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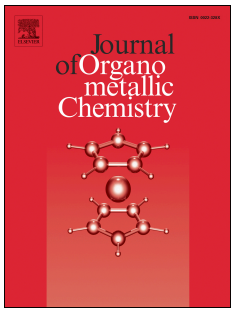

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Synthesis, characterization, photophysical properties of new fluorescent boron Schiff bases (BOSCHIBAs) and their application as cytoplasm staining dyes *in vitro*.

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Abstract

In this paper, we report a series of Schiff bases synthesized by the condensation reaction between 2-hydroxy-1-naphthaldehyde with aniline derivatives as well as their further coordination with diphenylborinic acid for giving the corresponding boron Schiff bases (BOSCHIBAs) (*E*)-N-((2-((di-phenylboryl)oxy)naphthalen-1-yl)methylene)pyridin-3-amine (**1a**), (*E*)-N-((2-((di-phenylboryl)oxy)naphthalen-1-yl)methylene)-2,6-di-methylaniline (**2a**), and (*E*)-N-((2-((di-phenylboryl)oxy)naphthalen-1-yl)methylene)-2,6-di-isopropylaniline (**3a**). The resulting BOSCHIBAs were characterized by NMR (^{1}H, ^{13}C, and ^{11}B), FT-IR, and atmospheric pressure ionization time-of-flight mass spectrometry. BOSCHIBA**1a** was structurally characterized by single-crystal. Structure analysis indicates that the boron atom adopts a tetrahedral molecular geometry into a six-membered ring with a half-chair conformation. BOSCHIBAs **1a**-**3a** and their ligands **1**-**3** exhibit relatively low quantum yields in a range from 1 to 3 %. In a bioimaging study by using the BOSCHIBAs **2a**, and **3a** as fluorescent staining dyes, those materials showed enhanced features in terms of low cytotoxicity, simple synthesis, photostability, hydrolytic stability and specific staining for cytoplasm structures.

Keywords: BOSCHIBAs, cytoplasm staining, low cytotoxicity, high photostability, fluorescent bioimaging.

Introduction

Nowadays fluorescence bioimaging (FBI) based on small organic molecules have gained tremendous attention as an indispensable tool in cellular biology and biomedical research because it provides a unique approach for the visualization of cell internal structures with morphological details in tissue at a subcellular resolution level. Hence, FBI might advance knowledge of cellular biology and disease at the molecular level: both *in vitro* and *in vivo* [1]. Nevertheless, this technique is not invasive, and it is revolutionizing the biomedical research and clinical practice [2].

On the flip side, the cytoplasm is a fluid thicker than water which contains all the cell organelles and it provides mechanical support to the internal cell structures. Moreover, the most important activities into the cell occur in the cytoplasm which include metabolic process, breakdown of waste, glycolysis, and cell division. In this sense, recently fluorescent cytoplasm markers have gained much interest since they can associated with the diseases and abnormal cellular functions [3]. As examples of fluorescence cytoplasm markers in the literature, there are reported antibodies, nanoparticles [4], quantum dots [5,6], lanthanide complexes [7], and small organic molecules such as organoboron compounds [8]. All these fluorophores must cover some important features for practical analyses which include solubility, low cytotoxicity, photostability, and degradability. In particular, the cytotoxicity, and photostability are two important parameters to be measurement before thinking about the practical purpose or application. It is remarkable that in recent years there has been considerable curiosity in the synthesis of organoboron compounds in areas such as supramolecular chemistry [9], medicinal chemistry [10], materials chemistry [11], imaging materials [12], organic light-emitting diodes [13],

organic field effect transistors [14], and photo-responsive materials [15,16]. Specially, the responsiveness has increased to those boron Schiff bases (BOSCHIBAs) due to they provide an interesting variety of molecular structural conformations and while electron withdrawing imine group (C=N) interacts with metal ions giving rise to materials with different optoelectronic properties [17] and several new applications [18]. Furthermore, bidentate Schiff bases could act as fluorescent molecular sensors for detection of toxic heavy metals in living cells by confocal microscopy [19,20]. In the literature there are many reports of the synthesis of BODIPY type boron compounds which have shown to be useful in FBI. These compounds display adequate permeability through cell membrane and it diffuses within the cytoplasm [21,22]. Likewise, to get FBIs *in vitro* or *in vivo* for practical situations, the photostability under UV-Vis light irradiation plays an important role. With the purpose to avoid the photobleaching, some strategies have been reported by the addition of antifading compounds, organic antioxidants, triplet state quenchers [23], and micrometer-sized polydimethylsiloxane wells [24]. However, it has scarcely been studied to use the bulky groups in organic dyes to reduce the photobleaching effect [25]. We have recently reported that mononuclear and binuclear BOSCHIBAs (Figure 1) [26] exhibit a low cytotoxicity level which is good for potential biomedical application but they have also a poor capacity for staining cells with a low quantum yield (Φ_F~1%) attributed to the boron atom is outside the ligand-plane.

Keeping this in mind, we designed new fluorescent BOSCHIBAs where the π-system delocalization was increased by the addition of the naphthyl group [27] and the photo-stability was also increased by the use of bulky groups (Scheme 1). All BOSCHIBAs **1a**-**3a** and their free ligand**1**-**3** were fully characterized by NMR (^{1}H, ^{11}B, and ^{13}C), ATR-

IR spectroscopy, and atmospheric pressure ionization time-of-flight mass spectrometry. In the case of BOSCHIBA **1a**, it was characterized by X-ray diffraction.

Figure 1. Here

Experimental Section

General marks

All starting materials were procured from Aldrich Chemical Company. Solvents were used without further purification. The diphenylborinic acid (Ph_2BOH) was prepared *in situ* as previously reported in the literature [28]. Melting points were confirmed by using an Electrothermal Mel-Temp apparatus. Infrared spectra were recorded using a Bruker Tensor 27 FT-IR spectrophotometer equipped with a Pike Miracle^{TM} ATR accessory with single reflection ZnSe ATR crystal. ^{1}H, ^{13}C, and ^{11}B NMR spectra were recorded in $CDCl_3$ on a Bruker advance DPX 400. Chemical shifts (ppm) are relative to $(CH_3)_4Si$ for ^{1}H and ^{13}C. ^{11}B NMR spectra were referenced externally to $BF_3{\cdot}OEt_2$. Atmospheric pressure ionization time-of-flight mass spectrometry(APCI-TOF-MS) in positive ion mode was acquired on an Agilent Technologies instrument.

X-ray crystallography

The X-ray crystallography data for **1a (**CCDC: 1520512**)**were measured at 100(2) K on a Bruker D8 Quest with a Photon 100 CMOS detector equipped with an Oxford Cryosystems 700 series cooler, a Triumph monochromator, and a Mo Kα fine-focus sealed tube (λ = 0.71073 Å). Intensity data were processed using the Bruker Apex II program

suite. All the calculations for the structure determination were carried out using the SHELXTL package (version 6.14). Initial atomic positions were located by direct methods using XS, and the structures of the compounds were refined by the least-squares method using SHELXL. Absorption corrections were applied by using SADABS. All the non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed in idealized positions and refined as riding atoms with relative isotropic displacement parameters.

Optical measurements

UV-Vis absorption spectra were measured on a UV spectra were obtained with a Perkin Elmer Lambda 365 UV/Vis spectrophotometer. The emission spectra have been recorded with a Fluorolog-3 research spectrometer, by exciting 10 nm below the longer wavelength absorption band. Fluorescence quantum yields (Φ_F) in solution were determined according to the procedure reported in the literature [29] and using quinine sulfate in H_2SO_4 0.1M (Φ= 0.54 at 310 nm) as the standard. Temperature was regulated at 25.0+0.5 °C with a water circulating bath. Three solutions with absorbance at the excitation wavelength lower than 0.1 were analyzed for each sample and the Φ_F was averaged.Φ_F measurements were measured by the relative method and the quantum yield of the unknown, Φ_x, is calculated according to the following equation:

$$\Phi_x = \Phi_R . \frac{A_R}{A_x} . \frac{E_x}{E_R} . \frac{I_R}{I_x} . \frac{n_x^2}{n_R^2} \qquad (\mathbf{1})$$

where Φ_R is the quantum yield of the standard, A is the absorbance of the solution, E is the corrected emission intensity, *I* is the relative intensity of the exciting light and n is the average refractive index of the solution. Subscripts R and X refer to the reference and unknown compound, respectively.

