J Fluoresc (2011) 21:347–354
DOI 10.1007/s10895-010-0723-0

ORIGINAL PAPER

Synthesis and Spectroscopic Characterization of Fluorescent Boron Dipyrromethene-Derived Hydrazones

Ozlem Dilek · Susan L. Bane

Received: 13 June 2010 / Accepted: 8 September 2010 / Published online: 1 October 2010

Abstract Derivatives of 4,4-difluoro-4-bora-3a,4a,diaza-s-indacene (BODIPY® or BDP) that possess a hydrazine substituent on position 5 are potential "turn-on" fluorophores for labeling aldehydes The unnatural amino acid L-3-formyltyrosine can be incorporated into a protein or peptide; thus, these hydrazines are potentially site specific labels for such polymers. In this work, model compounds were synthesized to assess whether the photochemical properties of the BDP-hydrazone would be suitable for protein labeling. Hydrazones were synthesized from the fluorophore 3-chloro-5-hydrazino-BDP and different aldehydes, and the absorption and emission spectra of the products were compared. The hydrazone of an unsubstituted aromatic aldehyde displays absorption and emission maxima (531 nm and 559 nm, respectively in dioxane) that are red shifted relative to those of a hydrazone from an aliphatic aldehyde (513 nm and 543 nm, respectively, in dioxane) and an increased quantum yield (0.21 vs. 0.11, respectively, in dioxane). The presence of a hydroxyl group *ortho*- to the aldehyde produces a hydrazone in which the absorption and emission maxima are slightly red shifted (528 nm and 564 nm, respectively in dioxane) from the unsubstituted aromatic hydrazone, but the quantum yields of the two hydrazones are equivalent. Thus, an *ortho*-hydroxy substituted aromatic aldehyde is a suitable electrophile for "turn on" protein labeling using the hydrazino-BDP. The specificity of this labeling reaction for the unnatural amino acid was demonstrated through fluorescent labeling of just the 3-formyltyrosine-containing α-subunit of α,β-tubulin.

Keywords BODIPY · Hydrazones · Bioorthogonal labeling · Fluorescent probes · Unnatural amino acids

O. Dilek · S. L. Bane (✉)
Department of Chemistry,
State University of New York at Binghamton,
Vestal Parkway,
Binghamton, NY 13902, USA
e-mail: sbane@binghamton.edu

Introduction

Boron dipyrromethene-based fluorescent dyes (BODIPY® or BDP) are versatile and valuable probes for many biological systems. Structural modifications of the core fluorophore can produce molecules with a large range of the absorption and emission energies—from visible light to the infrared and even microwave region of the electromagnetic spectrum [1, 2]. Absorption and emission energies of the dye can be tuned by the nature of the substituents on the dipyrromethene template and the boron atom. Rohand et al. showed that molecule **1** is amenable to substitution at the 3- and 5- positions by a variety of nucleophiles [3] and their photochemical properties of the BDP are highly dependent on the nature of these substitutents [4]. We are interested in exploiting this property of BDPs to create bioorthogonal protein labeling systems in which the BDP fluorescence is "turned on" by formation of the fluorophore-biomolecule covalent bond. Reactions between a modified biomolecule and its chemical partner can be exploited to selectively incorporate a reporter molecule into a chosen site within the biological target [5, 6]. For example, reactions between azides and phosphine ligands or alkynes have been successfully exploited to tag proteins, nucleic acid polymers and carbohydrates [7, 8].

We have chosen to focus on the reaction of aldehydes with hydrazine-containing fluorophores, since these reactions are known to occur in aqueous solution to form a

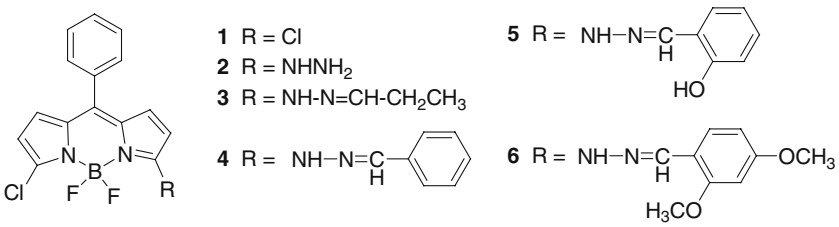

Fig. 1 Structures of BDP derivatives

stable product [9]. In addition, the optical properties of the fluorophore may change upon hydrazone formation by fluorophores in which the hydrazine functional group is directly bonded to the conjugated system. We have shown previously that while 5-hydrazino-BDP's are weakly fluorescent in solution, hydrazone formation causes a 30-45 fold increase in the BDP quantum yield [10]. Moreover, we found that the nature of the aldehyde affects the fluorescence of the hydrazone. Specifically, reaction of the BDP-hydrazine **2** with propanal yields the aliphatic hydrazone **3,** which has an emission maximum at higher energy than that of the aromatic hydrazone **4** (Fig. 1). This result indicates that a protein containing an aromatic aldehyde could be distinguished from endogenous aliphatic aldehydes that may be present in the biological system.

