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Labeling of Proteins by BODIPY-Quinone Methides utilizing Anti-Kasha Photochemistry.

Katarina Zlatić,[1] Ivana Antol,[1]* Lidija Uzelac,[2] Ana-Matea Mikecin Dražić,[2]† Marijeta Kralj,[2]* Cornelia Bohne,[3,4] and Nikola Basarić[1]*

[1] Department of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia
[2] Department of Molecular Medicine, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia
[3] Department of Chemistry, University of Victoria, Box 1700 STN CSC, Victoria BC, V8W 2Y2, Canada
[4] Centre for Advanced Materials and Related Technologies (CAMTEC), University of Victoria, Box 1700 STN CSC, Victoria BC, V8W 2Y2, Canada.

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Supporting Information Placeholder

ABSTRACT: A novel approach for the photolabeling of proteins by a BODIPY fluorophore is reported that is based on an anti-Kasha photochemical reaction from an upper singlet excited state (S_n) leading to the deamination of the BODIPY quinone methide precursor. On the other hand, the high photochemical stability of the dye upon excitation by visible light to S_1 allows for the selective fluorescence detection from the dye or dye-protein adduct, without concomitant bleaching or hydrolysis of the protein-dye adduct. Therefore, photolabeling and fluorescence monitoring can be uncoupled by using different excitation wavelengths. Combined theoretical and experimental studies by preparative irradiations, fluorescence and laser flash photolysis fully disclose the photophysical properties of the dye and its anti-Kasha photochemical reactivity. The application of the dye was demonstrated on photolabeling of bovine serum albumin.

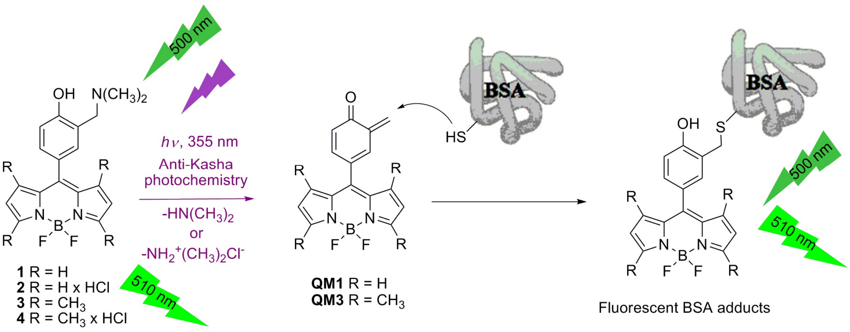
Introduction

BODIPY dyes are often employed in chemistry and biology due to their excellent spectroscopic and photophysical properties.[1,2] These dyes are generally characterized by excitation/emission spectra in the visible region with narrow emission bandwidths with high peak intensities, high fluorescence quantum yields (Φ_f), high ground-state molar absorption coefficients ($\varepsilon > 50\,000$ M^{-1} cm^{-1}), and usually small quantum yields of intersystem crossing (ISC).[3,4] Furthermore, these dyes have good thermal and photochemical stability as well as good solubility in many organic solvents. Easy synthetic modifications of BODIPY dyes allow for the preparation of a vast number of different molecules characterized by tunable spectral properties and different sensing and labeling applications.[5] In biological systems, BODIPY dyes are employed in specific protein labeling by attaching a BODIPY chromophore to amino acids which were incorporated into proteins.[6,7] A different labeling protocol is based on the reaction of lysine residues from a protein with activated succinimide-BODIPY derivatives.[8] Recent modification of BODIPY chromophores enabled new labeling protocols by reaction of the dyes with lysines,[9] tyrosines,[10] or cystein residues.[11] However, for the applications in biology it is desirable to develop photochemical activation protocols, which will enable the attachment of a label under mild, biologically acceptable conditions with temporal and spatial control.[12] In this respect, photochemical activation of BODIPY dyes for the covalent modification of proteins is hitherto mostly underdeveloped,[13,14] and there are only a few reports for the photochemical activation of BODIPY fluorescent labels.[15-17]

