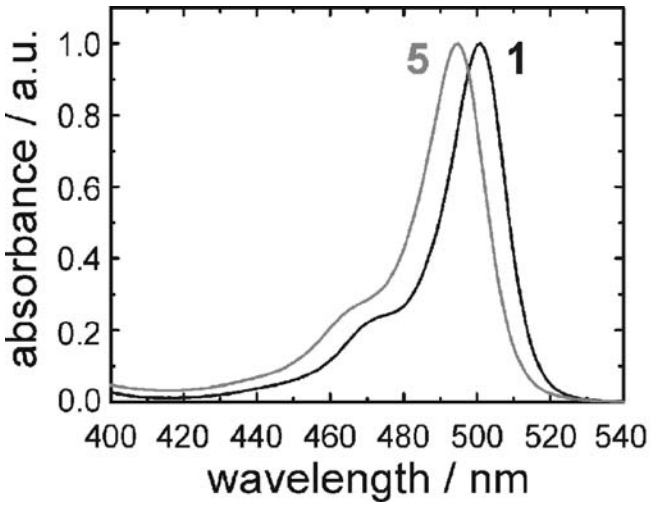
Results and discussion

Selection of educt

Bodipy dyes with hydroxyaryl substituents in *meso*-position were described several times [16–18]. Ionization of phenolic or naphtholic moieties with pK_a values between 7.5 and 10.5 was employed for pH measurements. Higher fluorescence quantum yields Φ_{Fl} up to 12% were detected for the neutral forms [18]. As pointed out in the introduction, we are interested in systems where both educt and product of a chemical reaction are appropriate for single-molecule spectroscopy. In this future application, the labelled phenol should be the reaction product and exhibit a higher Φ_{Fl} than the so far published compounds. Comparison of Φ_{Fl} of some of the mentioned dyes in aqueous solution has suggested that Φ_{Fl} could be increased further if more alkyl-substituents were attached to the Bodipy core [18]. In Scheme 1, this corresponds to raising the energetic level of the acceptor. We therefore synthesized **1**, which was described previously [16], as starting point for the phosphorylation.

Phosphorylation

Monoesters of **1** are preferred as any ester cleavage should be detectable by changes in the fluorescence properties. We applied three different procedures for the synthesis of phosphoesters and combined them with subsequent hydrolyzation (Scheme 2) [12–14]. In all cases, the conversion of **1** to the products **2**–**4** as the first reaction step in Scheme 2 was not quantitative, and TLC indicated a mixture of at least two compounds with the educt being one of these. The putative intermediates **2** and **3** were isolated but not further characterized. As expected, none of the fractions was water soluble. Hydrolyzing the double and triple esters by trimethylbromsilane was not successful and led to a complete loss of fluorescence [19]. However, hydrolyzing the esters by diluted basic solutions yielded green fluorescent aqueous solutions (Fig. 1). Despite the different access routes, hydrolyzation led to the same *one* final product according to optical spectroscopy and, more significant, its migration behaviour in TLC with different solvents. Further hydrolysation of the water soluble compound, however, led again to the educt which could subsequently be extracted