We are engaged in developing methodologies for incorporating aromatic aldehydes into proteins. In one manifestation of this work, we have shown that the aromatic amino acid L-3-formyltyrosine can be selectively bonded to the C-terminus of just one of the subunits of the tubulin heterodimer [11]. The aromatic aldehyde is therefore available for reaction with hydrazine-containing fluorophores. Since the electrophile on the protein is a substituted benzaldehyde, it is of interest to evaluate the effect of an *ortho*-substituent on the aromatic aldehyde on the reaction of 5-hydrazinyl-BDP's and the photochemical properties of the resulting product (Fig. 2).

Hydrazones formed between salicylaldehyde and aryl hydrazines are capable of undergoing tautomerization into quinoid structures, which are normally not fluorescent [12]. It was therefore important to assess the effect of hydroxyl substituent on the fluorescence spectra of the BDP-hydrazone. In this work, we synthesized two model compounds by allowing BDP-hydrazine **2** to react with salicylaldehyde or 2,4-dimethoxybenzaldehyde and compared their absorption and emission spectra with those of the previously characterized aliphatic and aromatic BDP-hydrazones.

It is also important to confirm that the hydrazone reaction occurs under conditions suitable for protein labeling. Kinetics of hydrazone formation at neutral pH was therefore examined using the model compound salicylaldehyde. Finally, the potential applicability of this fluorophore-unnatural amino acid reactive pair was assessed using 3-formyltyrosinated tubulin.

Experimental

Materials

Dry methanol and dichloromethane were purchased from Acros Chimica and Aldrich. Other solvents and aldehydes were also obtained from Acros or Aldrich and were used as received. Deuterated solvents were obtained from Cambridge Isotope Laboratories. All other chemicals are commercially available and were used without further purification. BDP compounds **1**–**4** were synthesized as described previously [10].

General Synthetic Procedures

^{1}H, ^{13}C, ^{11}B NMR spectra were recorded on instruments operating at a frequency of 360 MHz. ^{1}H and ^{13}C NMR spectra were referenced to $CDCl_3$ (7.26 ppm or 77.00 ppm). ^{11}B NMR spectra were referenced to $BF_3.OEt_2$ (0 ppm). Chemical shift multiplicities are reported as s=singlet, d=doublet, t=triplet, q=quartet and m=multiplet. Flash column chromatography was performed using Baker silica gel 60–200 mesh or 200–400 mesh. All flash columns were performed using the same solvent conditions used for TLC. All moisture and air sensitive reactions were carried out

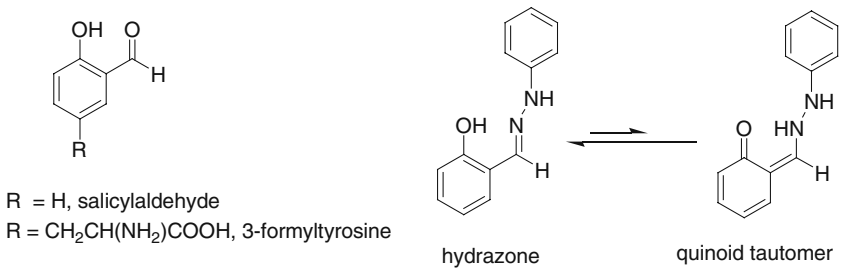

Fig. 2 Structures of aromatic aldehydes and hydrazone tautomers

under nitrogen or argon atmosphere using oven dried glassware.

Synthesis of BDP-hydrazones

3-Chloro-3a, 4a-diaza-4,4-difluoro-5-salicylidenehydrazino-8-phenyl boron dipyrromethene (5)

One equivalent of salicylaldehyde (6.6 mg) was added to 18 mg (0.054 mmol) of compound **2.** The mixture was stirred without solvent for 1.5 h. The color of the mixture changed immediately after addition of salicylaldehyde. The reaction was followed by TLC and UV-vis. The crude residue was purified by silica gel flash column chromatography to afford the desired product **5** as pink solid (16 mg, 76% yield); R_f=0.64 (100% CH_2Cl_2); IR (cm^{-1}, $CDCl_3$): υ 3256, 2950, 1609, 1481, 1412, 1197, 1096. ^{1}H NMR ($CDCl_3$): δ 6.25 (d, 1H, J=4.28 Hz), 6.49 (d, 1H, J= 4.28 Hz), 6.59 (d, 1H, J=4.70 Hz), 6.97 (d, 1H, J= 4.70 Hz), 7.00-7.24 (m, 2H, aromatic), 7.35 (m, 2H, aromatic), 7.50 (m, 5H, aromatic), 8.12 (s, 1H, CH), 9.29 (s, 1H, NH), 9.91 (s, 1H, OH) ^{13}C NMR ($CDCl_3$): δ 110.8, 113.9, 117.0, 120.1, 123.0, 128.0, 128.4, 129.8, 129.9, 130.4, 131.3, 132.1, 132.5, 133.0, 133.4, 133.6, 135.6, 150.0, 157.7, 164.7. ^{11}B NMR ($CDCl_3$): δ 0.50 (t, J= 33.5 Hz).