Photostability testing and stability in aqueous DMSO solutions

With the purpose to perform stability assay, BOSCHIBAs **1a-3a** were adjusted to give absorption of 0.5 u.a at the short wavelength absorption band. The samples were illuminated with 1.2 mW/cm^2 (365 nm) for 60 min with interval of 10 min at room temperature with air atmosphere. Each absorption spectrum was measured by using a Perkin Elmer Lambda 365 UV/Vis spectrophotometer in the region of 190-700 nm. Likewise, the stability in aqueous solutions at 1% v/v of DMSO was evaluated under the same experimental condition without photoirradiation. Both analyses are critical to assess how stable the dye is during imaging applications discussed later.

Cytotoxicity Assays

B16F10 murine melanoma cells (ATCC CRL-6475, Manassas, VA) were used to determine the cytotoxic effects of **1a**-**3a** complexes. Cells were maintained in GIBCO-DMEM/F12 culture media supplemented with 10% FBS and 1X antibiotic-antimitotic (all from ThermoFisher Scientific, Waltham, MA), at 37°C in an atmosphere of 5% CO_2. For the experiments cells were plated in 96-wells plates at a cell density of 2000 cells per well in 100 μL of media and let them undisturbed overnight before the treatments were added. Compounds were added at concentrations of 0.1, 1, 2.5, 5, and 10 μg/mL. Forty-eight hours later 10 μl of alamarBlue (Biosource Invitrogen Life Technologies, Carlsbad, CA) were added to each well to determine cell viability following manufacturer's instructions.

Bioimaging Assays

To assess the BOSCHIBAs capabilities to stain cells *in vitro*, B16F10 cells were plated at densities of $5x10^4$ cells per well in 500 μL of media on coverslips in 12 wells plates. After overnight incubation 10 μg/mL of each complex were added to each well and 2 hours later coverslips were mounted with Vectashield (Vector Laboratories, Inc. Burlingame, CA) and analyzed by confocal microscopy in a Leica TCS SP5 Confocal System at excitation wavelength of 405 nm and emission of 420-550 nm, or excitation of 488 nm and emission of 500-600 nm. DMSO treated cells were used as a control to determine endogenous fluorescence.

General procedure of synthesis of BOSCHIBAs 1a-3a and Schiff bases 1-3.

(E)-1-((pyridin-3-ylimino)methyl)naphthalen-2-ol (1)

A homogeneous mixture of 2-hydroxy-1-naphthaldehyde (0.50 g, 2.91 mmol) with 3-aminopyridine (0.27 g, 2.91 mmol) in acetonitrile was heated under reflux for 48 h. The reaction mixture was slowly cooled at room temperature and the precipitated product was filtrated and washed with hexane to give 0.57 g (2.30 mmol, 79 % yield) of **1** as a yellow solid. M.P.: 182° C; FT-IR$_{\upsilon max}$ (cm^{-1}): 3048, 1624 (C=N), 1562, 1484; UV/Vis (THF): $\lambda_{abs/max}$ (nm), [$\varepsilon_{max}*10^4$ ($M^{-1}cm^{-1}$)]: 377 [0.10], 322; Fluorescence ($CHCl_3$): λ_{fluor} (nm): 490; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.16 (1H, d, , ^3J = 8.0 Hz, H-3), 7.37 (2H, m, , H-7, H-14), 7.54 (1H t, ^3J = 16 Hz, H-8), 7.65 (1H, d, ^3J = 8.0 Hz, H-13), 7.75 (1H, d, ^3J = 8.0 Hz, H-6), 7.84 (1H, d, ^3J = 12 Hz, H-4), 8.13 (1H, d, ^3J = 8.0 Hz, H-9), 8.54 (1H, d, ^3J = 8.0 Hz, H-15), 8.63 (1H, d, ^3J = 4.0 Hz, H-17), 9.42 (1H, s, H-11), 14.93 (1H, s, OH); ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$) δ (ppm): 109.42 (C-1), 119.22 (C-9), 120.68 (C-3), 123.96

(C-14), 124.09 (C-7), 127.80 (C-13), 128.35 (C-8), 129.53 (C-6), 132.94 (C-10), 136.52 (C-4), 142.99 (C-17), 143.75 (C-12), 147.76 (C-15), 158.70 (C-11), 166.20 (C-2); $^{1}H/^{13}C$ HETCOR NMR δ (ppm): 7.16/120.54 (H-3/C-3), 7.36/123.84 (H-14/C-14), 7.54/128.21 (H-8/C-8), 7.64/127.66 (H-13/C-13), 7.75/129.41 (H-6/C-6), 7.84/136.39 (H-4/C-4), 8.12/119.09 (H-9/C-9), 8.54/147.64 (H-15/C-15), 8.64/142.89 (H-17/C-17), 9.42/158.57 (H-11/C-11); $^{1}H/^{1}H$ COSY NMR δ (ppm):7.89/7.15 (H-4/H-3), 7.55/7.38 (H-8/H-7), 7.64/7.38 (H-13/H-14), 7.73/7.38 (H-6/H-7), 8.12/7.54 (H-9/H-8), 8.54/7.38 (H-15/H-14).

(E)-1-(((2,6-dimethylphenyl)imino)methyl)naphthalen-2-ol (2)

Preparation of **2** was accomplished like that of **1** from 2-hydroxy-1-naphthaldehyde (0.50 g, 2.91 mmol) with 2,6-dimethylaniline (0.35 g, 2.91 mmol). The product was obtained as a yellow solid with yield of 75 % (0.60 g, 2.18 mmol). M.P.: 118° C. ATR-$IR_{\upsilon max}$: 3074, 2990, 2948, 1613 (C=N), 1573, 1161 cm^{-1}; UV/Vis (THF): $\lambda_{abs/max}$ (nm), [$\varepsilon_{max}*10^{4}$ ($M^{-1}cm^{-1}$)]: 364 [0.58], 315; Fluorescence ($CHCl_3$): λ_{fluor} (nm): 473;^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.13 (1H, d, ^{3}J = 8.0 Hz, H-3), 7.20 (3H, m, H-14, H-15, H-16), 7.36 (1H, t, ^{3}J = 16.0 Hz, H-7), 7.51 (1H, t, ^{3}J = 16.0 Hz, H-8), 7.77 (1H, d, ^{3}J = 8 Hz, H-6), 7.86 (1H, d, ^{3}J = 8.0 Hz, H-4), 8.00 (1H, d, ^{3}J = 8.0 Hz, H-9), 2.34 (6H, s, H-18, H-19), 9.14 (1H, s, H-11), 15.24 (1H s, OH); ^{13}C NMR {^{1}H}(100 MHz, $CDCl_3$) δ (ppm): 18.77 (C-18,C-19), 108.49 (C-1), 118.85 (C-9), 121. 61 (C-15), 123.50 (C-7), 125.70 (C-3), 127.41 (C-5), 128.14 (C-8), 128.70 (C-14, C-16), 129.39 (C-6), 129.70 (C-13, C-17), 133.27 (C-10), 136.06 (C-4), 145.86 (C-12), 161.32 (C-11), 168.02 (C-2); $^{1}H/^{13}C$ HETCOR NMR δ(ppm): 2.31/18.60 (H-18,19/C-18,19), 7.14/125.66 (H-3,/C-3), 7.20/128.69 (H-14, 16/C-14, 16), 7.23/121.56 (H-15/C-15), 7.37/123.46 (H-7/C-7), 7.52/128.10 (H-8/C-8),

7.79/129.36 (H-6/C-6), 7.87/136.04 (H-4/C-4), 8.01/118.79 (H-9/C-9), 9.16/161.27 (H-11/C-11); ^{1}H/^{1}H COSY NMR δ (ppm):7.86/7.13 (H-4/H-3), 7.77/7.38 (H-6/H-7), 7.51/7.38 (H-8/H-7), 7.99/7.51 (H-9/H-8).