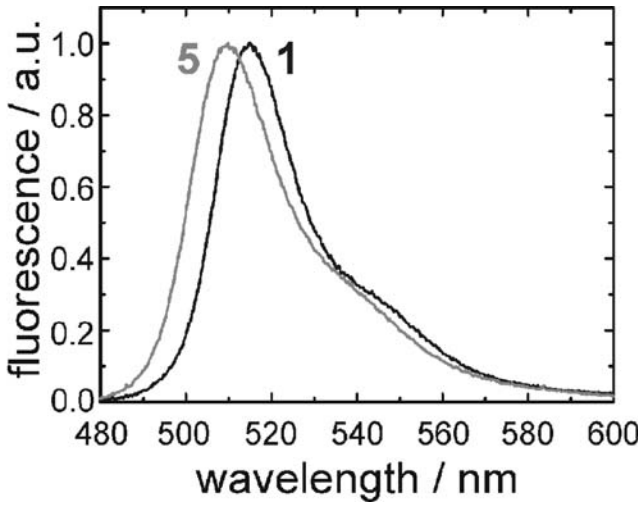


Fig. 1 Normalized absorption (*left*) and fluorescence emission spectra (*right*) of **1** in CH_2Cl_2 (*black*) and **5** at pH **7** (*grey*). The blue shift from **1** to **5** in absorption (λ_{max}(**1**)=501, λ_{max}(**5**)=495 nm) and emission (λ_{max}(**1**)=515, λ_{max}(**5**)=510 nm) is not significant as solvent dependent changes of the spectra of hydroxyaryl-Bodipy's in different solvents were noticed [20]

by CH_2Cl_2. From the mentioned solvation behaviour, we conclude that the water soluble fraction is the wanted phosphomonoester **5**. In agreement with these findings, hydrolyzation of the phosphodi- and triesters **2**–**4** by means of highly concentrated NaOH solutions (1 M) directly led back to the educt.

IR-Spectroscopy

Due to the low yields of the phosphomonoester and the experimental observation that complete removal of the solvent partially cleaved the ester, we performed IR-spectroscopy of the water soluble product under ATR-geometry for further support of our interpretation (Fig. 2). Comparison of the educt and the product revealed significant differences in the range between 1,000 and 1,600 cm^{-1}. First of all, the different bands of the product species appear much broader those of **1**. No coinciding absorbances between both spectra are detectable in this range. Furthermore, experimental Fourier transform IR-spectra of HPO_4^{2-} or $H_2PO_4^-$ showed no agreement with our spectra [20]. Most significant for the existence of a phosphoester is the product band at ~1,230 cm^{-1}, which is assigned to the P=O stretch vibration as in other aromatic phosphoesters [12].

Photophysical characterization

Figure 3 shows the comparison of TCSPC data of **1** and **5**. Excited **1** exhibits a very rapid fluorescence decay with a single lifetime τ_{Fl}=0.7 (±0.1) ns. By contrast, the excited