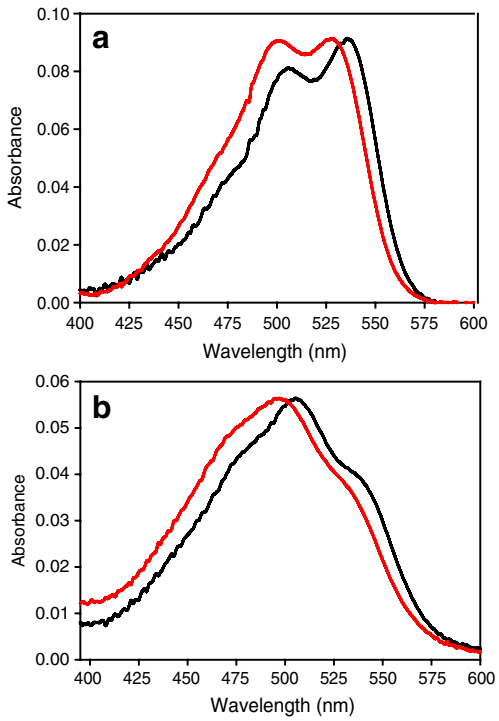

Fig. 3 Normalized absorption spectra of BDP hydrazones **5** (panel A) and **6** (panel B) in dioxane (*black*) and methanol (*red*)

3-Chloro-3a, 4a-diaza-4,4-difluoro-5-(2,4-dimethoxy) benzylidenehydrazino-8-phenyl borondipyrromethene (6)

One equivalent of 2,4-dimethoxybenzaldehyde (7.5 mg) was added to 15 mg (0.045 mmol) of compound **2** in 90 µl CH_2Cl_2. Neutral Alumina (Brockman Activity I, 0.14 g) was added to the mixture. The color of the mixture changed immediately after addition of 2,4-dimethoxybenzaldehyde. The reaction was followed by TLC and UV-vis. The crude residue was purified by silica gel flash column chromatography to afford the desired product **6** as pink solid (7 mg, 35 % yield); R_f=0.52 (100% CH_2Cl_2); IR (cm^{-1}, $CDCl_3$): υ 3324, 2926, 1727, 1573, 1469, 1412, 1276, 1084. ^{1}H NMR ($CDCl_3$): δ 3.86 (s, CH_3), 3.88 (s, CH_3), 6.18 (s, aromatic), 6.21 (d, 1H, J=4.40 Hz), 6.26 (d, 1H, J=5.50 Hz), 6.39 (d, 2H, J=4.40 Hz), 6.46 (s, aromatic), 6.55 (m, J=8.43 Hz), 6.82 (t, aromatic, J=5.86 Hz), 6.92 (d, 1H, J=5.50 Hz), 7.46 (m, 5H, aromatic), 7.87 (d, aromatic, J=8.43 Hz), 8.35 (s, CH), 9.29 (s, NH). ^{13}C NMR ($CDCl_3$): δ 55.4, 55.6, 97.9, 106.0, 112.5, 112.9, 114.5, 115.1, 117.7, 120.9, 127.5, 128.0, 128.3, 128.7, 129.3, 130.3, 130.5, 133.3, 134.9, 135.3, 143.8, 163.3. ^{11}B NMR ($CDCl_3$): δ 0.95 (t, J=36.6 Hz).

Absorption and Fluorescence Spectroscopy

Absorption spectra were obtained using an HP 8453 absorption spectrophotometer equipped with a circulating water bath for temperature control. Kinetic data were obtained using absorption difference spectroscopy [13]. Equal volumes of each reactant were placed separately placed in a dual chambered quartz cuvette. The instrument was blanked and the solutions were mixed by inversion of the cuvette. Absorption spectra were collected every 30 s until a steady state was achieved.

