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Wavelength dependent photochemistry of BODIPY-phenols and their applications in fluorescent labeling of proteins

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A series of BODIPY dyes were synthesized, that were at the 3, or 3 and 5 positions, substituted by photochemically reactive quinone methide (QM) precursor moieties. Fluorescence properties of the molecules were investigated and we demonstrated that the molecules undergo wavelength dependent photochemistry. Photodeamination to deliver QMs takes place only upon excitation to higher excited singlet states, showing unusual anti-Kasha photochemical reactivity. The findings were corroborated by TD-DFT computations. Laser flash photolysis experiments could not reveal QMs due to low efficiency of their formation, but enabled detection of the phenoxyl radicals. The applicability of the molecules for fluorescent labeling of bovine serum albumin as a model protein upon photoexcitation at 350 nm was demonstrated.

Introduction

BODIPY dyes are versatile chromophores, whose applications in fluorescence technology and microscopy gained importance over last 20 years.1 They are generally characterized by narrow excitation/emission spectra in the visible region with high molar absorption coefficients (ε > 50000 M-1 cm-1) and high fluorescence quantum yields (Φf), owing to usually small quantum yields of intersystem crossing (ISC).2 Moreover, BODIPY dyes are well soluble in many solvents and they are thermally and photochemically stable. Easy synthetic post-modifications of the BODIPY moiety allows for the preparation of a large number of derivatives characterized by tunable spectral properties and different applications.3 Thus, they were used for sensing,4 or intracellular labeling of different biomolecules.5 Different protocols were established for the *in vitro* labeling of proteins by covalent attachment to lysines,6 tyrosines,7 or cystein residues.8 Furthermore, BODIPY dyes were investigated as potential drugs in photodynamic therapy (PDT).9

Photochemical activation of fluorescent dyes in labeling leads to significant advances when used in biological systems, since it allows for spacial and temporal control of the activation event.10 In that context, several protocols for the photoactivation of BODIPY dyes for the fluorescent labeling of proteins have been discovered.11,12 Furthermore, we have recently reported protein photolabeling method, which is based on anti-Kasha photochemical reactivity of BODIPY-quinone methide (QM) precursors.13,14 QMs are reactive intermediates of phenols15 that have been intensively investigated in the last two decades due to their applications in synthesis16 and biological activity,17 which is connected to the reactivity with DNA18 and proteins.19 QMs can be generated in photochemical reactions under mild conditions,20 allowing for their use in photolabeling,14 or in the development of anticancer phototherapeutics.21 Use of QM precursors as phototherapeutics is advantageous compared to the classical PDT since it does not rely on dissolved oxygen in cells, and it is known that cancer cells are often hypoxic.22

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Chart 1. BODIPY-QM precursors.

Herein we report the synthesis of a series of BODIPY dyes **1**-**5** (Chart 1) that are substituted at the BODIPY 3, or 3 and 5 positions with the photochemically reactive phenol group(s) that undergo deamination upon excitation by light and deliver QMs.23 Photochemical reactivity was investigated by preparative irradiations, whereas photophysical properties of the molecules were characterized by steady state fluorescence measurements and corroborated by TD-DFT computations. To detect QMs or other plausible intermediates in the photochemistry we used laser flash photolysis. The main finding is that the molecules show wavelength dependent photochemical reactivity, with more efficient reactions taking place upon higher energy excitation, contrary to the Kasha’s law.24 The applicability of the dyes was investigated in fluorescent labeling of a model protein bovine serum albumin (BSA).

Results and discussion

Synthesis

The starting molecules for the synthesis of BODIPY QM precursors **1**-**5** (Scheme 1) are mono- or dichlorinated BODIPY derivatives **6** and **9** (Schemes 1 and 2). They were prepared by two synthetic protocols, by chlorination of the dipyrromethane followed by the transformation to BODIPY,25 or by preparation of the unsubstituted BODIPY and its chlorination by use of CuCl2.26 Arylation27 with Bn-protected boronic acid afforded mono- or bis-arylated derivatives in moderate to good yield, and the cleavage of the benzyl group was conducted in good yield by use of Et3SiH and Pd/C as a catalyst. We performed also a direct C-H arylation of the parent unsubstituted BODIPY according to the modification of the procedure.28 Although the direct C-H arylation shortened the synthetic protocol since it was not necessary to prepare the chlorinated derivative **6**, compound **7** was obtained in low yield of 36% only (see the experimental), precluding further direct C-H arylations. A treatment of the free phenolic compounds with the Eschenmoser's salt afforded the BODIPY-QM precursor **1** in good yield. Upon purification of **1** on preparative HPLC by use of solvent that contained trifluoroacetic acid (TFA), **1** was transformed into the corresponding salt **1×TFA**.

Synthesis of **2**-**5** followed the same protocol as described for **1**. Dichlorinated BODIPY **9** was arylated affording mono- and bis-arylated BODIPY derivatives **10** and **11**, respectively. Cleavage of the benzyl ethers from compound **11** by Et3SiH on Pd/C gave phenol **12** and mono-benzylated phenol **13**. Electrophylic substitution on **12** with the Eschenmoser’s salt gave a mixture of **2**-**5**, which were separated by preparative HPLC and characterized only in the form of TFA salts. ESI-MS and MALDI-MS analyses indicated that compounds **2×TFA-5×TFA** were monoprotonated. Attempt to prepare a larger amount of **4** followed a protocol where the benzylated compound **13** was treated with an access of the Eschenmoser’s salt, followed by the cleavage of the benzyl group. However, this procedure gave a complex mixture of products where the dimethylamino groups were also cleaved off.

Photophysical prperties

Photophysical properties of **1** and **2** and their salts, as model fluorophores, were investigated by UV-vis and steady-state fluorescence spectroscopy in nonaqueous CH3CN and aqueous solvent CH3CN-H2O (1:1 v/v). The measurements were conducted at pH 7 and 9 (sodium phosphate buffer, 50 mM), since it is known that at these pH values QM precursors exist in different prototropic forms, which reflects their photochemical reactivity.23 For all spectra see Figs S1-S10 in the ESI.

Absorption spectra of **1×TFA** and **2×TFA** in CH3CN (Fig S3) are characterized by a sharp and intensive absorption band with a maximum at 534 nm and 573 nm, respectively. It corresponds to the HOMO→LUMO transition and population of the S1, the typical for BODIPY derivatives with additional conjugation.2,4 Higher excited singlet states are well separated in energy, and excitation occurs at *λ* < 400 nm. The computations for **1** (*vide infra*) corroborate this assignment. Comparison of the absorption spectra of **7**, **8**, **1** and **1×TFA** show that all exhibit similar absorption properties with a small difference between the absorption maxima (Fig S1 and S2). The most batochromically shifted is the spectrum of **1**, which was explained by intramolecular H-bond between the amino group and the phenol oxygen, increasing the negative charge on the oxygen, and increasing the energy of the HOMO orbital. In the aqueous solution at pH 7 (Fig 1), in the absorption spectrum of **1**, an additional batochromically shifted shoulder shows up at ≈ 585 nm, indicating that at pH 7 the molecule is in an equilibrium with the phenolate. The assignment is corroborated by the spectrum at pH 9 (Fig S4), where the molecule is mostly in the phenolate form. The absorption spectrum of **2×TFA** in CH3CN is batochromically shifted compared to **1×TFA** by 39 nm due to additional conjugation, and it matches the spectrum of the parent bisphenol **12** (Fig S5). In the aqueous solvent at pH 7 and 9, in the absorption spectra of **2** there are no additional bands due to phenolate, and the spectra are only broader. Thus the width of the band at the half intensity in CH3CN is 1976 cm-1, at pH 7 is 2311 cm-1, whereas at pH 9 it is 2826 cm-1 (Fig S6).

Fluorescence spectra of **1×TFA** and **2×TFA** in CH3CN are mirror images of the absorption spectra and have maxima at 563 nm and 618 nm, respectively. The Stokes shifts are small (965 cm-1 and 1271 cm-1), the typical for the BODIPY derivatives.2,4 The maxima of the emission spectra of **7**, **8**, **1** and **1×TFA** in CH3CN overlap, indicating that all compounds have the same emitting state regardless of the substitution. The fluorescence quantum yields (ΦF) were measured using rhodamine B in CH3OH (ΦF = 0.66) as a reference.29 The ΦF for **7**, **8**, **1** and **1×TFA** in CH3CN are generally very low (< 2%), and the photochemically reactive molecules **1** and **1×TFA** are ten times less emissive compared to the parent phenols (Table 1). A lower ΦF in CH3CN for **1** compared to **8** is tentatively assigned to the excited state intramolecular proton transfer (ESIPT) to nitrogen atom, as seen with the phenolic BODIPY described in literature precedent.14 In aqueous solvent, fluorescence spectra of **8** and **1** practically overlap, and there are no changes in spectra by pH increase to 9 (Fig S7), indicating that the emissive state is the same. QM precursor **1** is the most emissive in aqueous solvent at pH 7 (ΦF = 0.008-0.009), when the intrinsic ESIPT is less efficient due to competition of H2O molecules that perturb intramolecular H-bond. Furthermore, increase of the pH reduces ΦF five times indicating that the phenolate is less emissive.