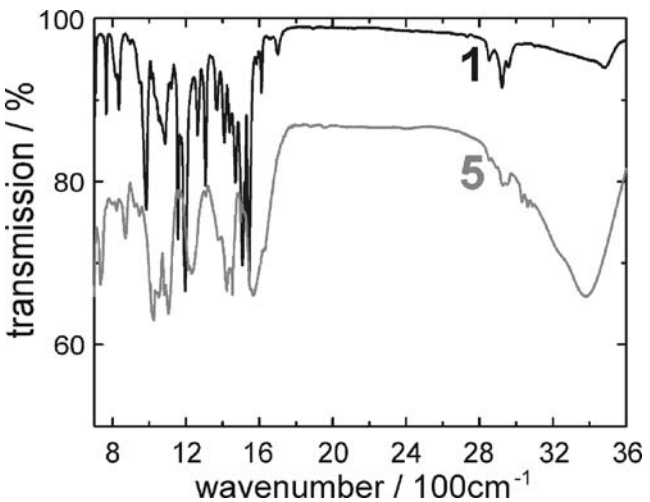


Fig. 2 ATR-IR spectra of **1** (*black*) and **5** (*grey*) in solid form

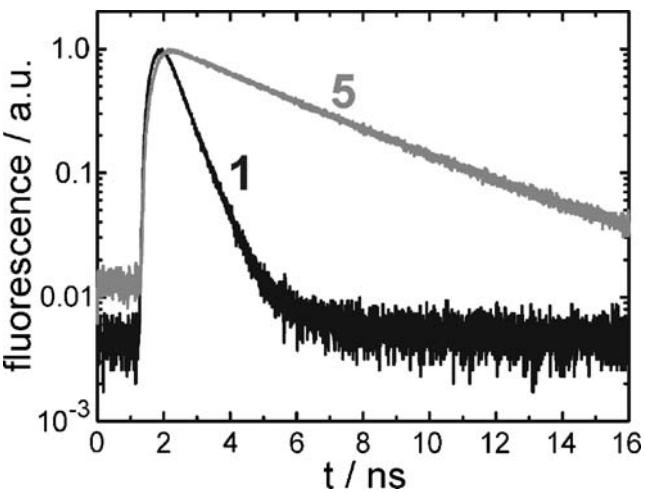


Fig. 3 Fluorescence lifetime measurement of **1** and **5** in water by time-correlated single photon counting. The prolongation of τ_{Fl} from **1** (τ_{Fl}=0.7 ns) to **5** (τ_{Fl}=3.7 ns) is explained by lowering the donor energy level in Scheme 1 by phosphorylation

state lifetime of its phosphorylated counterpart **5** is prolonged by roughly a factor of **5** (τ_{Fl}=3.7 (±0.1) ns). This latter value is close to the value obtained for 4,4-Difluoro-8-phenyl-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (τ_{Fl}=3.6 ns; B. Hinkeldey, G. Jung, unpublished results) which bears a phenyl-group in the *meso*-position. No pH-dependence of τ_{Fl} of **5** was detected.

The low Φ_{Fl} of different hydroxyaryl-substituted Bodipy-dyes was explained by fluorescence quenching due to photoinduced electron transfer (PeT) [18, 21]. The phenol acts as electron donor and the excited Bodipy core as electron acceptor. Probably, this also holds for **1** since its τ_{Fl} is considerably smaller than that of the phenyl-derivative. Thus, our interpretation of the prolonged τ_{Fl} of **5** compared to that of **1** is that phosphorylation of the hydroxyaryl-group cancels its donor properties and, consequently, PeT.

We finally performed FCS of **1** and **5** to judge their suitability for single-molecule experiments (Fig. 4). Only a few autocorrelation traces of **1** were recorded since this was very time consuming due to its low Φ_{Fl}, whereas acquisition of intensity dependent FCS data of **5** were quickly obtained. Comparison of curves at the same intensity (grey and black curves) shows a higher amplitude C for **1** than for **5**. This corresponds to a higher k_{23} for **1** than for **5** (Eq. 1), if one assumes that both dyes possess similar σ. On the basis of the available fluorescence lifetimes τ_{Fl}, i.e. the reciprocal values of k_{21}, we can estimate that the intersystem crossing quantum yield, $\Phi_{isc}=k_{23}/k_{21}$ is larger for **1** than for **5** by a factor of ~10. From the determined values for **5** (Fig. 4), we calculate Φ_{isc}=0.007.

The fact that FCS curves could be obtained for dyes **1** and **5** evidences their suitability for single-molecule research. The decrease of the diffusional time τ_{diff} of **5**, which is normally interpreted as photobleaching, also sets an upper limit for other imaginable photochemical reactions. Most relevant for future applications of **5** would be the cleavage of the here synthesized ester. Indeed, fluorescently labelled phosphoesters are employed for this so-called uncaging of phosphate, and such a reaction would exclude the use of **5** for measuring e.g. phosphatase activity [22]. However, it can be concluded from the curves that the light-induced release of phosphate in **5** occurs, if observable at all, with a very low quantum yield since otherwise the quality of the curves would drop considerably.

Conclusion

We have shown that the donor ability of phenol in PeT can be switched off by phosphorylation. This proof-of-principle sketches how phosphoester cleavage can be studied with dyes which are also appropriate for single-molecule detection. Different methods of obtaining **5** were tested, but there is still demand for improving its synthesis. The purification is the most challenging procedure and appears critical due to the decomposition of **5** upon drying. As the

