π-Extended BODIPY Analogues: Synthesis, Electronic Structure, Potential Utility for in vivo Imaging Applications and Cytotoxicity

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A series of 3,5-distyryl and meso-morpholino/hydroquinoline BODIPY dyes have been synthesized and characterized. The electronic structure was investigated by optical spectroscopy, electrochemistry and TD-DFT calculations. In addition, they fluoresce in the biological window and are hence potentially suitable for use as cellular imaging agents. The meso-1,2,3,4-isoquinoline substituted BODIPYs 3c-d interact strongly with DNA and this enhances their ability to act as bioimaging agents. In contrast, the meso-morpholine substituted BODIPYs 3a-b were found to have higher cell toxicity.

Keywords: BODIPY, electronic structure, cellular imaging, cell toxicity.
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Introduction

Ideal molecules used for bioimaging applications should absorb and emit strongly in the red/near-infrared (NIR) spectral region and have narrow emission bands, large Stokes shifts, long excited-state lifetimes, excellent photostability, and low biotoxicity. Research in this area has focused on chromophores, such as porphyrins, corroles and phthalocyanines.[4-8] More recently, there has been growing interest in the use of BODIPY (difluoroboradipyrromethenes) dyes in this context,[4-8] since they have high fluorescence quantum yields and intense absorption and fluorescence bands. In addition, the BODIPY chromophores could be facially modified through an extension of the π-conjugation system so that the fluorescence emission bands lie at the red end of the visible region or in the NIR in the optical window for biological tissues (650–1000 nm), making them potentially suitable for use as fluorescence cellular imaging agents.[9] Thus, there is currently considerable interest in the design and synthesis of novel BODIPY dyes with intense absorption and emission bands in this spectral region. In this study, styryl groups are introduced as substituents at the 3,5-positions on the pyrrole moieties through Knoevenagel condensation reactions (Scheme 1), since this is known to result in a large red shift of the main spectral bands into biological window.

Thus, there is currently considerable interest in the design and synthesis of novel BODIPY dyes with intense absorption and emission bands in this spectral region. In this study, styryl groups are introduced as substituents at the 3,5-positions on the pyrrole moieties through Knoevenagel condensation reactions (Scheme 1), since this is known to result in a large red shift of the main spectral bands into biological window. Moreover, morpholino and hydroquinoline and their derivatives were widely used in organic and biological systems. Along with meso-phenyl substituents that enhance the solubility of the dyes in aqueous solution and their DNA binding properties. The suitability of the dyes for use as bioimaging agents is investigated in HepG-2 cells along were studied for antitumor activity in vitro.

Experimental

General

All reagents were of reagent grade quality and were used as received unless noted otherwise. 1H and 19F NMR spectra were recorded on a Bruker AVANCE 400 spectrometer (400.03 MHz). Residual solvent peaks were used to provide internal references for the 1H NMR spectra (7.26 ppm for CDCl3 and 5.32 ppm for CD2Cl2). FT-IR spectra were measured with a Nicolet Nexus 470 FT-IR spectrophotometer over the 4000–400 cm-1 energy range. Fluorescence measurements were performed on a CARY Eclipse spectrophotometer (Varian, USA) in 1.0 cm quartz cells (λex=460 nm, slit width=5 nm). Electrospay ionization mass spectrometry (ESI-MS) data were obtained on a Finnigan LCQ mass spectograph. The UV and visible regions of the electronic absorption spectra were recorded with an HP 8453A diode array spectrophotometer. A glassy carbon disk, a platinum wire and an Ag/AgCl electrode were used as the working, counter and reference electrodes, respectively, during electrochemical measurements with a Chi-730D electrochemical working station at room temperature. An inert nitrogen atmosphere was introduced during all electrochemical measurements. BODIPY 3b-labelled HeLa cells were plated in a 24-well plate at a density of 2.4×104 cells mL-1. After incubating for 24 h with the complexes, the cells were stained with DAPI, and nuclei change was visualized under a fluorescence microscope (Nikon TE2000 inverted microscope).