Quinone methides (QMs) are reactive intermediates of phenols[18] that have attracted scientific interest due to their applications in synthesis[19,20] and reactivity with biomacromolecules, particularly with DNA[21,22] and proteins.[23] Popik et al. have reported selective modification of cystein residues in human serum albumins (HSA) in reactions with naphthalene QMs, which were photochemically generated.[23] Furthermore, QM chemistry was applied in fluorescent modification of surfaces where thiols attached to surfaces reacted with QMs that were photochemically generated.[24] An additional protocol for protein modification was based on a hetero-Diels-Alder reaction of alkenes attached to a surface with photogenerated QMs substituted with avidin,[25] and subsequent avidin-biotin recognition.[26] Alternatively, QM precursors can be bound to surfaces, and upon photoexcitation generate QMs that react with alkenes substituted with flurophores such as fluorescein or rhodamine.[27]

Photochemical labeling of proteins by use of QM chemistry is simple and elegant.[23-27] However, QM-nucleophile adducts are also photochemically reactive, and in principle, additions to QMs can be reversible. Reversibility of the nucleophilic additions to QMs was utilized in the reactivity with DNA,[28-31] but in protein labeling the reversible reactivity may lead to a loss of the fluorescent tag due to photo-initiated hydrolysis. Herein we report a novel approach in protein labeling that utilizes anti-Kasha photochemistry of BODIPY-QM precursors, where the reactivity occurs from a higher excited state of the precursor. The excitation of the BODIPY chromophore by visible light allows for the fluorescence readout without any modification to the chromophore, or chromophore-protein adduct. To photogenerate QMs and induce labeling, photoexcitation by UV light to a higher excited state is required (Scheme 1). Anti-Kasha photochemistry has been reported, but it is still rather rare since the majority of molecules deactivate faster from higher excited states by internal conversion (IC) than the time it takes for slow bimolecular photoreactions to compete with IC.[32] Photophysical properties of the dyes were investigated by fluorescence

Scheme 1. Anti-Kasha photochemical labeling of BSA (in the protonated compounds - × HCl, the amine was transformed to a salt).



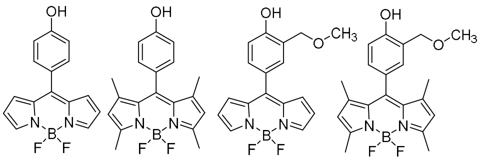
spectroscopy, whereas anti-Kasha photochemical reactivity was demonstrated by preparative irradiations with light of different wavelengths, and laser flash photolysis (LFP), that allowed for the detection of QMs. Photophysical properties and photochemical reactivity were rationalized by TD-DFT computations. The applicability of the anti-Kasha photochemical protocol in labeling proteins was demonstrated with photoinduced fluorescent labeling of bovine serum albumin (BSA).

Results and Discussion

Synthesis of BODIPY QM precursors **1**-**4** (Scheme 1) described in the SI (Schemes S1-S7) is based on the Mannich reaction of *meso*-BODIPY phenols **7** and **9** with Eschenmoser's salt.

Photophysical properties of the dyes were investigated by steady-state and time-resolved fluorescence spectroscopy in nonaqueous CH_3CN and aqueous CH_3CN-H_2O solvents (Figures S1-S13 and Table S2 in the SI). Compounds exhibit the typical BODIPY sharp absorption band at ≈ 496 nm, corresponding to the HOMO-LUMO transition, populating the S_1 state (see computational part below), and weaker intensity bands in the UV region populating higher singlet excited states (S_n). The emission spectra are the mirror image of the absorption with a small Stokes shift and a maximum at ≈505 nm. Fluorescence quantum yields (Φ_f) generally depended on the solvent (presence of H_2O) and the dye's molecular structure, being higher for the methylated derivatives. The comparison of the Φ_F for **3**, **4** and the corresponding phenol not bearing the methylammoium group (Table S2) indicates that the substituents at the phenol moiety affect the fluorescence properties of the whole molecule when excited to S_1 and suggests that the phenol and BODIPY moieties in **3** and **4** do not behave as two independent chromophores. In aqueous solution, salt **4** has Φ_f of 0.25-0.30, allowing its use as a fluorescent label in biological aqueous systems and in fluorescent microscopy.