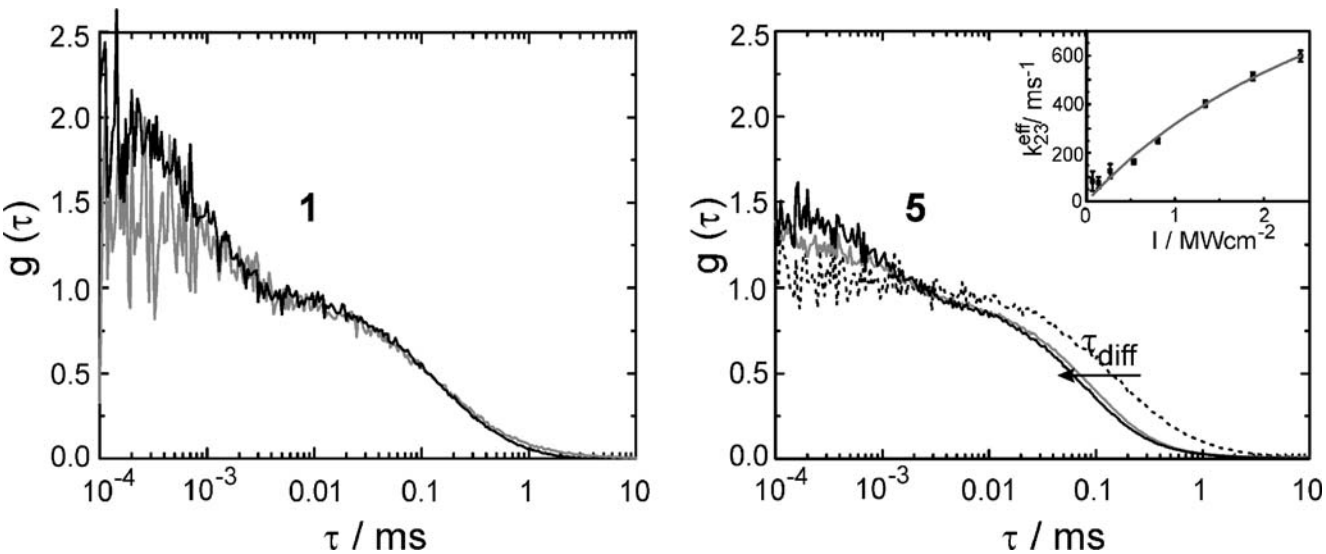


Fig. 4 Fluorescence correlation curves of **1** (*left*) and **5** (*right*) in water at different intensities (*dotted*: 67 kWcm^{-2}, *grey*: 0. 27 MWcm^{-2}, *black*: 0.80 MWcm^{-2}). The offset 1 in Eq. 1 was subtracted from the curves; subsequently the curves were normalized to N=1. The amplitude C of the decay on the (sub)microsecond time scale is a measure for the triplet population, and shows a stronger intersystem crossing for **1** than for **5** at the same intensities [15]. $k_{31}(5)=7.3\times10^5$ s^{-1} (±20%) and, from the intensity dependent k_{23}^{eff}, $k_{23}(5)=1.8\times10^6$ s^{-1} (±25%; *inset*) can be determined. Photobleaching which appears in a reduction of the diffusional time τ_{diff} is more eminent for **5** than for **1** and might indicate phosphate uncaging

proposed concept is not restricted to Bodipy dyes, we will turn to correspondingly modified Xanthene-dyes which are inherently more water soluble than Bodipy dyes. The enhanced water solubility favors these dyes for bioanalytical applications.