Computational Methods

The Gaussian 09 software package[10] was used to carry out DFT geometry optimizations for a meso-phenyltetramethylBODIPY model compound (BDY), 2a-b and 3a-d by using the B3LYP functional with 6-31G(d) basis sets. TD-DFT calculations were carried out by using CAM-B3LYP functional, which includes a long-range correction of the exchange potential, since this provides more accurate results for complexes that have excited states with significant intramolecular charge transfer character.[11,12]

Cytotoxicity Testing

Cytotoxicity assays were measured with HepG-2 cells in normal culture conditions. HepG-2 cells were seeded at a density of 4×104 cells mL-1 into sterile 96-well plates. 3a-d were added in DMSO and diluted with culture media. After 24 h, compounds were added into the cultured HepG-2 cells for a further 24 h. Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay by measuring the absorbance at 570 nm. Each test was performed in triplicate.

Synthesis

4-(4-(5,5-Difluoro-1,3,7,9-tetramethyl-5H-4λ,5λ-dipyrrrolo[1,2-c:2′,1′-f][1,3,2]diazaborinin-10-yl)benzyl) morpholine (2a). A 15 mL THF solution containing BODIPY 1 (372.1 mg, 1.0 mmol), morpholine (173.2 mg, 2.0 mmol) and 0.5 mL petroleum ether:ethyl acetate=5:1; v:v) as a red solid in 71.2 % yield (301.5 mg). Elemental analysis (%): Found: C 68.00, H 6.50, N 9.93, O 3.78, B 2.55, F 8.98. Calcd for C24H28BF2N3O: C 68.10, H 6.67, N 9.93, O 3.78, B 2.55, F 8.98. IR (KBr) ν cm-1: 2956, 2922, 2855, 2801, 1547, 1509, 1468, 1408, 1365, 1194, 1047, 980, 821, 754, 706. 1H NMR (400 MHz, CDCl3) δ ppm: 7.46–7.44 (d, J=8 Hz, 2H), 7.24–7.22 (d, J=8 Hz, 2H), 5.97 (s, 2H), 3.75–3.72 (t, J=6 Hz, 4H), 3.59 (s, 2H), 2.55 (s, 6H), 2.46 (s, 4H), 1.37 (s, 6H).

Scheme 1. The synthesis and molecular structures of 2a-b and 3a-d.
for C45H42BF2N3O2

Found: C 76.70, H 6.10, N 5.90, O 4.46, B 1.46, F 5.34. Calcd (%)

as a purple solid in 43.4 % yield (153.0 mg). Elemental analysis (%):

was used instead. The target compound was successfully obtained

3a was followed, but precursor

the procedure similar to that of

dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine (2a). A THF solution (15 mL) containing I

(372.1 mg, 1.0 mmol), 1,2,3,4-isouquinoline (160 mg, 1.2 mmol, 1.2 eq), K2CO3 (166.0 mg, 1.2 mmol), KI (34.2 mg, 0.2 mmol) and a catalytic amount of 18-crown-6 ether was stirred at 60 °C for 5 h under N2. After removal of the solvent, the residue was dissolved in 20 mL CH2Cl2, washed three times with brine and dried over Na2SO4. The target compound was obtained by silica gel column chromatography (eluent: petroleum ether:acetone = 10:1; ν1) as a red solid in 73% yield (542.5 mg). Elemental analysis (%): Found: C 74.11, H 6.55, N 8.83, B 2.38, F 8.15. Calcd (%) for C52H64BF2N4O2: 74.61, H 6.44, N 8.95, B 2.30, F 8.09, m/z (ESI-MS) 570.40 (Calcd for C52H64BF2N4O2: [M+H]+ = 570.39). IR (KBr) νmax cm⁻¹: 2919, 2851, 1538, 1463, 1305, 1304, 1282, 1187, 1154, 1070, 972, 806, 761, 693. 1H NMR (400 MHz, CDCl3) δ ppm: 7.55‒7.53 (d, J = 8 Hz, 2H), 7.26‒7.25 (d, J = 4 Hz, 2H), 7.14 (m, 3H), 6.98‒6.96 (d, J = 8Hz, 1H), 5.99 (s, 2H), 3.80 (s, 2H), 3.65 (s, 2H), 2.96‒2.93 (t, J = 6 Hz, 2H), 2.82‒2.79 (t, J = 6 Hz, 2H), 2.56 (s, 6H), 1.41 (s, 6H).