To probe for the photodeamination reaction of BODIPY derivatives **2**-**4**, we conducted preparative irradiations in the presence of CH_3OH. The deamination is anticipated to give QMs, which react with CH_3OH as a nucleophile giving methyl ethers solvolysis products (Schemes S8 and S9 in the SI).[33] Irradiations of **2** and **4** were performed in neat CH_3OH, as well as in buffered CH_3OH-H_2O (1:1) at pH 7 and 9, where a difference was expected at the different pH values since the molecules bear a positive charge, or are in neutral-zwitterionic form, respectively.[33] Irradiation with visible light which excites molecules to the S_1 state did not give methanolysis products. After irradiation for 16 h no decomposition of the molecules took place and the conversion to photoproducts was < 1%. On the contrary, upon excitation at 355, 300 or 254 nm, photomethanolysis took place giving ethers **10** and **11**, which were isolated and characterized (Table S1 in the SI). These results indicate that photodeamination takes place only upon excitation to an S_n state. For the methylated BODIPY **4**, which reacts more efficiently, the quantum yield for the photomethanolysis upon excitation at 254 nm is $\Phi_R = 0.19 \pm 0.04$ (see SI for details), a value that compares for example with the reported dehydration efficiency from naphthols used in biological systems, delivering QMs from S_1.[23] Taking molar absorption coefficients at 254 and 350 nm and product yields obtained by irradiating at these wavelengths, the estimated efficiency for the reaction at 350 nm is ≈ 0.03.



7 **9** **10** **11**

The photochemical formation of QMs was probed by LFP. Measurements for **2**-**4** were conducted in CH_3CN and CH_3CN-H_2O, since we expected differences based on literature precedent for the deamination of cresols (Figures S14-S22 in the SI).[33] The samples were excited at 500 nm or at 355 nm. Excitation at 500 nm (Figures S14, S16 and S19) gave rise to negative signals only, due to reversible bleaching of the precursor chromophore and fluorescence from the S_1 state. After the decay of the negative signal, no transient absorption was detected. In contrast, excitation at 355 nm gave rise to a long-lived transient absorbing with a maximum at ≈ 390 and ≈ 510 nm, decaying within milliseconds. This transient was assigned to QMs formed upon excitation to S_n. The QM transient absorption was stronger for the methylated BODIPY, in agreement with its more efficient photochemical reactivity. The same QM transient was detected for amine **3** and salt **4**, in CH_3CN and CH_3CN-H_2O

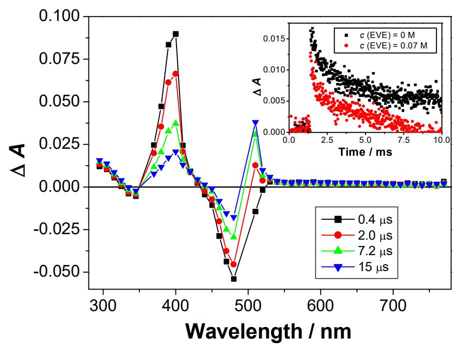


Figure 1. Transient absorption spectra of an O_2-purged solution of **4** in CH_3CN-H_2O (1:1), upon excitation at 355 nm. Inset: transient decays at 420 nm in the absence and presence of EVE.

(Figure 1). The transient assigned to QM decayed with a lifetime of 2.4 ± 0.5 ms. The assignment of the transient to a QM was based on the lack of quenching by O_2, but quenching by the nucleophiles NaN_3 ($k_q \approx 10^6$ M^{-1} s^{-1}) and ethyl vinyl ether (EVE, $k_q = 7.2\times10^3$ M^{-1} s^{-1}), which react with QMs in a Diels-Alder reaction.