E:\PODACI-Nikola\Papers\Papers 2021\BODIPY_PhOH\OBC\Figs\Scheme 1_new.tif

Scheme 1. Synthesis of BODIPY-QM precursor **1**.

E:\PODACI-Nikola\Papers\Papers 2021\BODIPY_PhOH\OBC\Figs\Scheme 2_new.tif

Scheme 2. Synthesis of BODIPY-QM precursors **2-5**.



Fig 1. Left: Normalized absorption spectra of **1** and **2** in CH3CN-H2O (1:1 v/v), at pH 7 (50 mM phosphate buffer). Right: Normalized fluorescence spectra in CH3CN (*λ*exc = 500 nm).

On the contrary to monophenolic compounds, bisphenolic derivatives **12**, **2×TFA** and **4×TFA** have generally higher ΦF (Table 1). In CH3CN solution, the ΦF for **12** is about 10 times higher than **2×TFA**, presumably due to photochemical reactivity or other nonradiative pathways for **2×TFA**. Furthermore, the ΦF for **12** in the aqueous solvent is lower compared to CH3CN, which may be attributed to the excited state proton transfer (ESPT), an ubiquitous pathway for phenols in the singlet excited state, taking place in the aqueous solvent only.30 However, the phenolate formed in the ESPT does not exhibit different maximum of the emission. Thus, the spectra at pH 7 and 9 have almost the same shape (Fig S8), but the fluorescence is quenched at higher pH. On the contrary, in the neutral aqueous solution, **2** has higher ΦF then in the CH3CN solution. The ΦF of 0.13, allows the use of **2** as a fluorescent label in biological aqueous systems and in fluorescent microscopy.

Table 1. Quantum yields of fluorescence (ΦF)a of model fluorophores in CH3CN and CH3CN-H2O (1:1 v/v)b.

|  |  |  |  |
| --- | --- | --- | --- |
| Comp. | ΦF (CH3CN) | CH3CN-H2O  (1:1 v/v, pH 7) | CH3CN-H2O  (1:1 v/v, pH 9) |
| **7** | 0.0210 ± 0.001 | - | - |
| **8** | 0.0170 ± 0.001 | 0.0160 ± 0.001 | - |
| **1** | 0.0010 ± 0.0005 | 0.0081 ± 0.0001 | 0.0016 ± 0.001 |
| **1×TFA** | 0.0031 ± 0.002 | 0.009 ± 0.001 | 0.001 ± 0.001 |
| **12** | 0.27 ± 0.01 | 0.21 ± 0.001 | 0.15 ± 0.03 |
| **2×TFA** | 0.0235 ± 0.004 | 0.13 ± 0.01 | 0.007 ± 0.002 |
| **4×TFA** | 0.046 ± 0.003 |  |  |

a Quantum yields of fluorescence were measured by use of rhodamine B in CH3OH as a reference (ΦF = 0.66).29 The samples were excited at three wavelengths, three quantum yields were calculated (See eq. S1 in the ESI) and the average value is reported. The quoted error corresponds to the maximum absolute deviation. b In the presence of sodium phosphate buffer, 50 mM.

Comparison of the absorption spectra of **2×TFA**, **3×TFA**, **4×TFA** and **5×TFA** in CH3CN indicates that their spectral properties are the same (Fig S10). However, the maximum of the emission spectrum of **4×TFA** is ≈ 40 nm batochromically shifted compared to **2×TFA**. Therefore, the number of methylamino groups does not affect the absorption properties of the molecules, but it affects the position of the emission maxima and ΦF. Note that the fluorescence properties are inversely correlated to the photochemical reactivity, and it was demonstrated that the introduction of additional methylamino group to the QM precursor increases the reactivity.23

Computations

The photophysical properties of BODIPY-QM precursor were rationalized by computations at TD-DFT level of theory using BPE0 density functional for molecule **1** (for all data see Figs S11-15 and Tables S3-S6 in the ESI). The computed absorption spectrum for **1** is shown in Fig S13, as well as frontier molecular orbitals important for the absorptions to S1 and Sn states (Fig S12). The low energy vertical excitation involves translocation of the electron density from the HOMO, which is delocalized over the phenolic functional group and the BODIPY core, to the LUMO where electronic density is decreased on the phenolic group including oxygen atom (Fig 2a). Therefore, the translocation of the electron density, upon population of S1, from the phenole to the BODIPY is expected. This is anticipated to drive ESIPT between the phenolic O (more acidic in the excited state than in the ground state) and the amine N in **1**, as demonstrated computationally for the model compound.31 Indeed, the optimization on the S1 excited state PES started in







Fig. 2. a) Computed HOMO and LUMO orbitals for **1** involved in the S0→S1 transition, computed at the PBE0/6-311+G(2d,p) level of theory; b) The ground state and the first excited singlet state optimized structures of molecule **1** using PBE0/6-311G(2d,p) method; c) Potential energy surface scan in the first excited singlet state (S1) for the deamination of **1** calculated at the TD PBE0/6-311G(2d,p) level of theory. Relative energies with respect to the ground state minimum energy of **1** are given in kcal mol-1

the FC point of **1**(S0 MIN geometry) lead to the S1 excited state minimum **1**(S1 MIN) with the proton bonded to the N atom (Fig 2b, calculated N-H distance is 1.09 Å). Along the ESIPT path, the ground state is strongly destabilized and the S0/S1 energy gap is decreased from 60.7 in FC point to 24.0 kcal mol-1 in the S1 MIN, increasing the probability of noradiative deactivation. Indeed, the experiment showed that the phototautomer is not emissive, and therefore, there are no batochromically shifted bands in the fluorescence spectra of **1** compared to **8**. The phototautomer formed by ESIPT does not undergo deamination (*vide infra*), due to an energy requirement for dissociation of the C-N bond along deamination reaction path on the S1 potential energy surface (See Fig 2c). This is in accordance with the phenolic BODIPY described in literature precedent.14

Photochemical reactivity

To probe for the photodeamination reaction of BODIPY derivatives **1**-**5**, we conducted preparative irradiations in CH3OH. The deamination is anticipated to give QMs, which react with CH3OH as a nucleophile giving methyl ethers (for all data see Schemes S1-S5 and Figs S16-S29). The irradiations were performed in neat CH3OH, as well as in buffered CH3OH-H2O (5:1) at pH 7 and 9, where difference was expected since the molecules bear a positive charge, or they are in neutral-zwitterionic form, respectively.23 The irradiation with visible light (cool white lamps) which excites molecules to the S1 state did not give methanolysis products. On the contrary, upon irradiation at 350 nm or 300 nm, photoproducts were detected by HPLC and HPLC-MS (Table 2), in line with the anti-Kasha photochemical reactivity, already reported for BODIPY-QM derivatives.14 The photoproducts **1-OCH3** and **2-OCH3** were isolated and characterized by NMR (Schemes 3 and 4).



Scheme 3. Photomethanolysis of **1**.



Scheme 4. Photomethanolysis of 2×TFA.

Molecules **3**-**5** bear more than one methylamino substituents on the phenols, enabling 2 or 3 successive photochemical reactions to take place. Due to complex mixtures after photolyses, isolation of the photoproducts was not conducted and the composition was characterized by HPLC and HPLC-MS only. Plausible photoproducts are given in Chart 2 and schemes S1-S5. The analysis of the mixture by HPLC-MS could identify how many methylamino groups were substituted in the photolysis, but we could not distinguish between **5a-OCH**3 and **5b-OCH3** or **5a-(OCH3)2** and **5b-(OCH3)2**, etc.

The reactions are not efficient, long irradiations were required to achieve conversion to photoproducts. Quantum yields for the methanolysis reaction of **1×TFA** and **2×TFA** were estimated by use of ferrioxalate actinometer (ΦR = 1.25)32 upon excitation at 300 nm. The measured ΦR for the methanolysis of compound **1×TFA** in CH3OHhas value of(6.7±0.3) ×10-3, whereas **2×TFA** reacts less efficiently and we only give an estimate ≈3×10-4. However, one generally observe higher reactivity upon irradiation in aqueous solvent, in line with previous reports on ammonium salts23 and upon use of higher energy excitation (shorter wavelength), which was reported for BODIPY-QM precurosors.14 The results obtained by photolyses are in accord with the photodeamination of molecules taking place upon excitation to Sn only, and delivering QMs.