4-((5,5-Difluoro-1,9-dimethyl-5H-4λ,5λ-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)benzyl)morpholine (3a). 2a (234.5 mg, 0.5 mmol) and p-methoxybenzaldehyde (170.5 mg, 1.3 mmol) were dissolved in 15 mL toluene, and 4-methylphenylsulfonic acid and piperidine (4.7 mg, 0.1 mmol) were slowly added. The mixture was refluxed at 120 °C under N2 for about 8 h. After removal of the solvent, pure target compound was obtained by silica gel column chromatography (eluent: petroleum ether:acetone = 3:1; ν1) as a blue purple solid in 36.4% yield (102.4 mg). Elemental analysis (%): Found: C 77.20, H 6.20, N 6.20, O 7.36, B 1.61, F 5.71. Calcd (%) for C42H48BF2N4O: C 73.57, H 6.11, N 6.37, O 7.28, B 1.51, F 5.46. Calcd (%) for C47H48BF2N5O: C 77.15, H 6.61, N 9.57, B 1.48, F 5.10. m/z (ESI-MS) 732.74 (Calcd for C42H48BF2N4O: [M+H]+ = 732.70). IR (KBr) νmax cm⁻¹: 3205, 2922, 2848, 2801, 1594, 1537, 1362, 1254, 1161, 989, 950, 820, 761, 689. 1H NMR (400 MHz, CDCl3) δ ppm: 7.59 (s, 1H), 7.55‒7.50 (m, 5H), 7.47‒7.43 (d, J = 8 Hz, 2H), 7.31‒7.29 (d, J = 8 Hz, 2H), 7.17 (s, 1H), 7.14‒7.12 (m, 3H), 6.98‒6.90 (d, J = 4 Hz, 1H), 6.73‒6.67 (d, J = 8 Hz, 4H), 6.60 (s, 2H), 3.79 (s, 2H), 3.65 (s, 2H), 3.04 (s, 12H), 2.96‒2.93 (t, J = 6 Hz, 2H), 2.81‒2.78 (t, J = 6 Hz, 2H), 1.46 (s, 6H).

Results and Discussion

Synthesis

Novel meso-morpholine and meso-1,2,3,4-isouquinoline substituted BODIPYs (2a and 2b) were prepared from BODIPY I by modifying the substituent on the meso-carbon (Scheme 1). The methyl group protons of 2a and 2b are sufficiently acidic to undergo Knoevenagel condensation reactions.[13,14] The introduction of styryl groups to form 3a-d is one of the main strategies for shifting the main spectral bands into the biological window.[2]

Optical Spectroscopy

When a comparison is made with the main absorption band of the parent meso-phenyl-BODIPY complex at ca. 500 nm,[2] it becomes clear that only very minor spectral changes are observed for 2a and 2b (Figure 1), since the main absorption bands lie at 503 and 502 nm, respectively. This is the pattern that would be anticipated since the meso-substituent lies orthogonal to the BODIPY core for steric reasons. When further structural changes are made to form 3b-d, the extent of the observed red shift is proportional to the electron donor ability of the styryl groups.[2] The main absorption bands of the meso-morpholine substituted 3a and 3b dyes lie at 642 and 693 nm (Figure 1), respectively, with a larger red shift observed when p-N,N-dimethylaminophenyl-styryl groups are introduced. When the meso-substituent is changed to 1,2,3,4-isouquinoline, there are only minor spectral changes, since the main spectral bands of 3c and 3d lie at 641 and 696 nm (Figure 1). 2a-b, and 3c-d exhibit moderately intense fluorescence in THF at room temperature (Table 1), while those of morpholine substituted distyrylBODIPYs 3a and 3b are significantly lower. The fluorescence lifetimes values are consistent with those that have been reported previously for structurally similar 3,5-distyrylBODIPY dyes,[15] which has methoxyphenylstyryl groups at the 3,5-positions and a meso-1,2,3,4-isouquinoline substituent, was found to have the highest
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fluorescence quantum yield value. The most likely explanation for the increase relative to 3d is that there is less intramolecular charge transfer character in the S1 state than is the case when p-N,N-dimethylaminophenylstyril groups are present. Larger Stokes shifts are observed for meso-1,2,3,4-isoquinoline substituted BODIPYs 3c-d than for 3a-b, due to changes in the structural dynamics in the excited states.