(E)-1-(((2,6-diisopropylphenyl)imino)methyl)naphthalen-2-ol (3)

Preparation of **3** was accomplished like that of **1** from 2-hydroxy-1-naphthaldehyde (0.5 g, 2.91 mmol) with 2,6-diisopropylaniline (0.51 g, 2.91 mmol). The product was obtained as a yellow solid with yield of 73 % (0.70 g, 2.12 mmol). M.P.: 192° C. AT-IR$_{\upsilon max}$ cm^{-1}: 2980, 2964, 2943, 1620 (C=N), 1575, 1172, UV/Vis (THF): $\lambda_{abs/max}$ (nm), [ε_{max}*10^{4} (M^{-1}cm^{-1})]: 362 [0.71], 315; Fluorescence ($CHCl_3$): λ_{fluor} (nm): 477; ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.13 (12H, d, ^{3}J = 8.0 Hz, H-19, H-20, H-22, H-23), 3.02 (2H, septet, ^{3}J = 28.0 Hz, H-18, H-21), 7.10 (1H , d, ^{3}J = 8.0 Hz, H-3), 7.15 (3H, m, , H-14, H-15, H-16), 7.24 (t, 1H, ^{3}J = 16 Hz, H-8), 7.39 (1H, t, ^{3}J = 16 Hz, H-7), 7.66 (1H, d, ^{3}J = 8.0 Hz, H-6), 7.76 (1H, d, ^{3}J = 8.0 Hz, H-4), 7.89 (1H, d, ^{3}J = 8.0 Hz, H-9), 8.98 (1H, s, H-11), 15.13 (1H, s, OH); ^{13}C NMR {^{1}H}(100 MHz, $CDCl_3$) δ (ppm): 22.66 (C-19, C-20, C-22, C-23), 27.27 (C-18, C-21), 107.30 (C-1), 117.70 (C-9), 120.34 (C-3), 122.39 (C-8), 122.49 (C-14, C-16), 125.21 (C-15), 126.38 (C-5), 127.07 (C-7), 128.30 (C-6), 132.15 (C-10), 134.87 (C-4), 139.22 (C-13, C-17), 142.94 (C-12), 160.61 (C-11), 166.35 (C-2); ^{1}H/^{13}C HETCOR NMR δ(ppm): 1.09/22.54 (H-19, 20, 22, 23/C-19, 20, 22, 23), 3.01/27.14 (H-18, 21/C-18, 21), 7.10/120.31 (H-3/C-3), 7.14/122.43 (H-14, 16/C-14, 16), 7.15/125.17 (H-15/C-15), 7.23/122.34 (H-8/C-8), 7.38/127.05 (H-7/C-7), 7.65/128.26 (H-6/C-6), 7.74/134.83 (H-4/C-4), 7.88/117.65 (H-9/C-9) 8.98/160 (H-11/C-11); ^{1}H/^{1}H COSY NMR δ (ppm):7.75/7.09 (H-4/H-3), 7.38/7.25 (H-7/H-8), 7.38/7.65 (H-7/H-6), 7.88/7.25 (H-9/H-8);

(E)-N-((2-((diphenylboryl)oxy)naphthalen-1-yl)methylene)pyridin-3-amine (1a)

A solution of (*E*)-1-((pyridin-3-ylimino)methyl)naphthalen-2-ol **1** (0.1 g, 0.40 mmol) with diphenylborinic acid (0.18 g, 1.0 mmol) in acetonitrile was heated under reflux for 48 h. The reaction mixture was slowly cooled to room temperature, and the precipitated product was filtrated and washed with hexane followed by recrystallization in a chloroform/hexane mixture to give 0.16 g (0.39 mmol, 95%) of BOSCHIBA **3a** as a yellow crystalline solid. M.P.: 222° C; FT-IR υ_{max} (cm^{-1}): 2962, 1624, 1608(C=N), 1550, 1458, 1312, 1204, 1140, 892, 878, 825, 795, 745, 702 (O-B),623,597; UV/Vis (THF): $\lambda_{abs/max}$ (nm), [$\varepsilon_{max}*10^4$ ($M^{-1}cm^{-1}$)]: 425 [0.51], 337; Fluorescence ($CHCl_3$): λ_{fluor} (nm): 530; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.98 (1H, d, ^3J = 4 Hz, H-3), 7.09 (7H, m, H-13, 4H-*m*, 2H-*p*), 7.27 (2H, m, H-7, H-8), 7.36 (4H, d, ^3J = 8.0 Hz, H-8, 4H-*o*), 7.43 (1H, t, ^3J = 16 Hz, H-14), 7.59 (1H, d, ^3J = 8.0 Hz, H-6), 7.77 (1H, d, ^3J = 8.0 Hz, H-4), 7.81(1H, d, ^3J = 12 Hz, H-9), 8.33 (1H, d, ^3J = 4.0 Hz, H-15), 8.37(1H, s, H-17), 8.86 (1H, s, H-11); ^{13}C NMR {^{1}H}(100 MHz, $CDCl_3$) δ (ppm): 111.56 (C-1), 119.27 (C-9), 121.52 (C-3), 123.04 (C-7), 124.70 (C-13), 126.79 (C-14), 127.27 (C-*m*, C-*p*), 127.75 (C-5), 129.42 (C-8), 129.72 (C-6), 132.31 (C-10), 132.60 (C-4), 133.73 (C-*o*), 141.29 (C-*i*), 142.56 (C-17), 144.99 (C-12), 148.82 (C-15), 157.75 (C-11), 165.96 (C-2); ^{11}B NMR (128 MHz, $CDCl_3$) δ (ppm): 7.38; APCI-TOF-MS in positive ion mode calc. for [($C_{28}H_{21}N_2O_4B$+H)$^+$]: 413.1700 u.m.a; Exp.: 413.1822 u.m.a

(E)-N-((2-((diphenylboryl)oxy)naphthalen-1-yl)methylene)-2,6-dimethylaniline (2a)

Preparation of BOSCHIBA **2a** was accomplished like that of **1a** from Schiff base **2** (0.1 g, 0.36 mmol) with diphenylborinic acid (0.17 g, 0.93 mmol). The product was

obtained as a yellow solid with a yield of 92 % (0.14 g, 0.32 mmol); M.P.: 212° C; FT-IR$_{\upsilon max}$ (cm^{-1}): 2964, 1624, 1605(C=N), 1549, 1458, 1312, 1205, 1140, 1000, 892, 879, 825, 796, 745, 702 (O-B), 635; UV/Vis (THF): $\lambda_{abs/max}$ (nm), [ε_{max}*10^4 (M^{-1}cm^{-1})]: 400 [0.39], 327; Fluorescence ($CHCl_3$): λ_{fluor} (nm): 480; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.82 (1H, d, ^3J = 8.0 Hz, H-3), 6.97 (7H, m, H-15, 4H-*m*, 2H-*p*), 7.16 (2H, d, ^3J = 12 Hz, H-14, H-16), 7.27 (1H, t, ^3J = 12 Hz, H-7), 7.32 (1H, d, ^3J = 8.0 Hz, 4H-*o*), 7.45 (1H, t, ^3J = 20 Hz, H-8), 7.67 (1H, d, ^3J = 8.0 Hz, H-6), 7.77 (1H, d, ^3J = 8.0 Hz, H-4), 7.86 (1H, d, ^3J = 8 Hz, H-9), 1.79 (2H, s, H-18, H-19), 8.64 (1H, s, H-11); ^{13}C NMR {^{1}H}(100 MHz, $CDCl_3$) δ (ppm): 18.12 (C-18, C-19), 110.45 (C-1), 118.22 (C-9), 120.72 (C-15), 123.20 (C-7), 125.16 (C-13), 125.47 (C-*m*, C-*p*), 126.59 (C-5), 126.70 (C-8), 127.47 (C-14, C-16), 128.00 (C-13, C-17), 128.53 (C-6), 132.10 (C-*o*), 132.67 (C-*i*), 133.67 (C-10), 138.88 (C-4), 144.01 (C-12), 159.89 (C-11), 163.70 (C-2); ^{11}B NMR (128 MHz, $CDCl_3$) δ (ppm): 4.76; APCI-TOF-MS in positive ion mode calc. for [($C_{31}H_{26}NO_4B$+H)$^+$]: 440.2100 u.m.a; Exp.: 440.2182 u.m.a

(E)-N-((2-((diphenylboryl)oxy)naphthalen-1-yl)methylene)-2,6-diisopropylaniline (3a)