QMs are known to react with ethyl vinyl ether (EVE) in hetero Diels Alder reaction providing chromane products.16,33 Therefore, we performed irradiations of **1×TFA** and **2×TFA** in CH3CN in the presence of EVE where the anticipated cycloaddition products would prove formation of QMs as intermediates in their photochemistry. However, only after long irradiation of **1×TFA** (10 h) when most of the QM precursor reacted, HPLC-MS could reveal a trace of the cycloadduct (Scheme S6 in the ESI). Most probably hydrolysis of the QMs takes place faster than the hetero Diels-Alder reaction, making it very inefficient.



Chart 2. Plausible photoproducts from **3×TFA** and **5×TFA**

Table 2. Irradiation conditions and yields of photoproducts.a

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Comp. | Solvent | 254 nm | 300 nm | 350 nm | vis |
| **1 or 1×TFA** | CH3OH | -b | 21 | 0 | 0 |
| CH3OH-H2O pH 7 | -b | 30 | 0 | 0 |
| **2×TFA** | CH3OH | -b | < 1 | 0 | 0 |
| CH3OH-H2O pH 7 | 33 | 16 | < 1 | 0 |
| **3×TFA** | CH3OH | -b | 13 (**3-OCH3**)  26 (**3-(OCH3)2**)  10h | 0 | 0 |
| CH3OH-H2O pH 7 | -b | -b | 26  (**3-OCH3**) | 0 |
| CH3OH-H2O pH 9 | -b | 12 (**3-OCH3**)  81 (**3-(OCH3)2**) | 26  (**3-OCH3**) | 0 |
| **4×TFA** | CH3OH | - | 41 (**4-OCH3**)  2 (**4-(OCH3)2**) | 26  (**4-OCH3**) | 0 |
| CH3OH-H2O pH 7 | - | 48 (**4-OCH3**)  28 (**4-(OCH3)2**) | 27  (**4-OCH3**)  1  (**4-(OCH3)2**) | 0 |
| CH3OH-H2O pH 9 | - | 53 (**4-OCH3**)  32 (**4-(OCH3)2**)  10 h | 36  (**4-OCH3**)  3  (**4-(OCH3)2**) | 0 |
| **5×TFA** | CH3OH | -b | 52 (**5-OCH3**)  7+28  (**5-(OCH3)2** +  **5-(OCH3)3** ) | 32  (**5-OCH3**) | 0 |
| CH3OH-H2O pH 7 | -b | 29 + 19  ((**5-(OCH3)+**  **5-(OCH3)2** )  25 **5-(OCH3)3** | 31  (**5-OCH3**)  3  (**5-(OCH3)2**) | 0 |
| CH3OH-H2O pH 9 | -b | 36 **5-(OCH3)3**  10 unknown  54 unknown | 23  (**5-OCH3**)  3  (**5-(OCH3)2**) | 0 |

a Irradiation was performed for 16 h (unless stated otherwise) in CH3OH or CH3OH-H2O (5:1) in the presence of sodium phosphate buffer (50 mM) at pH 7 or 9. Luzchem reactor equipped with 8 lamps (1 lamp 8 W) was used. The reported yields (%) correspond to ratio of products obtained by HPLC. Photoproducts from **3×TFA** and **5×TFA**, (Chart 2) are given in parenthesis. b Material decomposition.

Laser flash photolysis

To detect QMs or other plausible intermediates in the photochemical reactions of **1×TFA** and **2×TFA**, we conducted laser flash photolysis experiments. The samples were excited with a YAG laser at 355 nm, where photochemical reactivity was anticipated based on the cutoff wavelength when we start to observe photolyses. The experiments were conducted in CH3CN or CH3CN-H2O (1:1 v/v) in the presence of phosphate buffer (50 mM) at pH 7, and prior to the experiment the solutions were purged with Ar or O2, where O2 was expected to quench triplet excited states, but not quinone methides, phenoxyl radicals or radical-cations (For all data see Figs S31-S55 in the ESI).

**1×TFA** in the Ar- and O2-purged CH3CN solution gave very weak transients absorbing in the region 550-650 nm. The transient was not quenched by O2 and it decayed with the lifetime of τ ≈ 30-50 μs. However, a similar transient was detected also for compound **8** (Fig S39 in the ESI), precluding its assignment to QM. The observed transients were tentatively assigned to the phenoxyl radical cation **1-RC** or phenoxyl radical **8-R**, ubiquitous transients in the phenol photochemistry.34 On the contrary, **2×TFA** in the Ar- and O2-purged CH3CN and CH3CN-H2O solution gave relatively strong transients with a maximum at 680 nm. The transient was fit to a mono-exponential function with the lifetime of *τ* = 430 ± 20 μs, irrespective on the irradiation conditions (Ar or O2, aqueous or not). Since a similar transient was detected for molecule **12** that cannot generate QMs, the transients from **2×TFA** and **12** were analogously assigned to the radical cation **2-RC** or phenoxyl radical **12-R** (Fig 3 and Chart 3). Inability to detect QMs from **1×TFA** and **2×TFA** is in accord with low efficiency of their methanolysis reaction. However, transients such as phenoxyl radicals formed from **1×TFA** and **2×TFA** in photochemical reactions may react with biomacromolecules, affect cancer cells and potentially induce antiproliferative effects.



Fig 3. Transient absorption spectra measured in O2-purged CH3CN for **12** and **2×TFA** measured 3 μs after the laser pulse.



Chart 3. Plausible intermediates detected by LFP of **1×TFA**, **8**, **2×TFA** and **12**.

Labeling of proteins

Although QMs from **2×TFA** were not detected by LFP, photomethanolysis indicated their formation. Therefore, we investigated applicability of the most fluorescent compound from the investigated series, **2×TFA**, to fluorescently label proteins via QM intermediate upon photoactivation. As a model protein we have chosen bovine serum albumin, which is known to bind numerous molecules since it serves as a transport protein.35 Noncovalent binding of **2×TFA** to the protein was investigated by fluorescence titrations in CH3CN-H2O (1:4 v/v) in the presence of phosphate buffer (pH 7, 50 mM). Addition of BSA induced significant quenching of the emission, which was processed by nonlinear regression analysis providing stability constant of the complex **2×TFA@BSA** with 1:1 stoichiometry, log*β* = 4.00 ± 0.02 (Fig 4).





Fig 4. Left: Fluorescence titration (*λ*exc = 520 nm) of **2×TFA** (c = 5.4×10-6 M) with BSA in CH3CN-H2O (1:4 v/v) in the presence of phosphate buffer (pH 7, 50 mM); Right: Dependence of the fluorescence intensity at 610 nm on the BSA concentration. The black dots are experimental values and the red line is fit to the model involving complex formation with the stoichiometry 1:1.

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Fig 5. SDS-PAGE gel (10%) after photoinduced labeling of BSA with **2×TFA**. BSA (20 µg) was incubated with or without **2×TFA (***c* = 0.1 mM, or *c* = 1 mM), irradiated at 350 nm (30 min, and 60 min, 6 lamps) or not irradiated, and then subjected to SDS-PAGE. Top panel: The labeled BSA was visualized in the gel using a UV light (254 nm). Bottom panel: The same gel stained with Coomassie brilliant blue.

The covalent binding of **2×TFA** to BSA was probed by irradiating the dye in the presence of BSA, followed by an analysis by SDS PAGE electrophoresis (Fig 5). The dye in the concentration 1 mM after 30 or 60 min of irradiation at 350 nm made the bands visible in the gel. On the other hand, the bends were not visible if the sample was not treated with **2×TFA** or it was not irradiated, confirming that the dye attached covalently to the protein in a photochemical reaction. Involvement of QMs in the photolabeling of proteins, probably involving lysines or cysteines, is in accord with literature precedent.19 The lamps with the broad maximum output at ≈ 350 nm were chosen instead of 300 nm (as in the MTT experiment, *vide infra*), not to damage the protein, which can absorb the light at 300 nm. Note that the formation of QM from **2×TFA** requires the use of *λ* < 350 nm light, whereas for the fluorescence readout of the labeled proteins visible light can be used for the excitation, preventing photo-detachment of the dye from the protein. This is particularly important in the development of sensor and indicator molecules that do not detach from the targets or bleach during long laser experiments.

For the ultimate use of BODIPY dyes in fluorescent labeling it is also important to test their cytotoxicity. Therefore, we investigated antiproliferative effect of QM-BODIPY precursors by the standard MTT test on three human cancer cell lines MCF-7 (breast), H460 (lung) and HCT 116 (colon), with the cells kept in dark or irradiated (see Table S8 in the ESI). All compounds exhibited antiproliferative effect in the micromolar concentration range. The exposure of cells treated with compounds **2×TFA**, **4×TFA**, and **5×TFA** to visible light, induced significant enhancement of the activity, which cannot be correlated to the formation of QMs since it was initiated by visible light.

Note that **5×TFA** has three methylamino groups, allowing for the formation of long-lived QMs that are anticipated to undergo reversible alkylations of biomolecular targets leading to the stable cross-links, ultimately should be the most toxic event for the cell.36 Therefore, **5×TFA** represents a *lead* molecule for the further development of photoactivable BODIPY compounds. Consequently, further structure development should be devoted to make molecules like **5** reactive upon excitation with visible light.