Kinetic data were collected in 0.1 M phosphate buffer (pH 4.0 or 7.0) at 23°C. Experiments were performed by

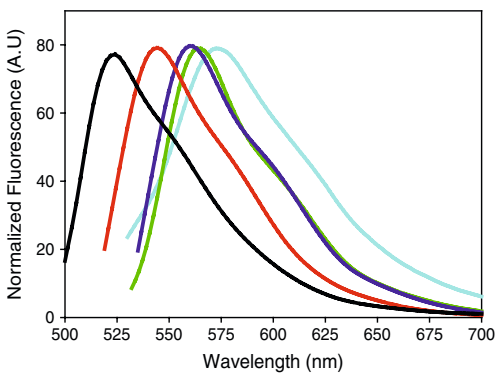

Fig. 4 Normalized fluorescence emission spectra of **2–6** in dioxane. From left to right, compound **2** (*black*), compound **3** (*red*), compound **4** (*blue*), compound **5** (*green*), compound **6** (*light blue*). Molecules were excited at their absorption maxima at room temperature (23°C)

mixing 65 μM hydrazine **2** with an excess of aldehyde (salicylaldehyde: 650 μM, λ_{max}=326 nm, ε_{326}=3400 cm^{-1} M^{-1}; propanal: 2 mM)

Fluorescence spectra were collected using a Spex FluoroMax-3 spectrofluorometer. The fluorescence quantum yields (ϕ_F) were determined in dilute solutions with an absorbance below 0.1 at the excitation wavelength. Quinine sulfate in 0.1 M H_2SO_4 (λex=347 nm, φ_F=0.57) was used as a standard (Lakowicz, 2006). All spectra were recorded at 23°C. Quantum yields were calculated using the following equation:

$$\phi_F^{sample} = \phi_F^{standard} \times \left(F^{sample} - F^{solvent}\right)/\left(F^{standard} - F^{solvent}\right) \times \left(\eta^{sample}/\eta^{standard}\right) \times \left(A^{standard}/A^{sample}\right)$$

where F denotes the area under the fluorescence band, A denotes the absorbance at the excitation wavelength, and η denotes the refractive index of the solvent. Integration of the emission bands was performed using SigmaPlot 10.0.

Covalent Labeling of α-tubulin with BDP-hydrazine 2

Tubulin was purified from bovine brain as described by Williams and Lee [14]. The carboxy terminal of α-tubulin was cleaved by carboxypeptidase A and was replaced with either L-tyrosine or L-3-formyltyrosine using tubulin tyrosine ligase as described in Banerjee et al. [11]. Retyrosinated tubulin was prepared in PME buffer (0.1 M PIPES, 1 mM $MgSO_4$ and 2 mM EGTA, pH 6.9 at 25°C). BDP-hydrazine **2** was dissolved in DMSO to a final concentration of 1 mM. While vortexing the protein solution, the dye **2** in DMSO was slowly added to each reaction solution to a final concentration of 100 μM (1:10 molar ratio of tubulin: dye). The reactions were incubated for 2.5 h at 25°C on a rocker. Unreacted dye was removed by rapid gel filtration using Sepharose G-25 columns [15]. After addition of sample buffer, the samples were boiled for 5 min and 6.5 μg of protein was loaded on an SDS-PAGE gel. After electrophoresis, the gel was placed in the destaining solution (1:5:4 acetic acid: methanol: dd-water) overnight and photographed. The next day, the gel was stained with Coumassie Blue, destained and photographed.

Results and Discussion

In a previous study, we showed that BDP's with a hydrazino substituent at the 5-position of BDP are weakly fluorescent, and that hydrazone formation induces an increase in fluorescence quantum yield and a red shift in the emission spectrum [10].

The emission energies and quantum yields are different for an aliphatic and aromatic hydrazine. Since the unnatural amino acid we hope to covalently label is a substituted benzaldehyde, it was necessary to ascertain whether a corresponding substituent on the aromatic ring affects the emission properties of the BDP-hydrazone. We therefore synthesized two model hydrazones (**5** and **6**).

Absorption and emission spectra of the two hydrazones were obtained in an apolar, aprotic solvent (dioxane) and a polar, protic solvent (methanol). The absorption spectra are broad (Fig. 3) and feature two narrower bands centered at about 500 nm and around 530 nm. The overall broadness may be attributed to the asymmetrical substitution of the dipyrromethene at the 3,5-positions [16] and the amine-like substituent on the 3-position. The absorption spectrum of a similarly substitute molecule is also broad in protic solvent and sharper in aprotic solvent [17] The relative intensity of these two bands is affected by solvent and by the substituent on the 5-position, but the shape of the emission is the same when the molecule is excited in either peak. Because both hydrazones possess these broad bands and since no quionoid tautomer was observed in the NMR

Table 1 Absorption and Emission Maxima and Quantum Yields of BDPs **3–6**

Solvent	Dioxane			Methanol		
Compound	λab_{max} (nm)	λem_{max}(nm)	Φ_F(at 23°C)	λab_{max} (nm)	λem_{max}(nm)	Φ_F(at 23°C)
3	513	543	0.11	502	539	0.064
4	531	559	0.21	521	555	0.098
5	500, 528[a]	564	0.20	506, 536 (sh)[b]	559	0.062
6	502, 534[a]	572	0.061	500, 533 (sh)[b]	545	0.006

[a] Absorption spectrum shows two peaks of similar intensity. [b] Absorption spectrum is broad and less structured than in dioxane

spectra of **5**, the two main components in the absorption bands are most likely the 0-0 and 0-1 vibrational bands of the lowest energy electronic transition [18].