The photophysical properties and anti-Kasha photochemistry of BODIPY-QM precursors were rationalized by computations. Computations at PBE0/6-311G(2d,p) level of theory were conducted for molecules **1** and **2**. The computed absorption spectrum for **1** is shown in Figure S23, as well as frontier molecular orbitals important for the absorptions to S_1 and S_n states (Figure S24). The low energy vertical excitation populates the Franck Condon state (FC state) at 72.4 kcal/mol (exp. value 495 nm, 57.8 kcal/mol). The relaxation on the S_1 surface involves two processes, torsional motion of the two aryl groups, giving a local minimum **1b** at 69.9 kcal/mol (Figure 2 and Figures S25-S27 in the SI). The second relaxation process on the S_1 surface involves excited state intramolecular proton transfer (ESIPT) from the phenolic OH to the amine nitrogen giving the **1a** local minimum characterized by the $\pi_{Ph}\rightarrow\pi_{BP}$* charge transfer character and an energy of 58.2 kcal/mol. Most probably, the two decay times detected for **3** in CH_3CN solution correspond to fluorescence from such minima on the S_1 surface. According to literature precedent, the CT state after ESIPT is anticipated to undergo a cleavage of the amine group to give the QM.[33,34] For BODIPY **1**, formation of QM on the S_1 surface requires an energy barrier of 40.7 kcal/mol (Figure S26 in the SI), and therefore, it is not plausible. All attempts to localize a minimum on the S_1 surface for protonated BODIPY **1H**$^+$ (model system for salt **2**) failed, probably due to the existence of conical intersections which cannot be assessed by TD-DFT. The molecular motion that leads to the CI with S_0 for **1H**$^+$ most probably involves a puckering of the BODIPY moiety.[35] Consequently, excitation to S_1 for **1** and **2** does not lead to the deamination and formation of QMs. On the other hand, excitation to S_n can provide the required energy for deamination to occur from these higher excited states. In particular, upon population of higher excited states S_8 and S_9 (Figures S23 and S24 and Tables S3-S6 in the SI), which correspond to $\lambda < 355$ nm, the excitation is localized on the phenolic moiety, leading to a change in electron density on the phenolic oxygen and methylamino nitrogen, which is a requirement to initiate the deamination process. Since the deamination is an ultrafast reaction taking place in one ps,[36] it can compete with fast IC from higher excited states. Therefore, excitation of **2-4** to S_n leads to the observed anti-Kasha photochemical reaction of deamination.

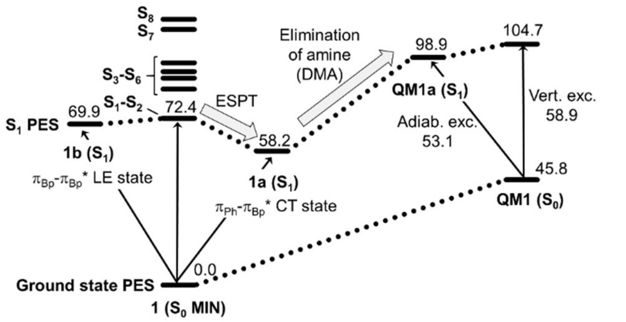


Figure 2. Energy diagram for the QM formation from **1**. Stationary points for the ground and the S_1 state were computed at the PBE0/6-311G(2d,p) level of theory. Relative energies are given in kcal mol^{-1}. Optimized structures (S_0 and S_1) are shown in the SI.

The observed anti-Kasha reactivity for **1** or **3** may, in principle, be due to non-conjugation of two molecular fragments, the BODIPY and the phenol, which would behave as two chromophores. However, computed spectra for **1**, **3**, and the fragments obtained by a disconnection of the BODIPY from the phenol moiety (see Figures S28-S30 in the SI) indicate that the sum of the absorption spectra of the fragments differs from the spectra of the whole molecules. Therefore, the fragments do not behave as two separate chromophores. Furthermore, computational results for **1** indicate that the system does not obey the Kasha rule. Optimization of the S_1 state for **1** gave two minima that involved geometry changes on the phenol moiety, possible only if the molecule behaves as one chromophore.

The applicability of BODIPY **4** for the photoactivable fluorescent labeling of proteins and fluorescence microscopy was investigated. Derivative **4** was chosen since it exhibits high Φ_f in aqueous solvent (0.25-030) and high Φ_R for the deamination upon excitation to S_n (0.19). First, we investigated if the dye can be used in fluorescent microscopy for staining cells. A confocal image of MCF-7 cells (human breast cancer carcinoma) showed staining with **4** (Figure 3, and Figures S31 and S32 in the SI). The dye enters cells, but remains in the cell cytoplasm, not entering the cell nuclei. The dye irradiation in cells at 490 nm for 30 min did not show any bleaching, indicating a high photochemical stability of the dye in intracellular media upon excitation to S_1. Cytotoxicity of BODIPY dyes was investigated by standard MTT tests, with and without irradiation at 300 nm (Table S16 in the SI). Dyes **2** and **4** exhibit cytotoxicity in the micromolar concentration range. However, the dyes at concentrations used for fluorescence microscopy do not affect cells during measurements taking place over several hours.