Conclusions

We have demonstrated that BODIPY-QM precursors **1**-**5** undergo wavelength dependent photochemistry. The deamination to QMs takes place only upon excitation to higher excited states at *λ*<350 nm. Due to low efficiency of the QM formation they were not detected by LFP. However, the LFP measurements enabled detection of phenoxyl radicals, which may also be involved in the photoinduced toxicity. Compounds bearing two phenol moieties are generally more fluorescent, and we have demonstrated that they can be used as fluorescent labels for proteins, which can be attached in the photochemical reaction involving QMs. The applicability of the dyes in photolabeling stems from the fact that different wavelength of light (350 nm) is used for the photoattachment of the dye and for the fluorescence readout (550 nm).

Experimental

General 1H and 13C NMR spectra were taken at rt on a Bruker Avance 300 MHz or a Bruker Avance 600 MHz spectrometer. CDCl3, or CD3OD was used as deuterated solvent. TMS (1H NMR) or deuterated solvent itself (13C NMR) was used as internal reference. Chemical shifts were reported in ppm. 13C NMR spectra were recorded in the APT technique or fully decoupled (denoted as COM in the ESI). Melting points were measured on an Original Kofler Mikroheiztisch apparatus and were not corrected. HRMS data were obtained on Applied Biosystems DE STR MALDI-TOF/TOF instrument. Chemicals were purchased from the usual commercial sources and used as received. Solvents for chromatographic separations were used as is from the supplier (p.a. or HPLC grade) or purified by distillation (CH2Cl2). Procedures for the preparation of known intermediates are reported in the ESI. Methods for the analysis of samples by HPLC and for the purification by semipreparative HPLC separations are reported in the ESI.

4,4-Difluoro-3-[4-(benzyloxy)phenyl]-8-phenyl-4-bora-3a,4a-diaza-s-indacen (7)13

Chloro-BODIPY (**6**, 1.2 g, 4 mmol) was dissolved in toluene (40 mL). Under N2 inert atmosphere to the solution was added *p-*benzyloxyphenyl boronic acid (2.0 g, 8.8 mmol), Pd(PPh3)4 (0.5 g, 0.4 mmol) and Na2CO3 dissolved in H2O (2.1 g / 2.5 mL H2O, 20 mmol). The reaction mixture was heated at the temperature of reflux overnight. To the reaction mixture H2O (20 mL) was added, and an extraction with CH2Cl2 was carried out (3 × 20 mL). The extracts were dried over anhydrous MgSO4, filtered and the solvent was removed on a rotary evaporator. The residue was chromatographed on a column of silica gel using CH2Cl2 as eluent, followed by another chromatography on a column of silica gel using CH2Cl2/hexane (1:1) as eluent to afford the pure product (1.1 g, 61%).

Purple crystals, mp 62-65°C; 1H NMR (CDCl3, 600 MHz) / ppm: 7.99 (d, *J* = 8.9 Hz, 2H), 7.81 (s, 1H), 7.59-7.56 (m, 3H), 7.54-7.51 (m, 2H), 7.47-7.46 (m, 2H), 7.43-7.40 (m, 2H), 7.36-7.35 (m, 1H), 7.10 (d, *J* = 8.9 Hz, 2H), 6.96 (d, *J* = 4.5 Hz, 1H), 6.80 (d, *J* = 4.0 Hz, 1H), 6.69 (d, *J* = 4.4 Hz, 1H), 6.49 (dd, *J* = 1.8 Hz, 2.2 Hz, 1H), 5,14 (s, 2H); 13C NMR (CDCl3, 150 MHz, APT) / ppm: 160.2 (s), 160.1 (s), 143.9 (s), 140.6 (d), 136.9 (s), 136.1 (s), 133.8 (s), 133.4 (s), 132.5 (d), 130.8 (d), 130.0 (d, 4C), 129.7 (d), 128.1 (d, 2C), 127.6 (d, 4C), 127.0 (d, 2C), 124.2 (s), 120.5 (d), 117.0 (d), 114.3 (d, 2C), 69.6 (t); HRMS (MALDI-TOF/TOF): calculated for C28H21BF2N2O 450.1715; found 450.1695.

4,4-Difluoro-3-[4-(benzyloxy)phenyl]-8-phenyl-4-bora-3a,4a-diaza-s-indacen (7) via C-H arylation

4,4-Difluoro-8-phenyl-4-bora-3a,4a-diaza-s-indacen (25 mg, 0.09 mmol), *p-*benzyloxyphenyl boronic acid (61 mg, 0.23 mmol), Pd(OAc)2 (1 mg, 0.005 mmol), K2CO3 (39 mg, 0.28 mmol), pivalic acid (PivOH, 3 mg, 0,03 mmol) and tricyclohexylphosphin tetrafluoroborate (PCy3HBF4, 3 mg, 0.009 mmol) were dissolved in anhydrous toluene (5 mL) under N2-inert atmosphere and heated at the temperature of reflux 30 h. To the cooled reaction mixture, Et2O (10 mL) was added and the mixture was washed with H2O (20 mL). The organic solution was dried over anhydrous MgSO4, filtered and the solvent was removed on a rotary evaporator. The residue was chromatographed on a column of silica gel using CH2Cl2/hexane (4:1) as eluent to afford the pure product (15 mg, 36%).

4,4-Difluoro-3,5-bis[4-(benzyloxy)phenyl)]-8-phenyl-4-bora-3a,4a-diaza-s-indacen (11)

According to the above procedure for the preparation of **7** by Suzuki coupling reaction, starting from dichloro-BODIPY **9** (1.0 g, 3 mmol), *p-*benzyloxyphenyl boronic acid (1.5 g, 6.5 mmol), Pd(PPh3)4 (0.3 g, 0.3 mmol) and Na2CO3 dissolved in H2O (1.1 g / 2 mL H2O), after chromatographic separation the reaction afforded pure products **10** (43 mg, 3%) and **11** (923 mg, 51%).

4,4-Difluoro-3-[4-(benzyloxy)phenyl)]-5-chloro-8-phenyl-4-bora-3a,4a-diaza-s-indacen (10)13 Purple crystals, mp 131-133 °C; 1H NMR (CDCl3, 600 MHz) / ppm: 7.89 (d, *J* = 8.9 Hz, 2H), 7.45-7.34 (m, 10H), 7.03 (d, 2H, *J* = 8.9 Hz), 6.89 (d, *J* = 4.1 Hz, 1H), 6.85 (d, *J* = 4.1 Hz, 1H), 6.64 (d, *J* = 4.4 Hz, 1H), 6.60 (d, *J* = 4.2 Hz, 1H), 5.1 (br s, 2H); 13C NMR (CDCl3, 150 MHz) / ppm: 160.3, 159.4, 158.0, 143.2, 136.8, 134.6, 133.0, 131.3, 130.3, 130.0, 129.5, 129.3, 128.7, 128.3, 128.2, 128.1, 127.6, 125.3, 121.0, 120.4, 114.8, 70.1; HRMS (MALDI-TOF/TOF): calculated for C28H20BClF2N2O 507.1223; found 507.1230.

4,4-Difluoro-3,5-bis[4-(benzyloxy)phenyl)]-8-phenyl-4-bora-3a,4a-diaza-s-indacen (11) Purple crystals, mp 213-216 °C; 1H NMR (CDCl3, 600 MHz) / ppm: 7.88 (d, *J* = 8.8 Hz, 4H), 7.58-7.52 (m, 5H), 7.46-7.44 (m, 4H), 7.41-7.39 (m, 4H), 7.35-7.34 (m, 2H), 7.03 (d, *J* = 8.8 Hz, 4H), 6.84 (d, *J* = 4.3 Hz, 2H), 6.60 (d, *J* = 4.3 Hz, 2H), 5.11 (s, 4H); 13C NMR (CDCl3, 150 MHz) / ppm: 160.1 (s, 2C), 143.5 (s, 2C), 136.8 (s, 2C), 136.4 (s), 135.4 (s), 134.7 (s, 2C), 125.5 (s, 2C), 131.2 (d), 130.6 (d), 130.4 (d), 129.9 (d), 128.6 (d), 128.2 (d), 128.1 (d), 127.6 (d), 120.5 (d), 114.7 (d), 70,1 (t); HRMS (MALDI-TOF/TOF) calculated for C41H31BF2N2O2 632.2447; found 632.2463.