The difference in the spectrum in dioxane and methanol is more pronounced for compound **5** than for compound **6**. The ability of the ortho substituent to be both a hydrogen bond donor as well as a hydrogen bond acceptor may explain the different behavior of the two molecules in solvent.

Figure 4 shows the normalized emission spectra of compounds **2**–**6** in dioxane. The emission maxima of all hydrazones are red shifted relative to the hydrazine **2**, and all of the aromatic hydrazines emit at longer wavelength than the aliphatic hydrazone **3**.

The quantum yields of the model hydrazones are compared in Table 1. Addition of the *ortho*-substituent to the benzaldehyde produces a small red shift in the emission maximum of the hydrazone in dioxane. Interestingly, an *ortho*-hydroxyl group does not affect the quantum yield of the hydrazone in this solvent, while the hydrazone possessing an *ortho*-methoxy group has a quantum yield that is about 1/3rd that of the other aromatic hydrazones. A similar trend in emission maxima and relative quantum yields of the hydrazones in methanol is observed, although the magnitudes of the quantum yields are lower methanol than in dioxane. This result indicates that the BDP hydrazones will be more effective "turn on" fluorophores in a hydrophobic than in a hydrophilic environment.

We therefore conclude that an *ortho*-hydroxyl group, such as in 3-formyltyrosine, will not adversely affect the photochemical properties of the BDP-hydrazone. Thus, the hydrazone formed between **2** and 3-formyltyrosine will be suitably fluorescent for BDP-hydrazine labeling of proteins.

Kinetics Studies

BDP-hydrazine 2 with Salicylaldehyde

The kinetics of hydrazone formation was examined in order to determine experimental conditions that could be used for protein labeling. Reaction of 3-chloro-5-hydrazino-BDP **2** with salicylaldehyde, the model compound for the unnatural amino acid 3-formyltyrosine, was followed by absorption difference spectroscopy. The reaction at non-physiological pH was measured first, since the maximum reaction rate of the hydrazone formation is near the pKa of the hydrazine [19]. The reaction was completed in 2 h and a red shift of the absorption was seen, as expected. Figure 5a displays the absorption difference spectrum produced as a function of time. The change in absorption at 550 nm as a function of time was fit to a pseudo 1st order reaction (Fig. 5b). The 2nd rate order constant calculated from the data is 37 $M^{-1}min^{-1}$.

The rate of hydrazone formation at pH 7 was also measured and it was slower than pH 4 (not shown). The 2nd rate order constant calculated from the data was 27 $M^{-1}min^{-1}$.

BDP-hydrazine 2 with Propanal

An isolated protein can be prepared to contain just an aromatic aldehyde. Cells, however, may also contain aliphatic aldehydes. It was therefore of interest to evaluate