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References

1. Ziessel R, Ulrich G, Harriman A (2007) The chemistry of Bodipy: A new *El Dorado* for fluorescence tools. New J. Chem 31:496–501
2. Loudet A, Burgess K (2007) BODIPY dyes and their derivatives: Syntheses and spectroscopic properties. Chem Rev 107:4891–4932
3. Gabe Y, Urano Y, Kikuchi K, Kojima H, Nagano T (2004) Highly sensitive fluorescence probes for nitric oxide based on Boron dipyrromethene chromophore—rational design of potentially useful bioimaging fluorescence probe. J Am Chem Soc 126: 3357–3367
4. Basaric N, Baruah M, Qin W, Metten B, Smet M, Dehaen W, Boens N (2005) Synthesis and spectroscopic characterisation of BODIPY based fluorescent off–on indicators with low affinity for calcium. Org Biomol Chem 3:2755–2761
5. Kollmannsberger M, Rurack K, Resch-Genger U, Daub J (1998) Ultrafast charge transfer in amino-substituted Boron dipyrromethene dyes and its inhibition by cation complexation: A new design concept for highly sensitive fluorescent probes. J Phys Chem A 102:10211–10220
6. Rurack K, Kollmannsberger M, Resch-Genger U, Daub J (2000) A selective and sensitive fluoroionophore for HgII, AgI and CuII with virtually decoupled fluorophore and receptor. J Am Chem Soc 122:968–969
7. Qi X, Jun E, Kim S, Hong J, Yoon Y, Yoon J (2006) New BODIPY derivatives as off–on fluorescent chemosensors and fluorescent chemodosimeter for Cu^{2+}: cooperative selectivity enhancement toward Cu^{2+}. J Org Chem 71:2881–2884
8. Li M, Wang H, Zhang X, Zhang H-S (2004) Development of a new fluorescent probe: 1,3,5,7-tetramethyl-8-(4′-aminophenyl)-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene for the determination of trace nitrite. Spectrochim Acta A 60:987–993
9. Kalai T, Hideg K (2006) Synthesis of new, BODIPY-based sensors and labels. Tetrahedron 62:10352–10360
10. Koutaka H, Kosuge J, Fukasaku N, Hirano T, Kikuchi K, Urano Y, Kojima H, Nagano T (2004) A novel fluorescent probe for Zinc ion based on Boron dipyrromethene (BODIPY) chromophore. Chem Pharm Bull 52:700–703
11. Urano Y, Kamiya M, Kanda K, Ueno T, Hirose K, Nagano T (2005) Evolution of fluorescein as a platform for finely tunable fluorescence probe. J Am Chem Soc 127:4888–4894
12. Tietze L, Eicher T (1991) *Reaktionen und Synthesen im organisch-chemischen Praktikum und Forschungslaboratorium*. Thieme, Stuttgart
13. Silverberg L, Dillon J, Vemishetti P (1996) A simple, rapid and efficient protocol for the selective phosphorylation of phenols with dibenzylphosphite. Tetrahedron Lett 37:771–774
14. Kaboudin B (1999) Phosphorylation of phenols with diethyl-chlorophosphonate on the surface of magnesia. J Chem Res 402–403, DOI 10.1039/a900183b
15. Widengren J, Mets Ü, Rigler R (1995) Fluorescence correlation spectroscopy of triplet states in solution: A theoretical and experimental study. J Phys Chem 99:13368–13379
16. Coskun A, Deniz E, Akkaya E (2005) Effective PET and ICT switching of boradiazaindacene emission: A unimolecular, emission mode molecular half-subtractor with reconfigurable logic gates. Org Lett 7:5187–5189
17. Gareis T, Huber C, Wolfbeis O, Daub J (1996) Phenol/phenolate-dependent on/off switching of the luminescence of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacenes. Chem Comm 1717–1718, DOI 10.1039/a703536e
18. Baruah M, Qin W, Basaric N, DeBorgraeve W, Boens N (2005) Bodipy-based hydroxyaryl derivatives as fluorescent pH-probes. J Org Chem 70:4152–4157
19. Lazar S, Guillaumet G (1992) A selective removal of benzyl protecting groups in arylphosphate esters with bromotrimethylsilane. Synth Comm 22:923–931
20. Klähn M, Mathias G, Kötting C, Nonella M, Schlitter J, Gerwert K, Tavan P (2004) IR spectra of phosphate ions in aqueous solution: Predictions of a DFT/MM approach compared with observations. J Phys Chem 108:6186–6194
21. Qin W, Baruah M, Stefan A, Van der Auweraer M, Boens N (2005) Photophysical properties of BODIPY-derived hydroxyaryl fluorescent pH probes in solution. ChemPhysChem 6:2343–2351
22. Goeldner M, Givens R (2005) Dynamic Studies in Biology. Wiley, Weinheim