4,4-Difluoro-3-(4-hydroxyphenyl)-8-phenyl-4-bora-3a,4a-diaza-s-indacen (8)13

Arylated compound 7 (200 mg, 0.4 mmol) was dissolved in anhydrous EtOH (15 mL). Under inert N2 atmosphere Pd/C (5%, 200 mg, 0.09 mmol Pd) and triethylsilane (Et3SiH, 0.6 ml, 4 mmol) were added to the solution. The reaction mixture was stirred at rt for 4.5 h and then it was filtered through filter paper (blue ribbon), the solid was washed with EtOH and EtOAc, and the solvent was removed on a rotary evaporator. The residue was chromatographed on a column of silica gel using CH2Cl2→CH2Cl2/EtOAc (1:1) as eluent to afford the product which still contained Et3SiH. The crude product was dissolved in CH2Cl2 and washed with H2O (3 × 20 mL). The organic solution was dried over anhydrous MgSO4, filtered and the solvent was removed on a rotary evaporator to afford the pure product (109 mg, 78%).

Purple crystals, mp 65-67 °C; 1H NMR (CDCl3, 600 MHz) / ppm: 7.93 (d, 2H, *J* = 8.7 Hz), 7.81 (s, 1H), 7.59-7.56 (m, 3H), 7.53-7.51 (m, 2H), 6.97 (d, *J* = 4.3 Hz, 1H), 6.94 (d, *J* = 8.7 Hz, 2H), 6.81 (d, *J* = 4.0 Hz, 1H), 6.68 (d, *J* = 4.4 Hz, 1H), 6.50 (dd, *J* = 1.7, 3.8 Hz, 1H), 5.26 (br s, 1H); 13C NMR (CDCl3, 150 MHz, APT) / ppm: 160.6 (s), 157.6 (s), 144.6 (s), 141.2 (d), 137.4 (s), 134.3 (s), 133.9 (s), 133.1 (d, 2C), 131.6 (d), 130.5 (d, 2C), 130.3 (d, 2C), 128.8 (d), 128.3 (d), 124.2 (s), 121.1 (d), 117.6 (d), 115.5 (d, 2C); HRMS (MALDI-TOF/TOF) calculated for C21H15BF2N2O 360.1246; found 360.1246.

4,4-Difluoro-3,5-bis-(4-hydroxyphenyl)-8-phenyl-4-bora-3a,4a-diaza-s-indacen (12)13

Arylated compound 7 (120 mg, 0.2 mmol) was dissolved in anhydrous EtOH (10 mL). Under inert N2 atmosphere Pd/C (10%, 100 mg, 0.1 mmol Pd) and triethylsilane (Et3SiH, 0.3 mL, 2 mmol) were added to the solution. The reaction mixture was stirred at rt and after 10 min Et3SiH (0.3 mL, 2 mmol) was added and the stirring was continued for 24 h. The next day Et3SiH (0.3 mL, 2 mmol) was added and the stirring was continued for 2 h. The reaction mixture was filtered through filter paper (blue ribbon), the solid was washed with EtOH and EtOAc, and the solvent was removed on a rotary evaporator. The residue was chromatographed on a column of silica gel using CH2Cl2→CH2Cl2/EtOAc (1:1) as eluent to afford pure products 12 (45 mg, 52%) and 13 (28 mg, 27%).

Purple crystals, mp 226-228 °C; 1H NMR (CDCl3, 300 MHz) / ppm: 7.84 (d, *J* = 8.7 Hz, 4H), 7.63-7.53 (m, 5H), 6.89 (d, *J* = 8.8 Hz, 4H), 6.87 (d, *J* = 4.4 Hz, 2H), 6.61 (d, *J* = 4.2 Hz, 2H), 5.10 (br s, 2H); 13C NMR (CDCl3, 75MHz, APT) / ppm: 131.4 (d, 4C), 130.6 (d, 2C), 130.5 (d, 2C), 129.9 (d, 2C), 128.3 (d), 120.5 (d, 2C), 115.3 (d, 4C), signals of quaternary C atoms were not observed; HRMS (MALDI-TOF/TOF): calculated for C27H19BF2N2O2 452.1508; found 452.1530.

4,4-Difluoro-3-(4-hydroxyphenyl)-5-chloro-8-phenyl-4-bora-3a,4a-diaza-s-indacen (13)13 Purple oily crystals, 1H NMR (CDCl3, 300 MHz) / ppm: 7.88 (d, *J* = 8.8 Hz, 2H), 7.83 (d, *J* = 8.9 Hz, 2H), 7.61-7.52 (m, 5H), 7.47-7.33 (m, 5H), 7.03 (d, *J* = 8.9 Hz, 2H), 6.88 (d, *J* = 8.9 Hz, 2H), 6.84 (d, *J* = 4.4 Hz, 2H), 6.62-6.59 (m, 2H), 5.11 (s, 2H); 13C NMR (CDCl3, 75MHz, APT) / ppm: 131.4 (d, 4C), 131.2 (d, 4C), 130.6 (d), 129.9 (d, 2C), 128.7 (d), 128.2 (d, 2C), 128.1 (d, 2C), 127.6 (d), 120.4 (d), 115.3 (d, 2C), 114.7 (d, 2C), 70.1 (t), signals of quaternary C atoms were not observed; HRMS (MALDI-TOF/TOF): calculated for C34H25BF2N2O2 542.1977; found 542.2000.

4,4-Difluoro-3-[3-(*N*,*N*-dimethylaminomethyl)-4-hydroxyphenyl]-8-phenyl-4-bora-3a,4a-diaza-s-indacen (1)13

Phenol 8 (163 mg, 0.5 mmol) was dissolved in toluene (25 mL). Under N2 inert atmosphere, the Eschenmoser’s salt ((CH3)2N+=CH2Cl-, 55 mg, 0.6 mmol) and anhydrous K2CO3 (82 mg, 0.6 mmol) were added. The reaction mixture was heated at the temperature of reflux overnight and it was filtered through a sinter funnel. The solids on the funnel were washed with EtOAc. The solvent was removed from organic filtrate and the residue was purified on a column of alumina (activity IV, 8.2% H2O) using EtOAc→EtOAc/MeOH (0-100%) to afford the pure product (112 mg, 60%).

Purple crystals, mp 150-151 °C;  1H NMR (CD3OD, 300 MHz) / ppm: 7.95-7.88 (m, 2H), 7.75 (br s, 1H), 7.63-7.58 (m, 5H), 7.01 (d, *J* = 4.5 Hz, 1H), 6.89-6.86 (m, 2H), 6.79 (d, *J* = 4.3 Hz, 1H), 6.54 (dd, *J* = 2.1, 4.1 Hz, 1H), 3.93 (s, 2H), 2.55 (s, 6H); 13C NMR (CDCl3, 75 MHz) / ppm: 161.2, 160.9, 143.6, 140.2, 137.5, 134.2, 133.6, 133.1, 130.9, 130.2, 130.1, 128.2, 127.9, 122.5, 121.7, 121.2, 117.2, 116.3, 62.6, 44.4; 13C NMR (CD3OD, 75 MHz, APT) / ppm: 161.5 (s), 141.2 (d), 135.5 (s), 134.4 (d), 133.3 (d), 132.9 (d), 131.7 (d, 2C), 131.5 (d, 2C), 129.6 (d), 128.8 (d), 123.3 (s), 122.5 (d), 122.0 (s), 118.3 (d), 117.3 (d), 61.5 (t), 44.1 (q), some signals of quaternary C-atoms were not detected.

4,4-Difluoro-3-[3-(*N*,*N*-dimethylaminomethyl)-4-hydroxyphenyl]-8-phenyl-4-bora-3a,4a-diaza-s-indacen (1×TFA)

Compound 1 (50 mg) was dissolved in anhydrous Et2O and a solution of HCl in Et2O was added dropwise as long as precipitate was being formed. The precipitate was filtered off on a sinter funnel and the solvent was removed from the filtrate on a rotational evaporator. Since the solid was not pure, additional purification by preparative HPLC was performed (for the chromatographic method see the ESI) to afford the pure 1×TFA.

Purple crystals, mp 74-77 °C;  1H NMR (CD3OD, 300 MHz) / ppm: 8.12 (d, *J* = 2.2 Hz, 1H), 7.94 (dd, *J* = 2.2, 8.4 Hz, 1H), 7.81 (s, 1H), 7.67-7.59 (m, 5H), 7.06 (d, *J* = 8.4 Hz, 1H), 7.04 (d, *J* = 4.4 Hz, 1H), 6.88 (d, *J* = 4.2 Hz, 1H), 6.86 (d, *J* = 4.4 Hz, 1H), 6.59 (dd, *J* = 2.0, 2.2 Hz, 1H), 4.38 (s, 2H), 2.94 (s, 6H); 13C NMR (CD3OD, 75 MHz, APT) / ppm: 160.8 (s), 159.8 (s), 159.7 (s), 146.6 (s), 142.9 (d), 138.6 (s), 135.4 (s), 135.3 (s), 134.7 (d), 134.2 (d), 131.7 (d), 131.6 (d), 130.4 (d), 129.9 (d), 129.6 (d), 125.3 (s), 122.0 (d), 119.1 (d), 117.7 (s), 116.5 (d), 58.2 (t), 43.4 (q); HRMS (MALDI-TOF/TOF) calculated for C26H23BF5N3O3 418.1902; found 418.1910.