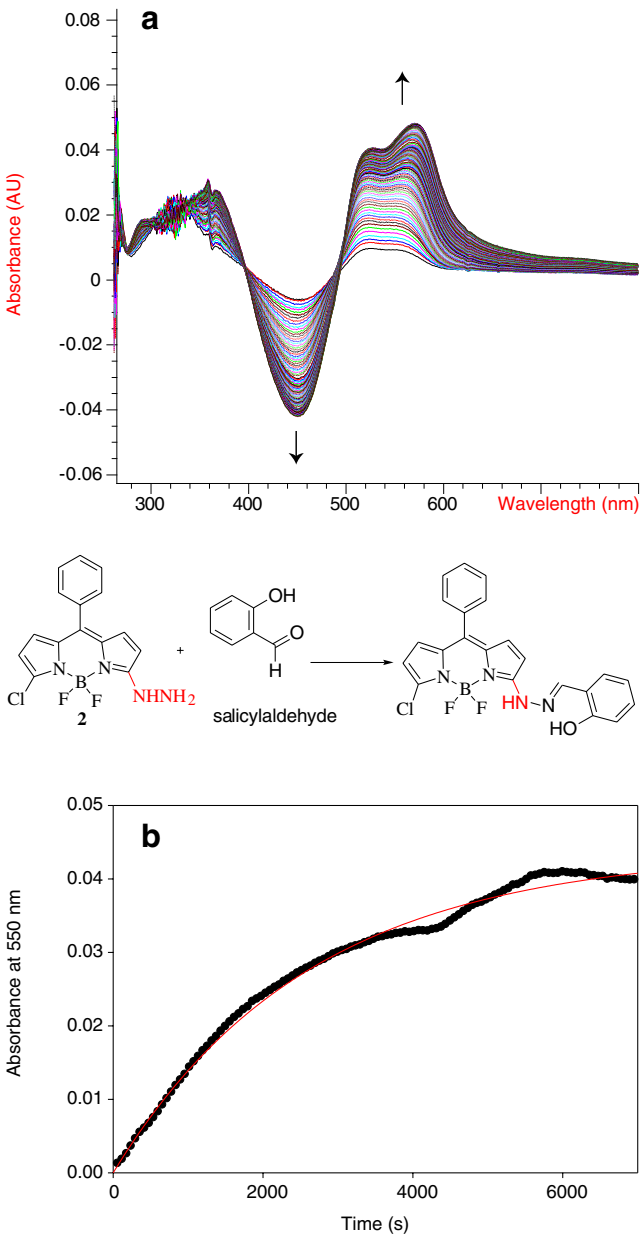

Fig. 5 **a** Absorption difference spectra for the reaction of 650 μM salicylaldehyde with 65 μM BDP **2** in 0.1 M phosphate buffer at pH 4 at room temperature. Spectra shown were collected immediately after mixing and at 30 s intervals thereafter. **b** Plot of the change in absorbance at 550 nm as a function of time. Solid line: fit of the data as a pseudo-first order reaction

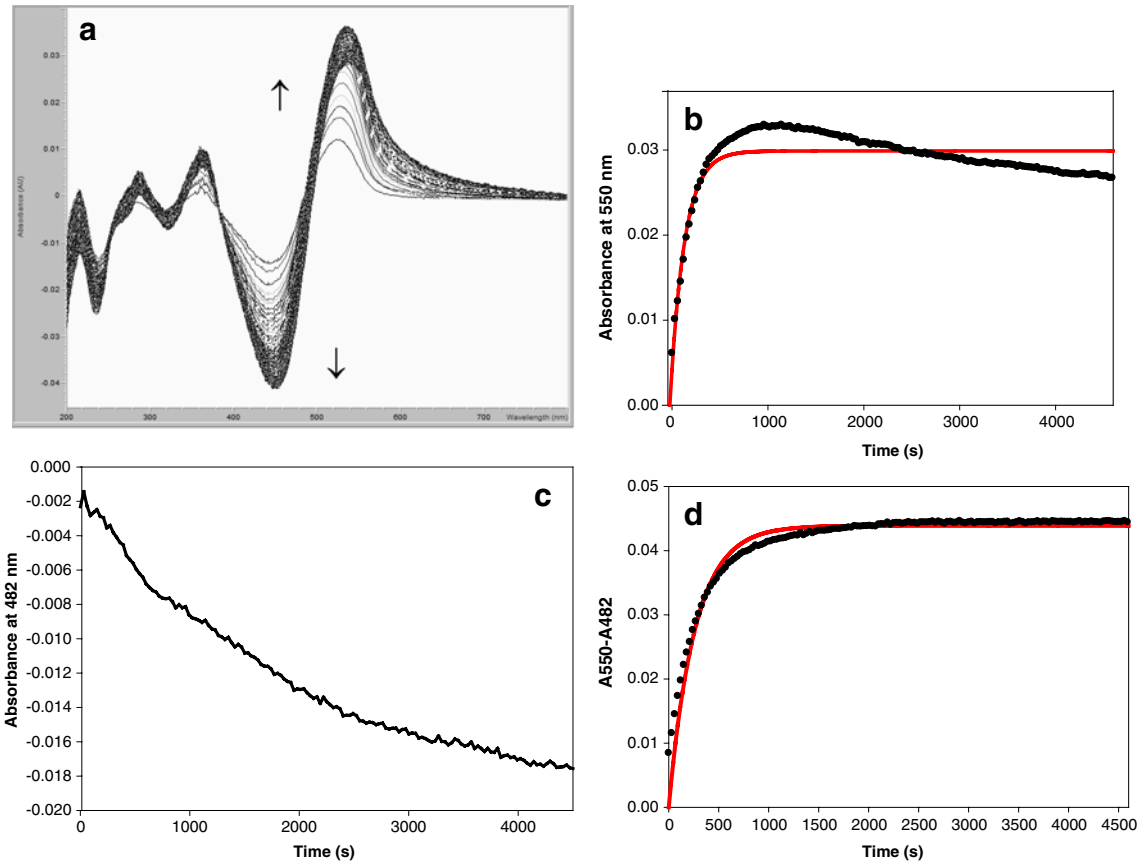

Fig. 6 **a** Absorption difference spectra for the reaction of 2 mM propanal with BDP **2** in 0.1 M phosphate buffer at pH 4 at room temperature. Spectra shown were collected immediately after mixing and at 30 s intervals thereafter. **b** Change in absorption at 550 nm as a function of time. Solid line: Data fit as a pseudo-first order reaction. **c** Change in absorption at 482 nm as a function of time. **d** Data from panel C subtracted from data in panel B. Solid line: Data fit as a pseudo-first order reaction

the kinetics of hydrazone formation with an aliphatic aldehyde.