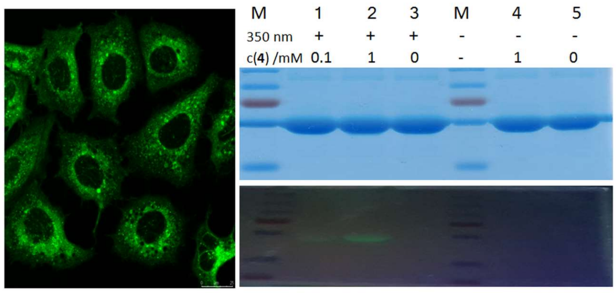


Figure 3. Left: Confocal image of live MCF-7 cells stained with **4** ($c = 10^{-5}$ M, $\lambda_{exc} = 490$ nm, $\lambda_{em} = 510$-540 nm). Right: SDS-PAGE gel (10%) after photoinduced labeling of BSA with **4**. BSA (20 µg) was incubated with or without **4**, irradiated at 350 nm (30 min, 6 lamps) or not irradiated, and then subjected to SDS-PAGE: lane 1 with **4** ($c = 0.1$ mM) lanes 2 and 4 with **4** ($c = 1$ mM), lanes 3 and 5 without **4**; lanes 1, 2, 3 irradiated at 350 nm for 30 min, lanes 4 and 5 not irradiated. Bottom panel: The labeled BSA was visualized in the gel using a UV lamp (254 nm). Top panel: Coomassie brilliant blue staining of gel. Lane M - Precision Plus Protein™ Standard.

Prior to the photochemical labeling of proteins, non-covalent binding of dyes **2** and **4** to BSA was investigated by fluorescence titrations (Figures S33 and S34 in the SI). Upon addition of BSA tothe solution of both dyes the fluorescence of the dyes was quenched. However, data could be processed for dye **4** only (Figures S33 and S34 in the SI). Multivariate nonlinear regression analysis based on a model for complex formation with a 1:1 stoichiometry revealed the binding constant of logβ = 4.70 ± 0.08. Irradiation of the complex **4@BSA** at 350 nm and subsequent denaturing gel electrophoresis indicated that the dye was covalently attached to the protein upon photochemical activation. Control experiments where the dye was kept in the dark did not give rise to any labelled protein (Figure

S35 in the SI). Thus, the most probable mechanism for the photolabeling involves photochemical formation of QMs upon excitation to S_n, and the reaction of the QMs with the cystein or lysine residues in the protein (Scheme 1). This proposal is supported by the well-known reactivity of QMs with cysteins[23] and lysines in proteins.[37] Alkylation of BSA by the QMs generated from **2** and **4** was also demonstrated by mass spectrometry. MALDI TOF/TOF experiments allowed for the detection of molecular ions corresponding to BSA covalently labeled with two molecules of **2** or **4** (increase of *m/z* =709, or 825, respectively, Table S17 in the SI).

Conclusion

In conclusion, we have designed new BODIPY dyes that are photochemically stable and highly fluorescent when excited to S_1. However, excitation to S_n triggers anti-Kasha photochemistry delivering QMs which can react with proteins and can be used in fluorescent labeling. This strategy is suitable for the use of the same molecule to photolabel proteins and to track the free dye and the dye-protein complex by fluorescence, without any photodecomposition happening when the dye is excited in the tracking mode.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. It contains detailed synthetic procedures, UV-vis and fluorescence spectra, LFP data, computational data, and details on biological investigations.

AUTHOR INFORMATION

Corresponding Author

IA iantol@irb.hr; MK Marijeta.Kralj@irb.hr; NB nbasaric@irb.hr

Present Addresses

†A.-M. M.D. Division of Experimental Hematology, German Cancer Research Center (DKFZ), HI-STEM gGmbH Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.

Notes

The authors declare no competing financial interests.

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ORCID

Marijeta Kralj 0000-0002-7165-2722
Cornelia Bohne: 0000-0001-9996-0076
Nikola Basarić: 0000-0001-9412-9734

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SYNOPSIS TOC.