Preparation of 2×TFA-5×TFA

Phenol 12 (184 mg, 0.4 mmol) was dissolved in toluene (20 mL). Under N2 inert atmosphere, the Eschenmoser’s salt (100 mg, 1.1 mmol) and anhydrous K2CO3 (152 mg, 1.1 mmol) were added. The reaction mixture was heated at the temperature of reflux overnight and it was filtered through a sinter funnel. The solids on the funnel were washed with EtOAc and CH3OH. The solvent was removed from organic filtrate and to the residue Et2O was added. To the ethereal solution a solution of HCl in Et2O was added dropwise as long as precipitate was being formed. The solid was filtered off, and both the solid and the solution were purified by preparative HPLC (for the method see the ESI) to afford the following products: regenerated starting material 12 (20 mg, 11%), 2×TFA (20 mg, 10%), 4×TFA (3 mg, 1%), 5×TFA (10 mg, 4%). Product 3×TFA was isolated in traces after preparative HPLC of fractions enriched in its content from three reactions.

4,4-Difluoro-3-[3-(*N*,*N*-dimethylammoniummethyl)-4-hydroxyphenyl]-5-(4-hydroxyphenyl)-8-phenyl-4-bora-3a,4a-diaza-s-indacene trifluoroacetate (2×TFA)Purple crystals, mp 155-157 °C;  1H NMR (CD3OD, 600 MHz) / ppm: 8.08 (d, *J* = 2.0 Hz, 1H), 7.79 (d, *J* = 8.8 Hz, 2H), 7,75 (dd, *J* = 2.0, 8.6 Hz, 1H), 7.63-7.58 (m, 5H), 7.02 (d, *J* = 8.4 Hz, 1H), 6.92 (d, *J* = 4.4 Hz, 1H), 6.88 (d, *J* = 4.0 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, 2H), 6.75 (d, *J* = 4.5 Hz, 1H), 6.72 (d, *J* = 4.4 Hz, 1H), 4.35 (s, 2H), 2.89 (s, 6H); 13C NMR (CD3OD, 75 MHz, APT) / ppm: 160.9 (s), 160.6 (s), 159.1 (s), 157.7 (s), 144.0 (s), 137.7 (s), 137.2 (s), 135.6 (s), 135.4 (d), 134.4 (d), 132.5 (d), 132.4 (d), 131.7 (d), 131.4 (d), 131.0 (d), 129.5 (d), 126.1 (s), 125.1 (s), 122.2 (d), 120.7 (d), 117.2 (s), 116.2 (d), 116.0 (d), 58.1 (t), 43.4 (q); HRMS (MALDI-TOF/TOF) calculated for C30H27BF2N3O2+ 510.2164; found 510.2172.

4,4-Difluoro-3,5-bis[3-(*N*,*N*-dimethylammoniummethyl)-4-hydroxyphenyl]-8-phenyl-4-bora-3a,4a-diaza-s-indacene trifluoroacetate (3×TFA) Purple crystals, mp 121-123 °C;  1H NMR (CD3OD, 300 MHz) / ppm: 7.77 (dd, *J* = 2.2, 6.2 Hz, 2H), 7.63 (d, *J* = 2.1 Hz, 2H), 7.63-7.50 (m, 5H), 6.87 (d, *J* = 8.8 Hz, 2H), 6.80 (d, *J* = 4.2 Hz, 2H), 6.59 (d, *J* = 4.5 Hz, 2H), 3.70 (s, 4H), 2.36 (s, 12H); 13C NMR (CD3OD, 150 MHz, APT) / ppm: 159.6 (s), 158.9 (s), 144.5 (s), 137.5 (s), 135.5 (s), 135.0 (d, 2C), 134.1 (d, 2C), 131.9 (d, 2C), 131.7 (d, 2C), 131.5 (d, 2C), 129.5 (d), 125.8 (s), 121.6 (d, 2C), 118.0 (s), 116.4 (d, 2C), 58.3 (t, 2C), 43.4 (q, 2C); HRMS (MALDI-TOF/TOF) calculated for C33H34BF2N4O2+ 567.2743; found 567.2737.

4,4-Difluoro-3-[3,5-bis(*N*,*N*-dimethylammoniummethyl)-4-hydroxyphenyl]-5-(4-hydroxyphenyl)-8-phenyl-4-bora-3a,4a-diaza-s-indacene trifluoroacetate (4×TFA) Purple oily crystals, 1H NMR (CDCl3, 300 MHz) / ppm: 7.78 (s, 2H), 7.68 (d, *J* = 8.4 Hz, 2H), 7.58-7.55 (m, 3H), 7.54-7.51 (m, 2H), 6.93 (d, *J* = 8.4 Hz, 2H), 6.87 (d, *J* = 4.4 Hz, 1H), 6.83 (d, *J* = 4.4 Hz, 1H), 6.62 (d, *J* = 4.4 Hz, 2H), 4.01 (s, 4H), 2.58 (s, 12H); 1H NMR (CD3OD, 300 MHz) / ppm: 7.99 (s, 2H), 7.78 (d, *J* = 8.3 Hz, 2H), 7.71-7.58 (m, 5H), 6.95 (d, *J* = 4.2 Hz, 1H), 6.89 (d, *J* = 4.2 Hz, 1H), 6.85 (d, *J* = 8.4 Hz, 2H), 6.77 (m, 2H), 4.36 (s, 4H), 2.87 (s, 12H); 13C NMR (CDCl3, 150 MHz, APT) / ppm: 134.4 (s), 133.0 (d), 131.3 (d), 131.0 (d), 130.5 (d), 130.0 (d), 128.3 (d), 129.6 (d), 123.9 (s), 123.6 (s), 120.8 (d), 118.9 (d), 118.1 (s), 115.5 (d), 58.2 (t), 53.5 (q), 45.6 (t), 43.1 (q), signals of all quaternary C atoms were not observed, 13C NMR (CD3OD, 150 MHz, APT) / ppm: 160.7 (s), 156.3 (s), 144.2 (s), 137.9 (s), 137.2 (s), 136.6 (d), 135.5 (s), 132.8 (d), 132.6 (d), 131.8 (d), 131.4 (d, 2C), 130.8 (d), 129.6 (d), 125.0 (s), 122.5 (d), 120.6 (d), 120.0 (s), 116.1 (d), 58.5 (t), 43.4 (q), signals of all quaternary C atoms were not observed.

4,4-Difluoro-3-[3,5-bis(*N*,*N*-dimethylammoniummethyl)-4-hydroxyphenyl]-5-(3-(*N*,*N*-dimethylammoniummethyl)-4-hydroxyphenyl)-8-phenyl-4-bora-3a,4a-diaza-s-indacene trifluoroacetate (5×TFA) Purple oily crystals, 1H NMR (CDCl3, 300 MHz) / ppm: 7.94 (d, *J* = 8.5 Hz, 1H), 7.88 (s, 2H), 7.76 (d, *J* = 2.2 Hz, 1H), 7.60-7.57 (m, 5H), 7.00 (d, *J* = 8.6 Hz, 2H), 6.89 (d, *J* = 4.4 Hz, 1H), 6.87 (d, *J* = 4.3 Hz, 1H), 6.76 (d, *J* = 4.4 Hz, 1H), 6.72 (d, *J* = 4.2 Hz, 1H), 4.29 (s, 2H), 4.19 (s, 4H), 2.81 (s, 6H), 2.75 (s, 12H); 13C NMR (CD3OD, 150 MHz, APT) / ppm: 163.0 (s), 159.4 (s), 159.1 (s), 158.4 (s), 137.7 (s), 137.4 (s), 135.7 (d, 2C), 135.5 (s), 135.2 (d), 134.2 (d), 132.1 (d, 2C), 131.7 (d, 2C), 131.4 (d), 129.6 (d, 2C), 126.0 (s), 121.7 (d), 121.5 (d), 120.8 (s), 117.6 (s), 116.4 (d), 59.7 (t, 2C), 57.9 (t), 43.4 (q, 4C), 43.2 (q, 2C); HRMS (MALDI-TOF/TOF) calculated for C36H41BF2N5O2+ 624.3321; found 624.3305.

Irradiation experiments - general

A solution of BODIPY compound (3 mg) was dissolved in 10 mL of CH3OH, CH3OH-H2O (5:1 v/v) in the presence of phosphate buffer (50 mM at pH 7.0 or 9.0) and placed in quartz test tubes. The solutions were purged with N2 for 15 min and irradiated in a Luzchem photoreactor equipped with 8 lamps with the maximum output at 254 nm, ≈ 300 nm, ≈ 350 nm, or cool white (1 lamp 8 W). The irradiated solutions were placed in the center of the irradiated chamber, 7.5 cm separated from each lamp. CH3OH for the irradiations was of HPLC purity, and mQ H2O was used. The course of the reaction was followed by HPLC (for the method see the ESI) and HPLC-MS. Material obtained after several irradiations of 1×TFA or 2×TFA was joined and chromatographed on a column of silica gel using EtOAc as eluent to isolate the photoproducts. For the MS characterization of other photoproducts see the ESI.