Theoretical Calculations

The observed spectral changes can be readily explained through a comparison with the results of TD-DFT calculations (Figure 2 and Table 2) at the CAM-B3LYP/6-31G(d) level of theory for B3LYP optimized geometries of the BDY model compound, 1, 2a-b and 3a-d, since similar trends are observed in the energies of the main visible region spectral bands. In each case the HOMO → LUMO transition provides the predominant contribution to the lowest energy transition (Table 2). As has been reported previously,[2,15,16] the red shift that is caused by the introduction of electron donating styryl groups at the 3,5-positions of the BODIPY core is related to a relative destabilization of the HOMOs of 3a-d (Figure 3), since the HOMO has larger MO coefficients at this position in the HOMO than in the LUMO resulting in a greater mesomeric interaction with the π-system of the styryl group that is co-planar with that of the BODIPY core. This results

Table 1. Spectroscopic data of 2a-b and 3a-d.

<table>
<thead>
<tr>
<th></th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt; (max/nm)</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt; (max/nm)</th>
<th>Stokes shift (cm&lt;sup&gt;–1&lt;/sup&gt;)</th>
<th>ε&lt;sup&gt;×10&lt;sup&gt;4&lt;/sup&gt;&lt;/sup&gt; (L·mol&lt;sup&gt;–1&lt;/sup&gt;·cm&lt;sup&gt;–1&lt;/sup&gt;)</th>
<th>τ (ns)</th>
<th>Φ&lt;sub&gt;F&lt;/sub&gt; (%)</th>
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<tr>
<td>2a</td>
<td>503</td>
<td>512</td>
<td>349</td>
<td>7.5</td>
<td>2.30±0.01</td>
<td>0.42±0.02</td>
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<tr>
<td>2b</td>
<td>503</td>
<td>512</td>
<td>349</td>
<td>8.3</td>
<td>2.29±0.01</td>
<td>0.48±0.02</td>
</tr>
<tr>
<td>3a</td>
<td>642</td>
<td>661</td>
<td>448</td>
<td>9.7</td>
<td>4.05±0.02</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>3b</td>
<td>641</td>
<td>656</td>
<td>357</td>
<td>9.9</td>
<td>4.03±0.01</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>3c</td>
<td>698</td>
<td>753</td>
<td>1046</td>
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<td>2.26±0.01</td>
<td>0.41±0.02</td>
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<tr>
<td>3d</td>
<td>696</td>
<td>743</td>
<td>909</td>
<td>7.9</td>
<td>2.29±0.01</td>
<td>0.34±0.03</td>
</tr>
</tbody>
</table>
Table 2. Calculated wavelengths (λ), oscillator strengths (f) and the related wavefunctions of BDY, 2a-b and 3a-d at the CAM-B3LYP/6-31G(d) level of theory.

<table>
<thead>
<tr>
<th>State</th>
<th>λ_{cal} (nm)</th>
<th>λ_{exp} (nm)</th>
<th>f</th>
<th>Wavefunction</th>
</tr>
</thead>
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<tr>
<td>BDY</td>
<td>411</td>
<td>503</td>
<td>0.54</td>
<td>H → L (97%)</td>
</tr>
<tr>
<td>1</td>
<td>412</td>
<td>–</td>
<td>0.53</td>
<td>H → L (97%)</td>
</tr>
<tr>
<td>2a</td>
<td>411</td>
<td>503</td>
<td>0.52</td>
<td>H → L (97%)</td>
</tr>
<tr>
<td>2b</td>
<td>411</td>
<td>502</td>
<td>0.52</td>
<td>H → L (97%)</td>
</tr>
<tr>
<td>3a</td>
<td>539</td>
<td>642</td>
<td>1.01</td>
<td>H → L (96%)</td>
</tr>
<tr>
<td>3b</td>
<td>562</td>
<td>698</td>
<td>1.10</td>
<td>H → L (95%)</td>
</tr>
<tr>
<td>3c</td>
<td>539</td>
<td>641</td>
<td>1.00</td>
<td>H → L (96%)</td>
</tr>
<tr>
<td>3d</td>
<td>562</td>
<td>696</td>
<td>1.09</td>
<td>H → L (95%)</td>
</tr>
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</table>


Figure 3. The MO energies of the BDY model compound, 1, 2a-b and 3a-d calculated at the CAM-B3LYP/6-31G(d) level of theory (BOTTOM). The angular nodal patterns of the HOMO and LUMO of BDY and 3a (TOP).
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in a narrowing of the HOMO–LUMO gap and hence a large red shift of the main spectral bands.