Preparation of BOSCHIBA **3a** was accomplished like that of **1a** from Schiff base **3** (0.1 g, 0.30 mmol) with diphenylborinic acid (0.13 g, 0.71 mmol). The product was obtained as a green solid with a yield of 30 % (0.044 g, .091 mmol); M.P.: 200° C; FT-IR$_{\upsilon max}$ (cm^{-1}): 2967, 1623, 1607(C=N), 1547, 1456, 1343, 1312, 1206, 1141, 999, 894, 878, 825, 796, 745, 702 (O-B), 634; UV/Vis (THF): $\lambda_{abs/max}$ (nm), [ε_{max}*10^4 (M^{-1}cm^{-1})]: 398 [0.81], 329; Fluorescence ($CHCl_3$): λ_{fluor} (nm): 480;^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.13 (6H, d, ^3J = 8.0 Hz, H-22, H-23), 1.16 (6H, d, ^3J = 8.0 Hz, H-19, H-20,), 3.57 (2H,

septeto, ^{3}J = 28 Hz, H-18, H-21), 7.20 (1H, d, ^{3}J = 8.0 Hz, H-3), 7.29 (3H, m, , H-14, H-15, H-16), 7.35 (6H, m, 4H-*m*, 2H-*p*), 7.49 (1H, t, ^{3}J = 16 Hz, H-7), 7.54 (5H, m, H-8, 4H-*o*), 8.00 (1H, d, ^{3}J = 12 Hz, H-6), 8.14 (1H, d, ^{3}J = 12 Hz, H-4), 8.28 (1H, d, ^{3}J = 8.0 Hz, H-9), 8.90 (1H, s, H-11); ^{13}C NMR {^{1}H}(100 MHz, $CDCl_3$) δ (ppm): 23.40 (C-19, C-20, C-22, C-23), 28.28 (C-18, C-21), 108.82 (C-1), 119.12 (C-9), 121.42 (C-3), 124.13 (C-14, C-16), 124.80 (C-7), 127.31 (C-15), 128.04 (C-*m*, C-*p*), 129.05 (C-5), 129.37 (C-8), 129.79 (C-6), 130.87 (C-10), 132.74 (C-4), 135.16 (C-13, C-17),135.69 (C-*o*), 140.2234 (C-*i*), 144.33 (C-12), 161.06 (C-11), 163.21 (C-2); ^{11}B NMR (128 MHz, $CDCl_3$) δ (ppm): 4.42; APCI-TOF-MS in positive ion mode calc. for [$(C_{35}H_{34}NO_4B+H)^{+}$]: 496.2700 u.m.a; Exp.: 496.2807 u.m.a

1-naphthylmethyllidenebenzoilhydrazone-phenyl-boron(4a).

A solution of (E)-N'-((2-hydroxynaphthalen-1-yl)methylene)benzohydrazide (0.29 g, 1 mmol) and phenylboronic acid (0.122 g, 1 mmol) in acetonitrile were heated under reflux for 48 hours. The reaction mixture was slowly warmed to room temperature, the solvent is evaporated and adding ethyl acetate, it was allowed to evaporate slowly at 10 days, giving a yellow solid. Yield of 0.28 g (74%). M. P.: 152 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.09 (1H m,H-3), 7.33 (1H, m,H-15), 7.39 (1H, m, H-7), 7.43 (1H, m, H-m), 7.45 (2H,m,H-*o*, *p*), 7.52 (1H, t,H-14), 7.57 (1H, m, H-8), 7.67 (1H,m,H-16), 7.87 (2H, m, H4,H-6), 8.11 (1H,d,H9), 8.71 (1H,s,H-11); ^{13}C NMR {^{1}H}(100 MHz, DMSO-d_6) δ (ppm):110.43 (C1), 119.79 (C*p*), 121.03 (C4), 123.74 (C7), 127.16 (C5), 127.68 (C15), 128.32 (C3), 128.42 (C10), 128.76 (C14), 128.97 (C8), 129.27 (C6), 131.45 (C*m*), 131.53 (C13), 132.12 (C16), 137.47 (C9), 141.53 (C11), 155.61 (C2), 170.89 (C12).

Results and Discussion

Synthesis

The BOSCHIBAs **1a-3a** were obtained by condensation of diphenylborinic acid (synthesized *in situ*) with the corresponding free ligand under reflux in acetonitrile (Scheme 1). In order to carry out a comparative study of the photophysical properties, the corresponding Schiff bases **1-3** were also prepared by a condensation reaction between 2-hydroxy-1-naphthaldehyde with the corresponding aniline. All the BOSCHIBAs and their ligands were obtained in nearly quantitative yield (73 to 95%) with high atom economy (**1a**: 92; **2a**: 91, and **3a**: 93%) and low environmental E factor (**1a**: 0.05; **2a**: 0.09), except **3a**, that showed a poor chemical yield (30%) after the final purification step as well as a high E value (2.30) (See electronic supporting information). In general, the condensation reactions demonstrated to be a cheap, simple, faster, and reproducible synthetic route with a high atomic economy and low E values.

Scheme 1. Here

Spectroscopic and spectrometric characterization

The formation of the N→B coordinate bond for BOSCHIBAs **1a-3a** was evidenced in the first instance by Boron NMR spectroscopy (See electronic supporting information Figures S1-S3). For example, one broad signal can be observed in a range from 7.38 to 4.42 ppm, corresponding to tetracoordinated boron atoms respectively (See Table 1). In the ^{13}C NMR spectra of BOSCHIBAs **1a-3a**, the signals for C-11 are shifted to low frequencies (157.7-161.0 ppm) with respect to the Schiff bases **1-3** (158.7-161.3 ppm) owing to the

coordination to boron [30]. ^{1}H NMR spectra confirmed the formation of the Schiff bases **1-3** with simple signals in the range from 8.64 to 9.42 ppm which are typical for imine groups (See electronic supporting information figures S4-S21) [31]. In the solid state, the FT-IR spectral analysis showed the stretching vibration bands attributed to imine group (C=N) for BOSCHIBAs **1a-3a** which were shifted to lower wavenumbers in comparison with the free ligands **1-3**, demonstrating a decrease in strength when the new coordination bond is formed (See electronic supplementary information Fig. S22-S27) [32]. Atmospheric pressure ionization time-of-flight mass spectrometry(APCI-TOF-MS) analysis in positive ion mode for BOSCHIBA **1a-3a** confirm the molecular ion peak (**1a**: 413.1822, **2a**: 440.2183, and **3a**: 496.2807) which correspond to the expected molecular ion mass (See electronic supplementary information Fig. S28-S30). As a characteristic for BOSCHIBAs **2a**, and **3a**, both molecules show a fragment due to the loss of one ring of benzene bonded to boron atom as well as the formation of boratropylium ion is attributed to loss of a fragment from 86 Da to 33 Da. In addition **3a** shows a fragment at *m/z* 157 that is related to loss of 2,6-diisopropylaniline fragment from 175 Da (Scheme 2).

Table 1. Here

Scheme 2. Here

X-ray analyses.

The crystal structure for BOSCHIBA **1a** is represented in Figure 2. BOSCHIBA **1a** belongs to the monoclinic space group P21/*c*, and its crystal structure analysis reveals that contains a tetra-coordinated boron atom and a three-ring-fused skeleton with a N→B

coordination bond length of 1.632(1) Å where boron atom adopt typical tetrahedral geometry. According to Höpfl formula, the boron atom has a strong dative bond from nitrogen atom corroborated by a tetrahedral character equal to 94.35 % [34]. The bond length of B-O is 1.505(1) Å, which is similar to those of the organoboron complexes reported previously [35,36]. The imine bond length for **1a** is 1.310 (2) Å, a value closer to that single bonds due to the formation of new N-B coordination bond. Moreover, the imine bond shows a configuration *E* with regard to the naphthalene group due to the quelate ring formation. Figure 3 exhibits the formation of a dimer through [C-H(13)···π-system(N-2) 2.5912 Å] as well as the formation of a 1D chain through [C-H(21)···π-system(O-2) intermolecular interactions. BOSCHIBA **1a** has a non-planar structure which is deduced from the dihedral angle (41.08°) calculated between naphthalene and pyridine rings. An aspect of relevance of the structure **1a** is the deviation (θ) of the boron atom from the naphthalenimino-plane that has a value equal to 0.336 Å, which is less than those previously reported by our group research. In this study, we found that boron compounds derivatives from salicylidenebenzohydrazide have great θ from the salicylidenimino-plane with values in a range from 0.488 to 0.749 Å [37]. Most likely this parameter could increase the luminescence properties as it has been reported for fluorescent borinates derived from benzoxazoles and benzothiazoles [38]. In those structures, the authors found two phenyl groups attached to the boron atom affectively keep luminescent rigid-fused π-conjugated skeletons apart, making these molecules highly emissive.

Figure 2. Here

Figure 3. Here

Table 2. Here

Photophysical properties.

The optical properties of BOSCHIBAs **1a-3a** and their free ligands **1-3** were obtained in spectroscopic grade tetrahydrofuran (Table 3), as it is a common solvent that dissolves all molecules reported here. Figure. 4 shows the electronic absorption spectra of BOSCHIBAs **1a-3a**, and (Inset) those corresponding ligands. The BOSCHIBAs **1a-3a** show a main absorption band in a range from 328 to 341 nm attributed to the π-π* electronic transition through the molecule. Moreover by making comparison with their corresponding free ligands, this π-π* intraligand electronic transition is observed in the BOSCHIBAs at longer wavelengths relative likely because a less distortion of the naphtyl imine system after complexation (torsion angle of C12-N1-C11-C1 is 171.91^{o} (9)). When the BOSCHIBAs are formed (Figure 4), the bands are red-shifted due to the larger electronic delocalization because of the formation of the imine system, in according to the displacement observed for H-11 protons in ^{1}H NMR analysis (Table 1).