The kinetics reaction between propanal and 3-chloro-5-hydrazino-BDP **2** was monitored by absorption difference spectroscopy. Figure 6a shows absorption difference spectra. The change in absorption at 550 nm was plotted as a function of time (Fig. 6b).

Note that the reaction with the aliphatic aldehyde does not show a sharp isosbestic point (Figs. 5a vs 6a). Also, the change in absorption as a function of time displays normal saturation kinetics for the aromatic aldehyde, but change in absorption intensity for the aliphatic aldehyde reaction reaches a maximum and then slowly decreases with time. Hydrazones are known to be susceptible to oxidation [9] and aliphatic hydrazones oxidize 10-100-fold more rapidly than aromatic hydrazones [20]. We hypothesized that the formation of an oxidation product might account for the lack of isosbestic point and decrease in absorption at 550 nm. Plotting the decrease of absorption at 482 nm, the isosbestic point early in the reaction time course, produced a curve that fit a first order decay (Fig. 6c). Subtracting the absorption at 482 nm from the change in absorption at 550 nm yielded a curve that reasonably fit a pseudo-first order reaction (Fig. 6d). The 2nd rate order constant for hydrazone formation is therefore estimated to be about 110 $M^{-1}min^{-1}$. The rate of hydrazone formation at pH 7 was

Table 2 Rate constants for hydrazone formation of BDP **2** with the aliphatic and aromatic aldehyde

Reactants	pH	2nd order rate constant, $M^{-1}min^{-1}$	1st order decomposition constant, min^{-1}
BDP **2**+propanal	4	~110	0.03
BDP **2**+propanal	7	~30	0.01
BDP **2**+salicylaldehyde	4	37	–
BDP **2**+salicylaldehyde	7	27	–

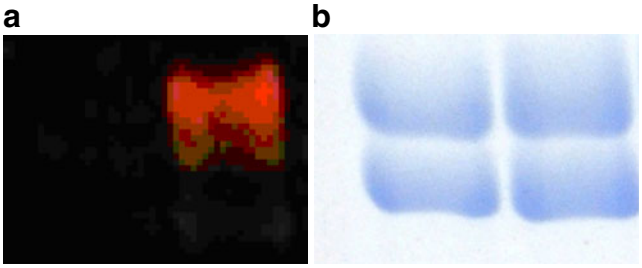

Fig. 7 SDS-PAGE of tyrosinated tubulin (Lane 1) and 3-formyltyrosinated tubulin (Lane 2) after treatment with BDP **2**. The gel was photographed in Panel A under irradiation with long wavelength UV light and in Panel B after staining with Coumassie blue

also measured and it was significantly slower than at pH 4. The data were analyzed in the same manner as for the pH 4 reaction, and the results are shown in Table 2.

Fluorescent Labeling of α-tubulin with BDP 2

The data with the model compounds have shown that the quantum yield of aromatic hydrazone of BDP **2** is essentially unaffected by an *ortho*- hydroxyl substituent, indicating that a covalent bond between 3-formyltyrosinated tubulin and BDP **2** will produce a fluorescent product. Furthermore, the reaction takes place at room temperature and neutral pH in a reasonable time, conditions that will not normally denature a protein such as tubulin. Finally, although an aliphatic aldehyde reacts more quickly with BDP **2** than the aromatic aldehyde, there is no evidence that the aromatic hydrazone oxidizes under the reaction conditions.

The unnatural amino acid 3-formyltyrosine was covalently attached to the carboxy terminus of α-tubulin using the method recently described [11]. The formyltyrosinated protein and a tyrosinated control were incubated with BDP **2** and the samples were subjected to SDS-PAGE. Figure 7a shows the gel irradiated with long wavelength UV light from a hand-held lamp. The only fluorescent band in the gel is α-tubulin in from the formyltyrosinated protein, indicating that covalent bond formation is formed between **2** and 3-formyltyrosine.

Conclusion

We have shown previously that the hydrazino derivative of BDP, compound **2**, reacts with an unsubstituted benzaldehyde, and that the fluorescence of the hydrazone is stronger and considerably red shifted from that of the hydrazine. We show here that the presence of an *ortho*-hydroxy substituent on benzaldehyde does not adversely alter the spectroscopic properties of the hydrazone. Moreover, hydrazone formation between **2** and the model compound salicylaldehyde occurs at neutral pH at a modest rate, and the product does not appear to oxidize under the reaction conditions. Reaction of **2** with 3-formyltyrosinated tubulin fluorescently labels just the subunit that possesses the hydroxybenzaldehyde functional group. Therefore, the reactive pair BDP **2** and 3-formyltyrosine should be generally useful for site specifically fluorescent labeling of proteins.