**Electrochemistry**

In order to gain further insight into the electronic structures of 2a-b and 3a-d, cyclic and differential pulse voltammetry (CV and DPV) measurements were carried out in o-dichlorobenzene (o-DCB) containing 0.1 M tetra-n-butylammonium perchlorate (TBAP) as a supporting electrolyte (Figure 4), so that redox potential ($E_{1/2}$) values could be derived from both CV and DPV measurements. The electrochemical properties of 2a-b are similar to those reported previously for other BODIPYs, with a reduction step observed at −1.14 V. When electron-donating p-methoxyphenylstyril and p,N,N-dimethylaminophenylstyril groups are introduced at the 3,5-positions, two reversible processes are observed at −0.93 and −1.68 V for 3a and −1.01 and −1.68 V for 3b. 1,2,3,4-Isoquinoline substituted BODIPYs 3c-d exhibit slight negative shifts in the reduction values at −1.00 and −1.76 V for 3c, and −1.02 and −1.77 V for 3d. The decrease in the first oxidation potentials of 3a-d relative those of 2a-b is consistent with what would be anticipated based on the destabilization of the HOMO in the theoretical calculations (Figure 3).

**Interaction with ctDNA**

BODIPYs 3a-b are suitable for use as “turn-on” type fluorescence enhancing bioimaging agents, because the morpholine moieties enhance their lipophilicity. The complex formed between ethidium bromide (EB) and ctDNA (ctDNA-EB) has an intense fluorescence band at 602 nm

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**Figure 4.** CV and DPV measurements of 2a-b and 3a-d in o-dichlorobenzene (o-DCB) containing 0.1 M TBAP.
Upon addition of 3a-b, the fluorescence intensity is significantly decreased due to the interaction between 3a-b and ctDNA. According to the Stern-Volmer equation \( F/F_0 = 1 + K_{sv}[Q] \), \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of the quencher, respectively, \( K_{sv} \) is the linear Stern–Volmer quenching constant, and \([Q]\) is the concentration of the quencher. 3a and 3b were found to have binding constants of 7130 and 7380 M\(^{-1}\), respectively, on this basis.

**Living Cell and Mitochondrial imaging**

After treatment with HepG-2 cells for 4 h at 37°C and washing with PBS solution, it can be clearly demonstrated that 3b passed through the cell membrane and exhibited strong fluorescence inside the cell. Thus, BODIPY 3b is suitable for use as a cell imaging agent. The targeting properties of the BODIPY complexes were analyzed by comparing the imaging effects of the 3b complexes with those of Mito Tracker Green FM on mitochondria (Figure 6). The red fluorescence images overlap completely with the green fluorescence images of Mito Tracker Green FM, indicating that BODIPY 3b can target mitochondria.

**Cytotoxicity**

3a-d were studied for antitumor activity in vitro by determining the inhibition of the growth of HepG-2 cells using MTT assays (Figure 7). The evaluation of the cytotoxicity of 3a-d at low concentrations (25 μM) suggests that the cell viability values of meso-morpholine substituted 3a-b are lower than those for the meso-1,2,3,4-isouinoline substituted 3c-d compounds, since the 1,2,3,4-isouinoline group readily reacts with oxygen and/or nitrogen containing compounds in the cell to enhance the cell toxicity. Upon increasing the concentration of 3a-d in the same solutions to 50, 75 and 100 μM, the cell viability ratio steadily decreases as would be anticipated on this basis. 3a-b have lower IC\(_{50}\) values for HepG-2 cell inhibition than 3c-d.

**Conclusions**

A series of 3,5-distyryl BODIPY dyes have been prepared that fluoresce in the biological window and are hence potentially suitable for use as cellular imaging agents. The meso-1,2,3,4-isouinoline-substituted BODIPYs 3a-d interact strongly with DNA and this enhances their ability...
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Figure 7. Inhibition activities of BODIPY derivatives 3a-d on the proliferation of HepG-2 cells. HepG-2 cells in the absence of the compounds was used as the control.

to act as bioimaging agents. In contrast, the meso-morpholine-substituted BODIPYs 3a-d were found to have slightly higher cell toxicity. Considering low toxic bio-inspired fluorescent molecules have a wide range of application, this study may enhance the understanding of the electronic structure of π-extended BODIPYs and their rational molecular design towards efficient biological applications.

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References


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