Figure. 4 Here

The fluorescence spectra of the BOSCHIBAs **1a-3a** and their free ligands are shown in the figure. 5. BOSCHIBAs **2a** and **3a** show a broad blue emission bands at 492, and 493 nm without an apparent change in the fluorescence emission maximum with respect to their

free ligands. In fact, both emission wavelengths are practically identical which indicates that the addition of bulky substituents can not tuned the emission wavelength in the visible spectrum (See table 3).Conversely, BOSCHIBA **1a** presents a broad band centered at 528 nm with a moderated red shift of 92 nm with respect its free ligand. This change implies that the complexation with boron affects the geometry in the excited state of the naphthalene fluorophore when it is substituted with a heterocyclic compound. An interesting aspect is that the emission can be tuned from the blue (**1**) to the green **(3a**) regions by changing from free ligand to complex. As a general remark, the fluorescence quantum yield (Φ_F) is very weak for all molecules, except **3a**, that exhibits a moderate increase of 3%. It is important to mention that **1a** showed the highest Φ_F might be the coplanarity of heterocyclic system. In all cases, large Stoke's shift ($\Delta\nu$) indicates that the geometry of the molecule changes dramatically after excitation and non-radiative losses are probably due to an internal conversion process, according to the previously reported studies by our research group [36] and could also be related to the low Φ_F measured for all molecules under our experimental conditions.

Figure 5. Here

Table 3. Here

Photostability testing and stability in aqueous DMSO solutions

For bioimaging applications, it is desirable that the molecules demonstrate that the light exposure does not change their chemical-optical properties. The photostability curves

for the BOSCHIBAS **1a-3a** are given in the electronic supplementary information (Fig. S31-S33). Figure 6 shows the degradation plots of BOSCHIBAs at different exposure time under UV light irradiation at 365 nm. Based on our strategy of bulky groups, the molecules were irradiated at 365 nm under ambient temperature with air atmosphere by time intervals from 10 to 60 min, and they were monitored by UV spectroscopy. BOSCHIBA **1a**, without bulky organic groups, shows a degradation of less than 15% at 20 min, affording the free ligand **1** after the light exposure. In the case of BOSCHIBAs **2a** and **3a**, with bulky groups, an insignificant degradation is observed after being exposed for 40 min at the same wavelength. The spectral analysis revealed that there are not remarkable changes in the main absorption bands for **2a** and **3a** which suggests that chemical modification using bulky groups increase the photostability. In order to compare the effect of bulky groups on the photostability of BOSCHIBAs **1a-3a,** a BOSCHIBA identified as **4a** with an unsubstituted phenyl group was synthesized (Scheme 1). Photo-stability studies, performed at 365 nm, revealed that **4a** shows a slightly lower resistance to degradation (~10% at 40 min) in comparison with BOSHIBAs **2a** and **3a** (~5% at 40 min) when they are exposed at this long wavelength. Therefore according with the behavior of experimental data measured, the inclusion of a benzene ring and its substitution with bulky groups increase the resistance to degradation compared with pyridyl group which is less stable and more reactive than benzene (Figure 6). Likewise, the stability in aqueous solutions at 1% v/v of DMSO for BOSCHIBAs were evaluated under the same experimental condition without photoirradiation. All the BOSCHIBAs **1a-3a** demonstrated to be highly stable in aqueous DMSO solution after 60 min because there were not any important changes in the absorption peaks (See electronic supporting information Fig. S35 and S36).

Figure 6. Here

Cytotoxicity assay

The cytotoxicity of BOSCHIBAs **1a**-**3a** were assessed by reassuring assay, using DMSO as solvent at different concentrations (0.1 to 10 μg/mL) for 24 hours (Figure 7). Following incubation of 0.1 μg/mL for 24 hours, it is observed that less than 9% of B16F10 cells died. The same happened when the concentration is increased to 2.5 μg/mL. When the concentration of 1a was increased to 10 μg/mL, cell viability remained above 60 %, while for **2a** and **3a** it is above 50%. Therefore, BOSCHIBAs **1a**-**3a** showed relatively low cytotoxicity in the concentration range and incubation times examined in this study. In general, the cytotoxicity, cell-permeable characteristics of fluorescent materials are critical to the bio-imaging application, since the analysis of the viability of cytotoxicity showed BOSCHIBAs are not toxic to the corresponding cells.

Figure 7. Here.

Bioimaging cells.

The capacity of BOSCHIBAs **1a-3a** to produce fluorescent staining in cells was tested on B16F10 cells. Figure 8 shows on the top row that DMSO treated cells do not fluoresce at either wavelength (Figure 8-B and 8-C). When cells were treated with **1a** a very weak blue cytoplasm fluorescent stain was observed (Figure 8-E), but a strong green stain was observed throughout the whole cell including nucleus (Figure 8-F). **2a**, and**3a** produced a strong cytoplasm blue stain with a few brighter spots in each cell (Figure 9 a

and b), which could suggest an endocytic internalization pathway. On the other hand, **2a** and **3a** also produced a weak green stain in the cells; however, this stain is generalized to the whole cell (Figure 8-I). Cells treated with **3a** show a similar fluorescent stain pattern as described for **2a**, only the blue staining is significantly stronger when **2a** is used (Figure 8-K and 8-L). The fact that blue and green stains compartmentalize differently could mean that once the original BOSCHIBAs reach the cell interior are being metabolized to at least two different molecules, one with capacity to get all the way into the nucleus, staining the whole cell; while the other can only reach the cytoplasm. By comparison, the BOSCHIBAs **2a** and **3a** with bulky aryl groups showed better fluorescent staining while **1a** exhibits a poor staining.

Figure 8. Here

Figure 9. Here

Conclusions

In summary, we have reported the design, synthesis and chemo-optical characterization of three BOSCHIBAs with good chemical yields based on easy and cheap condensation reactions. BOSCHIBAs **2a** and **3a** showed a low fluorescence emission (Φ_F~1%) while that inclusion of bulky groups induces a higher resistance to degradation compared with unsubstituted cyclic and heterocyclic analogs. All the BOSCHIBAs **1a**-**3a** demonstrated to be high stability in aqueous solution. Bioimaging studies for **2a** and **3a** revealed their potential application as enhanced cytoplasmic staining dyes with a low

cytotoxicity, a better staining (from 0.1 to 2.5 μg/mL), a high stability against UV radiation and hydrolytic stability.

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Supplementary Information

Appendix A Supplementary Data

The CCDC 1520512 contains the supplementary crystallographic data. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif or from the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, CB2 1EZ, UK).

Appendix B Supplementary Data

Supplementary figures and calculation of green chemistry metrics of the BOSCHIBAs **1a-3a**.

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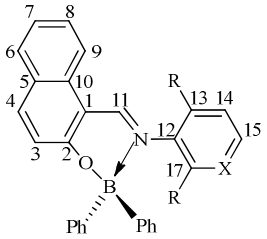
Table 1. Selected ^{1}H, ^{13}C, and ^{11}B NMR (ppm), and IR (cm^{-1}) spectroscopic data for BOSCHIBAs **1a**-**3a**, and their ligands **1**-**3**.



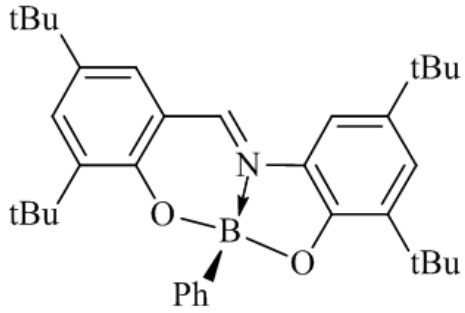
Comp.	^{1}H	^{13}C				^{11}B	FT-IR
	H-11	C-2	C-13/C-17	C-11	C-12		C=N
1	9.42	166.20	127.80/143.00	158.70	143.70	-	1624
2	9.14	168.00	129.70	161.30	145.80	-	1613
3	8.98	166.30	139.22	160.60	142.90	-	1620
1a	8.86	165.90	124.70/142.56	157.70	144.90	7.38	1608
2a	8.64	163.70	128.00	159.80	144.00	4.76	1605
3a	8.90	163.20	135.16	161.00	144.30	4.42	1607

Table 2. Crystal data for BOSCHIBA **1a**.