Acknowledgments Thanks to Dr. Jürgen Schulte for helping collecting ^{11}B NMR spectra and to Dr. Abhijit Banerjee for providing the formyltyrosinated tubulin and for assistance with the protein. We thank Professor Rebecca Kissling for helpful discussions and valuable scientific assistance. We also thank to David Tuttle for photography. Financial support from NIH (R01 CA69571 and R15 GM 093941) is gratefully acknowledged.

References

1. Loudet A, Burgess K (2007) BODIPY dyes and their derivatives: Syntheses and spectroscopic properties. Chem Rev 107:4891–4932
2. Ziessel R, Ulrich G, Harriman A (2007) The chemistry of Bodipy: A new El Dorado for fluorescence tools. New J Chem 31:496–501
3. Rohand T, Baruah M, Qin WW, Boens N, Dehaen W (2006) Functionalisation of fluorescent BODIPY dyes by nucleophilic substitution. Chem Commun 266–268.
4. Qin WW, Rohand T, Baruah M, Stefan A, Van der Auweraer M, Dehaen W, Boens N (2006) Solvent-dependent photophysical properties of borondipyrromethene dyes in solution. Chem Phys Lett 420:562–568
5. van Swieten PF, Leeuwenburgh MA, Kessler BM, Overkleeft HS (2005) Bioorthogonal organic chemistry in living cells: novel strategies for labeling biomolecules. Org Biomol Chem 3:20–27
6. Prescher JA, Bertozzi CR (2005) Chemistry in living systems. Nature Chem Biol 1:13–21
7. Agard NJ, Baskin JM, Prescher JA, Lo A, Bertozzi CR (2006) A comparative study of bioorthogonal reactions with azides. ACS Chem Biol 1:644–648
8. Wang CCY, Seo TS, Li ZM, Ruparel H, Ju JY (2003) Site-specific fluorescent labeling of DNA using Staudinger ligation. Bioconjug Chem 14:697–701
9. Smith PAS (1983) Derivatives of Hydrazine and Other Hydronitrogens Having N-N Bonds. The Benjamin/Cummings Publishing Company Inc, Canada
10. Dilek Ö, Bane SL (2008) Synthesis of boron dipyrromethene fluorescent probes for bioorthogonal labeling. Tetrahedron Lett 49:1413–1416
11. Banerjee A, Panosian TD, Mukherjee K, Ravindra R, Gal S, Sackett DL, Bane, SL (2010) Site-specific orthogonal labeling of carboxy terminus of alpha –tubulin. ACS Chem Biol 5 (in press).
12. Ball P, Nicholls CH (1982) Azo-hydrazone tautomerism of hydroxyazo compounds—a review. Dyes Pigm 3:5–26
13. Shanker N, Bane SL (2008) Basic aspects of absorption and fluorescence spectroscopy and resonance energy transfer methods. Methods Cell Biol 84:213–242
14. Williams RC, Lee JC (1982) Preparation of tubulin from brain. Methods in Enzymol 85 Pt B:376–85.
15. Penefsky HS (1977) Reversible binding of inorganic phosphate by beef heart mitochondrial adenosine triphosphatase. J Biol Chem 252:2891–2899
16. Qin WW, Leen V, Dehaen W, Cui J, Xu C, Tang XL, Liu WS, Rohand T, Beljonne D, Van Averbeke B, Clifford JN, Driesen K, Binnemans K, Van der Auweraer M, Boens N (2009) 3, 5-Dianilino

substituted difluoroboron dipyrromethene: Synthesis, spectroscopy, photophysics, crystal structure, electrochemistry, and quantum-chemical calculations. J Phys Chem C 113:11731–11740
17. Liras M, Prieto JB, Pintado-Sierra M, Arbeloa FL, Garcia-Moreno I, Costela AS, Infantes L, Sastre R, Amat-Guerri F (2007) Synthesis, photophysical properties, and laser behavior of 3-amino and 3-acetamido BODIPY dyes. Org Lett 9:4183–4186
18. Qin W, Baruah M, Van der Auweraer M, De Schryver FC, Boens N (2005) Photophysical properties of borondipyrromethene analogues in solution. J Phys Chem A 109:7371–7384
19. Jencks WP (1964) Mechanism and catalysis of simple carbonyl group reactions. Prog Phys Org Chem 2:63–128
20. Harej M, Dolenc D (2007) Autoxidation of hydrazones. Some new insights. J Org Chem 72:7214–7221