4,4-Difluoro-3-[3-(methoxymethyl)-4-hydroxyphenyl]-8-phenyl-4-bora-3a,4a-diaza-s-indacene (1-OCH3) 1 mg (30%), Purple oily crystals, 1H NMR (CD3OD, 600 MHz) / ppm: 7.89 (d, *J* = 2.5 Hz, 1H), 7.82 (dd, *J* = 2.1, 8.4 Hz, 1H), 7.68-7.67 (m, 1H), 7.54-7.48 (m, 5H), 6.93 (d, *J* = 4.6 Hz, 1H), 6.81 (d, *J* = 8.3 Hz, 1H), 6.78 (d, *J* = 4.3 Hz, 1H), 6.72 (d, *J* = 4.0 Hz, 1H), 6.45 (dd, *J* = 1.8, 4.0 Hz, 1H), 4.47 (s, 2H), 3.35 (s, 3H); HRMS (MALDI-TOF/TOF) calculated for C23H19BF2N2O2+ 404.1508; found 404.1512.

4,4-Difluoro-3-[3-(methoxymethyl)-4-hydroxyphenyl]-5-(4-hydroxyphenyl)-8-phenyl-4-bora-3a,4a-diaza-s-indacene (2-OCH3) 1 mg (30%), Purple oily crystals, 1H NMR (CD3OD, 600 MHz) / ppm: 7.82 (d, *J* = 2.2 Hz, 1H), 7.73 (d, *J* = 8.7 Hz, 2H), 7.66 (dd, *J* = 2.3, 8.3 Hz, 1H), 7.53-7.47 (m, 5H), 6.78 (d, *J* = 8.7 Hz, 1H), 6.76 (d, *J* = 3.9 Hz, 2H), 6.73 (d, *J* = 8.7 Hz, 2H), 6.62 (d, *J* = 4.4 Hz, 2H), 4.46 (s, 2H), 3.32 (s, 3H); HRMS (MALDI-TOF/TOF) calculated for C29H23BF2N2O3+ 496.1770; found 496.1752.

Quantum yield of methanolysis

Quantum yields for the photomethanolysis reactions for **1xTFA** and **2xTFA** were determined by using ferrioxalate actinometer upon excitation at 300 nm (ΦR = 1.25).32 Solutions of **1xTFA** and **2xTFA** in CH3OH and the actinometer were freshly prepared and their concentrations adjusted to have absorbances of 0.4-0.8 at 300 nm. After the adjustment of the concentrations and measurement of the corresponding UV-vis spectra, the solutions were purged with a stream of Ar (20 min), and then, sealed with a cap. The cells were placed in a holder which ensured equal distance of all samples from the lamp and were irradiated at the same time in the reactor with 1 lamp at 300 nm for 6 h (1 min for the actinometer). Before and after the irradiation, the samples were taken from the cells by use of a syringe and analyzed by HPLC to determine photochemical conversions. From the conversion of the actinometer, irradiance was calculated according to Eqs. S2-S4 reported in the ESI. The average value of two measurements was reported.

UV-vis and fluorescence measurements

UV-vis measurements were performed on a PG T80/T80+ instrument in CH3CN or CH3CN-H2O (1:1 v/v) in the presence of phosphate buffer (50 mM at pH 7.0 or 9.0). Fluorescence measurements were performed on an Agilent Cary Eclipse fluorometer by use of slits corresponding to the bandpass of 2.5 nm for the excitation and 5.0 nm for the emission. Alternatively, fluorescence spectra were measured on an Edinburgh FS5 instrument by use of slits corresponding to the bandpass of 2 nm for the excitation and emission. The spectra were corrected for the intensity of the Xe lamp and optics. Fluorescence quantum yields were determined by use of rhodamine B in methanol (Φf = 0.66) as a reference.29 Absorbance at the excitation wavelength was < 0.1. One fluorescence measurement was performed by exciting sample at three different wavelengths (500, 510 and 520 nm), and the average value was calculated (see Eqs. S1-S5 in the ESI). Prior to the measurements, the solutions were purged with N2 for 15 min. The measurement was performed at rt (25 °C).

Fluorescence titration with BSA

A solution of 2×TFA (*c* = 5.4×10-6 M) in 2 mL of CH3CN-H2O (1:4, v/v) in the presence of phosphate buffer (50 mM, pH 7) was placed in a quartz cell. To the solution aliquots (50 μL) of the solution of BSA (*c* = 5.0×10-4 M) in aqueous phosphate buffer (50 mM, pH 7) were added. After each addition, the solution was allowed to equilibrate for 15 min and fluorescence spectrum was taken. The samples were excited at 520 nm and the emission was collected in the range 550-800nm. The excitation slit was set to the bandpass of 5 nm, and the emission to 10 nm. The measurement was performed at 25 °C. Fluorescence data obtained by titration was processed by global nonlinear regression analysis implemented in the Specfit software to reveal the complex association constant.

Fluorescent labeling of BSA

Samples containing BSA (20 μg, Albumin standard, Thermo scientific, USA) were incubated with (or without) BODIPY 2×TFA (0.1 mM or 1 mM). The mixtures were irradiated (or not) in a Luzchem reactor (6 lamps, 350 nm) for 30 or 60 min. After the treatment and irradiation, the samples were run on a SDS-PAGE gel (10%). After the separation, proteins were visualized by UV light at 254 nm (Image Master VDS, Pharmacia Biotech, Sweden). Subsequently the same gel was stained with Coomassie brilliant blue.

Laser flash photolysis

The measurements were performed on a LP980 Edinburgh Instruments spectrometer. For the excitation the third harmonic of a Qsmart Q450 Quantel YAG laser at 355 nm was used. The energy of the laser pulse at 355 nm was set to 20 mJ and the pulse duration was 7 ns. Absorbances at the excitation wavelength were optically matched to 0.3. The static cells were used and they were frequently exchanged to assure no absorption of light by photoproducts. The solutions were purged for 20 min with Ar or O2 prior to the measurements.

Computational Methods

For the theoretical insight to photophysical and photochemical properties of molecule 1 we used density functional (DFT) and time dependent density functional theory (TD-DFT) approaches37 since it is today well established that TD-DFT performs well for calculations of excited states.38,39 The DFT and TD-DFT optimizations of geometries in the ground (Fig S10) and the first excited singlet state (Fig. SI14), respectively, were carried out with PBE040 density functional based on global hybrid generalized gradient approximation that include 25% fraction of HF exchange. On the basis of the optimized geometries in the ground state as well as in the first excited state, frequency calculations were carried out to confirm the nature of the stationary points as minima with real vibrational frequencies only. The same method was used to calculate ZPVE values (Table S3). Furthermore, photodeamination process was probed by fully relaxed potential energy surface scan in the first excited singlet state S1 following the C-N stretch reaction coordinate (Fig 2c). Regarding basis sets, for the ground state and the excited state optimizations and the S1 PES scanning, triple-ξ 6-311G(2d,p) Pople style basis set which include polarization functions on both heavy atoms and hydrogens was used. On the other hand, the 6-311+G(2d,p) basis set, which includes diffuse functions on heavy atoms was used for calculating 40 vertical excitation energies for singlet states and their oscillator strengths (Tables S1 and S2). These data were, subsequently, used for the simulations of absorption spectra shown in Figure S12. The theoretical procedure utilizes here was found to be a good compromise between accuracy and computational efficiency for adequate description of excited states and photodeamination process in similar BODIPY derivatives14 and it enable direct comparison of the results. All calculations were carried out with Gaussian09 program package,41 structure visualization, plotting orbitals and geometry manipulation with Molden42 program. Simulation of absorption spectra was produced by simple convolution fit with Gaussian functions with a half-width of 0.4 eV on the calculated excitation energies and oscillator strengths by using in-house created Perl script written by Dr. Mario Barbatti.43

Author Contributions

KZ was the main author involved in the investigation and formal analysis (synthesis, photophysics, photochemistry, binding to BSA). The investigation and formal analysis was partly conducted by MC (synthesis), IA (computations), LU (biology). MK was involved in supervision and validation for biological part and BM provided LFP resources. Conceptualization and original draft writing was performed by NB, and review and editing by all authors.

Conflicts of interest

There are no conflicts to declare.