Empirical formula	$C_{28}H_{21}BN_2O$
Formula weight	412.29
Temperature, K	100(2)
Wavelength	1.54056
Crystal system	Monoclinic
Space group	*P*2(1)/*c*
a, [Å]	17.2063(14)
b, [Å]	8.1494(7)
c, [Å]	16.6279(13)
α, [°]	90.00
β, [°]	114.3280(10)
γ, [°]	90.00
V [Å^3]	1367.5(8)
Z	4
$\rho_{calc,mg.cm}^{-3}$	1.289
μ, mm^{-1}	0.078
2θ range for data collection	1.27 – 27.00°
No. of reflns collected	20996
No. of indep reflns	4322
[R_{int}]	[R_{int}]
Goodness of fit	1.048
*R*1, w*R*2 (I>2σ(I))	0.0366; 0.0993
*R*1, w*R*2 (all data)	0.0388; 0.1019

Table 3. Photophysical properties of BOSCHIBAs **1a**-**3a** and their ligands **1**-**3** in THF.

Compound	λ_{abs} [nm]	$\varepsilon *10^4$ [$M^{-1}cm^{-1}$]	λ_{em} [nm]	$\Delta\nu$ [cm^{-1}]	Φ_F [%]
1	324 (378)	0.10 (0.12)	490	6047	0.05
2	316 (366)	0.58 (0.52)	461	5630	0.03
3	316 (364)	0.71 (0.60)	475	6420	0.01
1a	341 (426)	0.51 (0.38)	528	4535	3.80
2a	328 (402)	0.39 (0.21)	492	4550	1.07
3a	328 (402)	0.81 (0.44)	493	4592	1.01



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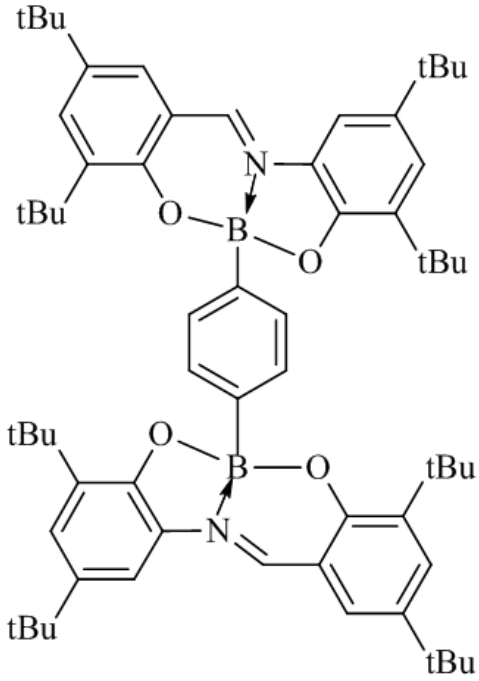

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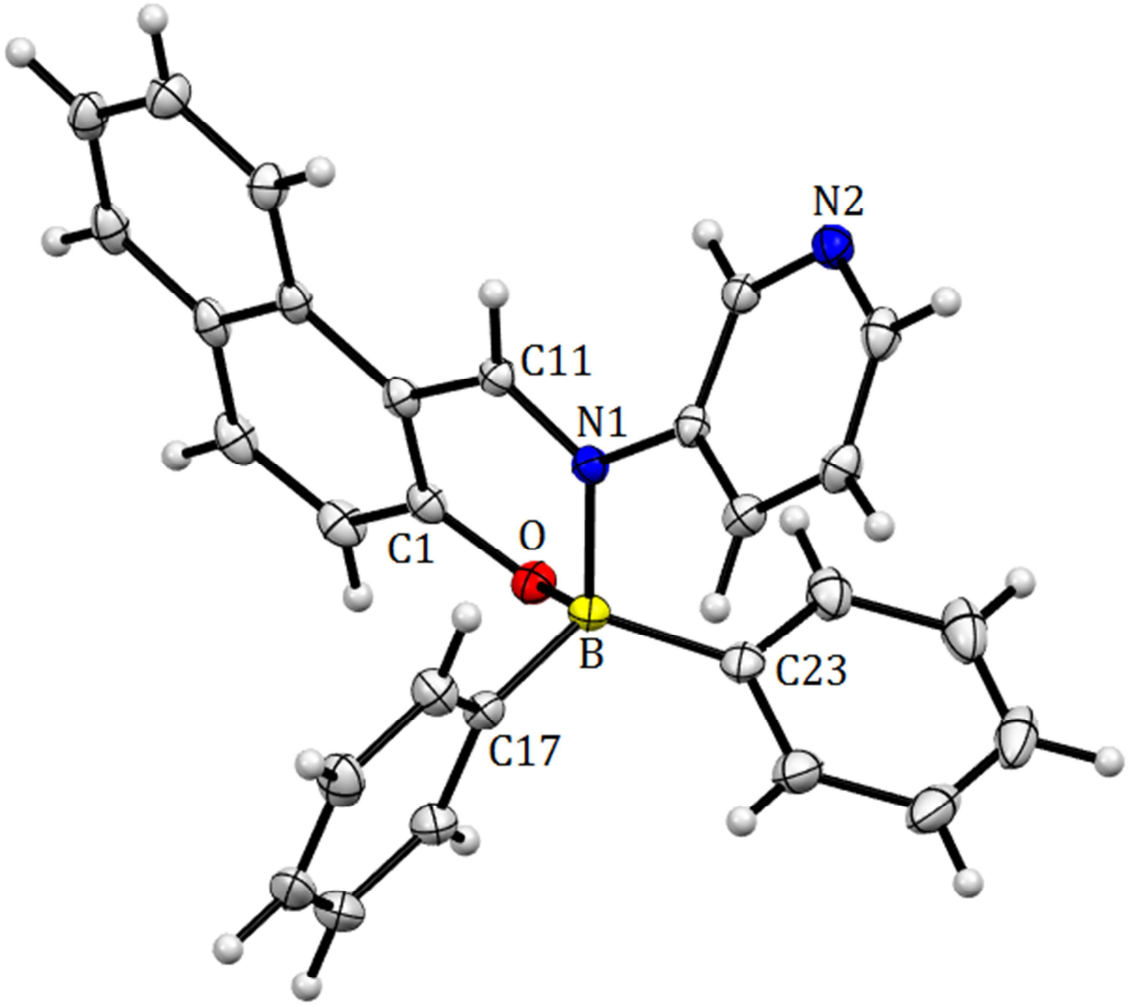
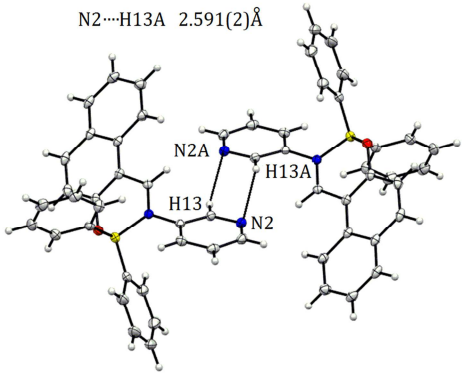
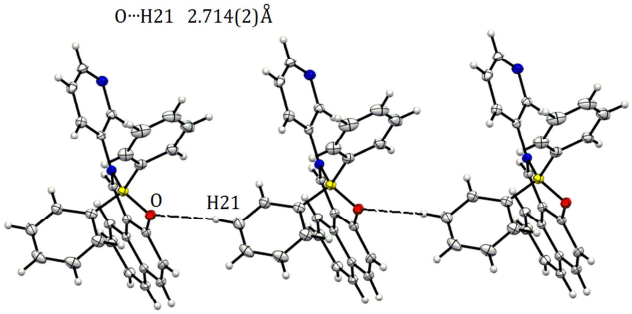
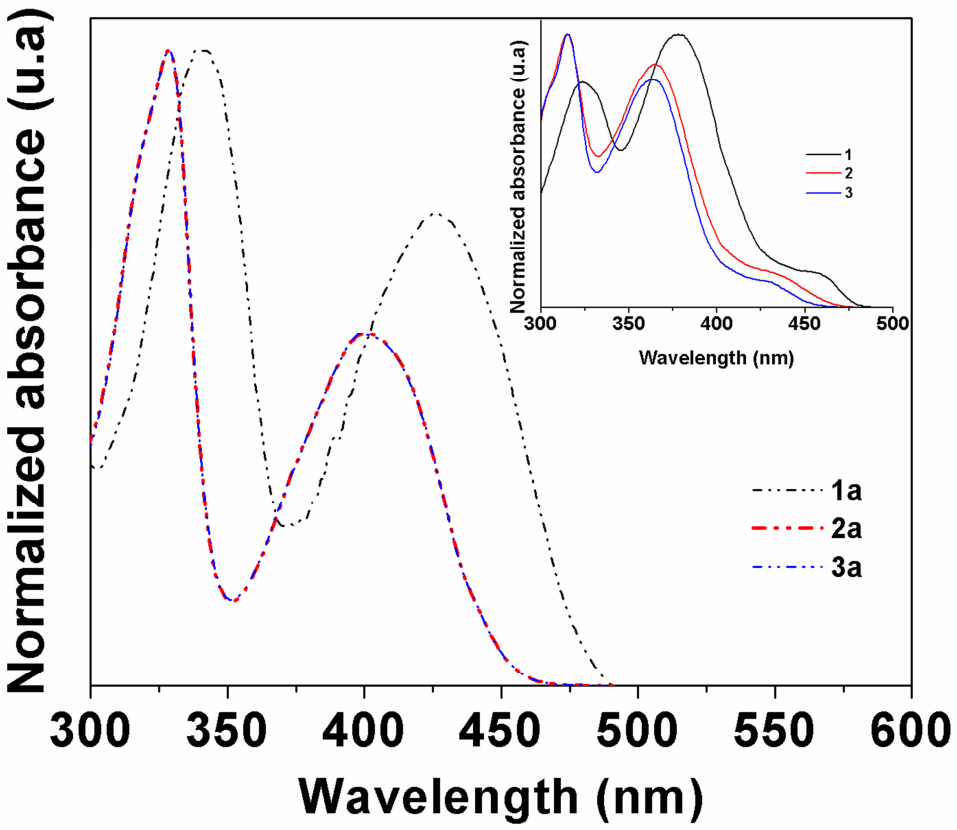
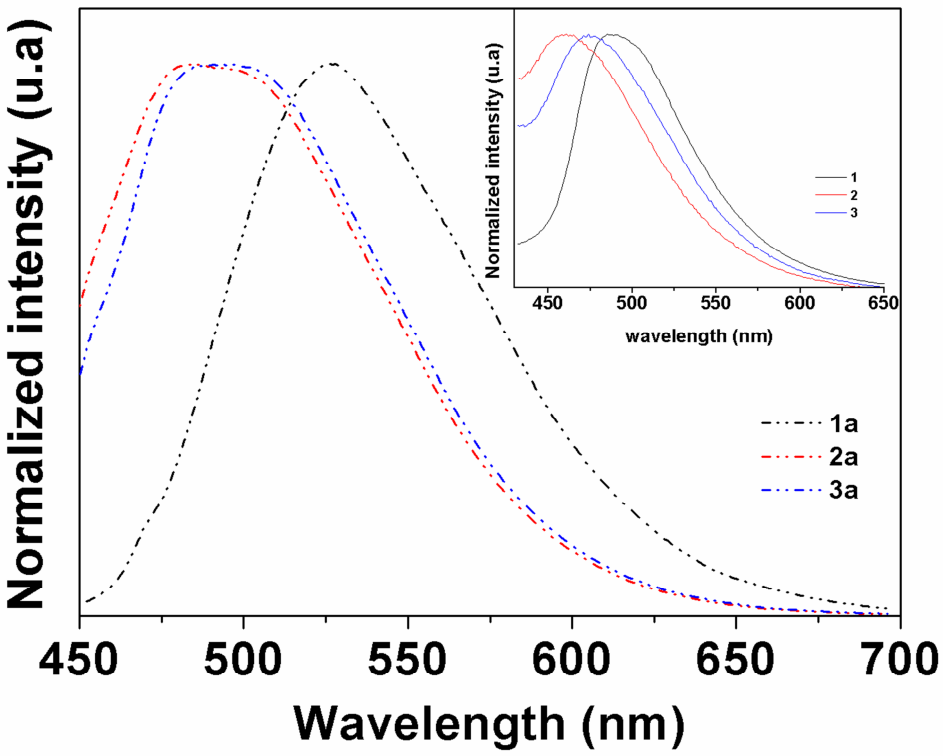
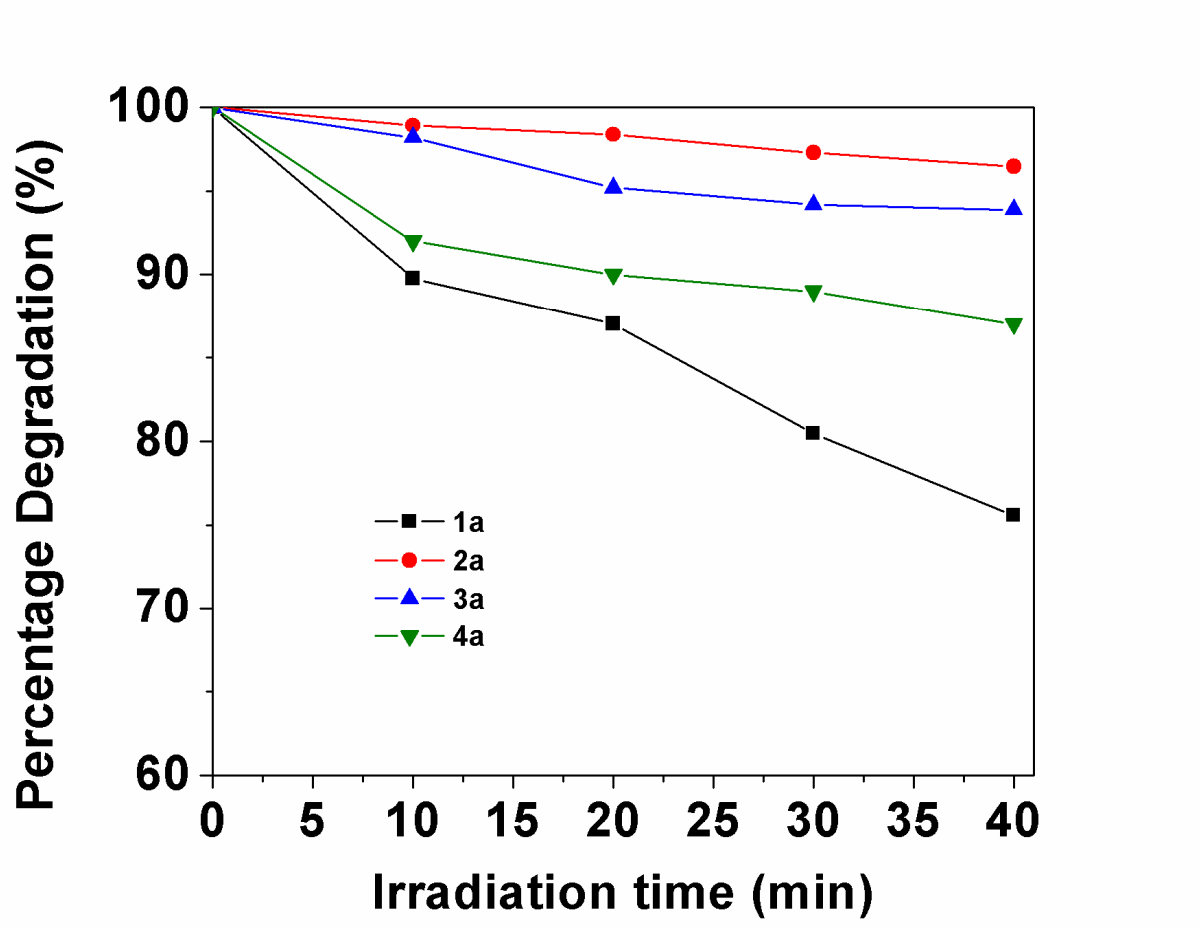