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**Notes and references**

1. (a) R. Ziessel, G. Ulrich, A. Harriman, *New J. Chem.*, 2007, **31**, 496-501. (b) M. Benstead, G. H. Mehl, R. W. Boyle, *Tetrahedron*, 2011, **67**, 3573-3601.
2. (a) A. Loudet, K. Burgess, *Chem. Rev.*, 2007, **107**, 4891-4932. (b) G. Ulrich, R. Ziessel, A. Harriman, *Angew. Chem. Int. Ed.*,2008, **47**, 1184-1201.
3. N. Boens, B. Verbelen, W. Dehaen, *Eur. J. Org. Chem*., **2015**, 6577-6595.
4. N. Boens, V. Leen, W. Dehaen, *Chem. Soc. Rev*., 2012, **41**, 1130-1172.
5. T. Kowada, H. Maeda, K. Kikuchi, *Chem. Soc. Rev*., 2015, **44**, 4953-4972.
6. D. Kim, D. Ma, M. Kim, Y. Jung, N. H. Kim, C. Lee, S. Won Cho, S. Park, Y. Huh, J. Jung, K. Han Ahn, *J. Fluoresc*., 2017, **27**, 2231-2238.
7. M. H. Y. Cheng, H. Savoie, F. Bryden, R. W. Boyle, *Photochem. Photobiol. Sci*., 2017, **16**, 1260-1267.
8. Y. Chen, K. Tsao, S. L. Acton, J. W. Keillor, *Angew. Chem. Int. Ed*., 2018, **130**, 12570-12574.
9. A. Kamkaew, S. Hui Lim, H. Boon Lee, L. Voon Kiew, L. Yong Chung, K. Burgess, *Chem. Soc. Rev.*, 2013, **42**, 77-88.
10. D. P. Murale, S. C. Hong, Md. M. Haque, J.-S. Lee, *Proteome Sci.*, 2017, **15**, 1-34.
11. (a) E. Deniz, M. Battal, J. Cusido, S. Sortino, F. M. Raymo, *Phys. Chem. Chem. Phys*., 2012, **14**, 10300-10307. (b) S. S. Ragab, S. Swaminathan, E. Deniz, B. Capitan, F. M. Raymo, *Org. Lett*., 2013, **15**, 3154-3157. (c) T. Aotake, M. Suzuki, D. Kuzuhara, N. Aratani, N. Tamai, H. Yamada, *Chem. Eur. J*., 2015, **21**, 4966-4974.
12. D. P. Murale, S. Cheol Hong, J. H. Yun, C. N. Yoon, J.-S. Lee, *Chem. Commun*. 2015, **51**, 6643-6646.
13. N. Basarić, M. Kralj, A. M. Mikecin, M. Cindrić, PCT/HR2017/000005.
14. K. Zlatić, I. Antol, L. Uzelac, A.-M. Mikecin Dražić, M. Kralj, C. Bohne, N. Basarić, *ACS Appl. Mater. Interfaces*, 2020, **12**, 347-351.
15. *Quinone Methides*, ed. S. E. Rokita, Wiley, Hoboken, USA, 2009.
16. (a) W. J. Bai, J. G. David, Z.-G. Feng, M. G. Weaver, K.-L. Wu, T. R. R.Pettus, *Acc. Chem. Res*., 2014, **47**, 3655-3664. (b) M. S. Singh, A. Nagaraju, N. Anand, S. Chowdhury, *RSC Adv*., 2014, **4**, 55924-55959.
17. (a) M. Freccero, *Mini Rev. Org. Chem*., 2004, **1**, 403-415. (b) P. Wang, Y. Song, L. Zhang, H. He, X. Zhou, *Curr. Med. Chem.*, 2005, **12**, 2893-2913.
18. (a) H. Wang, M. S. Wahi, S. E. Rokita, *Angew. Chem. Int. Ed.*, 2008, **47**, 1291-1293. (b) H. Wang, S. E. Rokita, *Angew. Chem. Int. Ed.*, 2010, 49, 5957-5960. (c) C. S. Rossiter, E. Modica, D. Kumar, S. E. Rokita, *Chem. Commun*., 2011, **47**, 1476-1478. (d) F. Fakhari, S. E. Rokita, *Nature Commun*. 2014, **5**, 5591 doi: 10.1038/ncomms6591
19. (a) S. Arumugam, J. Guo, N. E. Mbua, F. Fiscourt, N. Lin, E. Nekongo, G. J. Boons, V. V. Popik, *Chem. Sci*., 2014, **5**, 1591-1598. (b) R. Pérez-Ruiz, O. Molins-Molina, E. Lence, C. González-Bello, M. A. Miranda, M. Consuelo Jiménez, *J. Org. Chem*., 2018, **83**, 13019-13029.
20. (a) C. Percivalle, F. Doria, M. Freccero, *Curr. Org. Chem*., 2014, **18**, 19-43. (b) N. Basarić, K. Mlinarić-Majerski, M. Kralj, *Curr. Org. Chem*., 2014, **18**, 3-18.
21. L. Uzelac, Đ. Škalamera, K. Mlinarić-Majerski, N. Basarić, M. Kralj, *Eur. J. Med. Chem*., 2017, **137**, 558-574.
22. B. S. Sørensen, M. R. Horsman, *Front Oncol.* **2020** doi: 10.3389/fonc.2020.00562. eCollection 2020.
23. (a) Đ. Škalamera, C. Bohne, S. Landgraf, N. Basarić, *J. Org. Chem*., 2015, **80**, 10817-10828. (b) J. Ma, M. Šekutor, Đ. Škalamera, N. Basarić, D. L. Phillips, *J. Org. Chem*., 2019, **84**, 8630-8637.
24. A. P. Demchenko, V. I. Tomin, P.-T. Chou, *Chem Rev*., 2017, **117**, 13353-13381.
25. M. Baruah, W. Qin, N. Basarić, W. M. De Broggraeve, N. Boens, *J. Org. Chem*., 2005, **70**, 4152-4157.
26. X. Zhou, C. Yu, Z. Feng, Y. Yu, J. Wang, E. Hao, Y. Wei, X. Mu, L. Jiao, *Org. Lett.*, 2015, **17**, 4632-4635.
27. T. Rohand, W. Qin, N. Boens, W. Dehaen, *Eur. J. Org. Chem.* **2006**, 4658-4663.
28. L. Wang, B. Verbelen, C. Tonnelé, D. Beljonne, R. Lazzaroni, V. Leen, W. Dehaen, N. Boens, *Photochem. Photobiol. Sci*., 2013, **12**, 835-847.
29. N. Boens, W. Qin, N. Basarić, J. Hofkens, M. Ameloot, J. Pouget, J.-P. Lefèvre, B. Valeur, E. Gratton, M. van de Ven, N. D. Silva, Jr., Y. Engelborghs, K. Willaert, A. Sillen, G. Rumbles, D. Phillips, A. J. W. G. Visser, A. van Hoek, J. R. Lakowicz, H. Malak, I. Gryczynski, A. G. Szabo, D. T. Krajcarski, N. Tamai, A. Miura, *Anal. Chem*., 2007, **79**, 2137-2149.
30. K. M. Solntsev, D. Huppert, N. Agmon, L. Tolbert, *J. Phys. Chem. A* 2000, **104**, 4658-4669.
31. Y. Yang, Y. Ma, Y. Zhao, Y. Li, *J. Phys. Chem. A*, 2018, **122**, 1011-1018.
32. H. J. Kuhn, S. E. Braslavsky, R. Schmidt, *Pure Appl. Chem*., 2004, **76**, 2105-2146.
33. (a) S. Arumugam, V. V. Popik, *J. Am. Chem. Soc*., 2011, **133**, 5573-5579. (b) S. Arumugam, V. V. Popik, *J. Am. Chem. Soc*., 2011, **133**, 15730-15736. (c) S. Arumugam, S. V. Orski, J. Locklin, V. V. Popik, *J. Am. Chem. Soc*., 2012, **134**, 179-182.
34. G. Siano, S. Crespi, S. M. Bonesi, *J. Org. Chem*., 2020, **85**, 14012-14025.
35. A. M. Merlot, D. S. Kalinowski, D. R. Richardson, *Front Physiol*., 2014, **5**, 299.
36. S. R. Rajski, R. M. Williams, *Chem. Rev*., 1998, **98**, 2723-2795.
37. E. Runge, E. K. U. Gross, *Phys. Rev. Lett*., 1984,**52**, 997.
38. D. Laurent, D. Jacquemin, *Int. J. Quantum Chem*., 2013, **113**, 2019-2039 and references therein.
39. C. Fang, B. Durbeej, *J. Phys Chem A*, 2019, **123**, 8485-8495.
40. C. Adamo, V. Barone, *J. Chem. Phys*., 1999, **110**, 6158.
41. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, et al, Gaussian 09, revision D.01; Gaussian, Inc.: Wallingford, CT, **2009**.
42. G. Schaftenaar, J. H. Noordik, *J. Comp. Aided Mol. Design*, 2000, **14**, 123-134.
43. I. Antol, Z. Glasovac, D. Margetić, R. Crespo-Otero, M. Barbatti, *J. Phys. Chem. A*, 2016, **120**, 7088-7100.

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