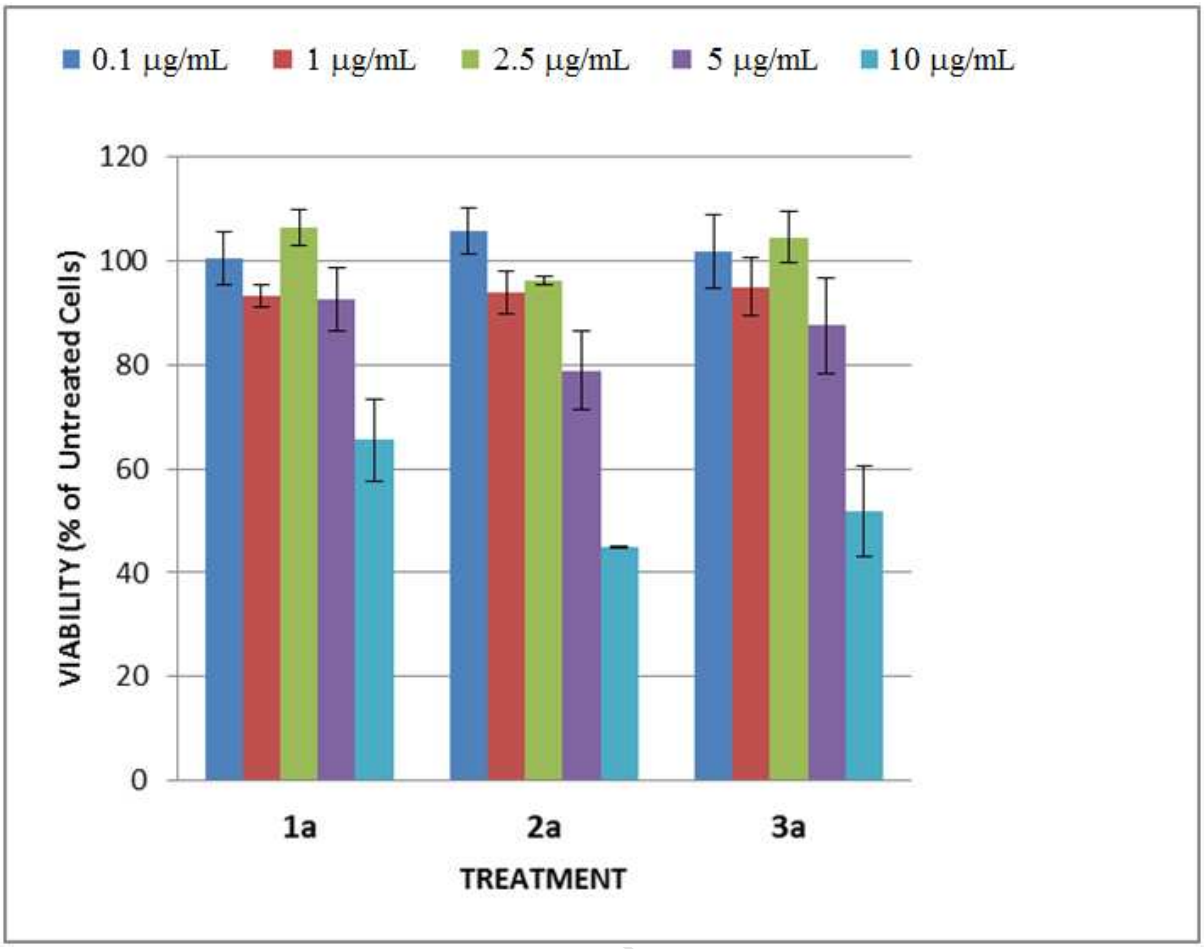


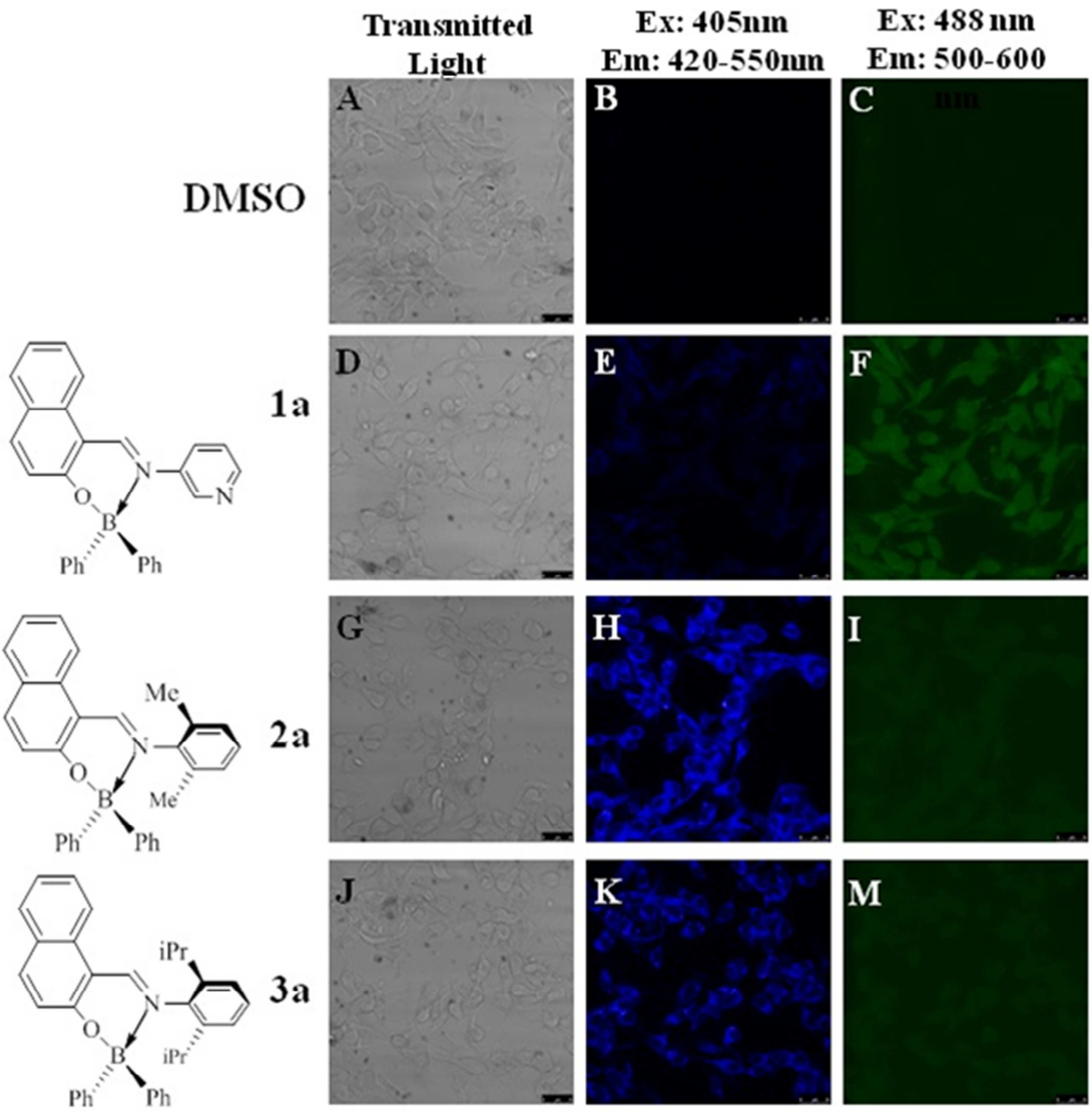




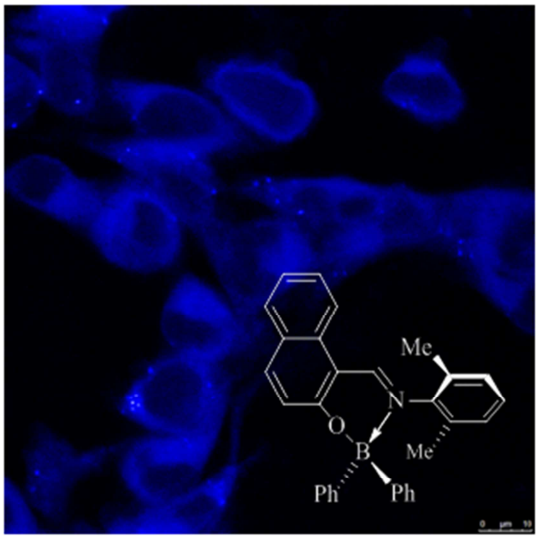




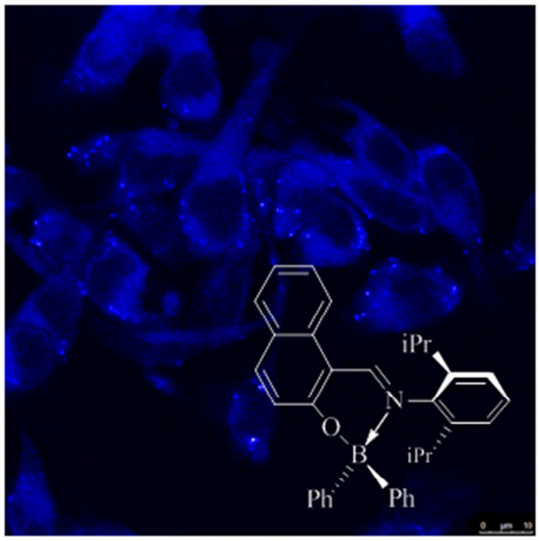


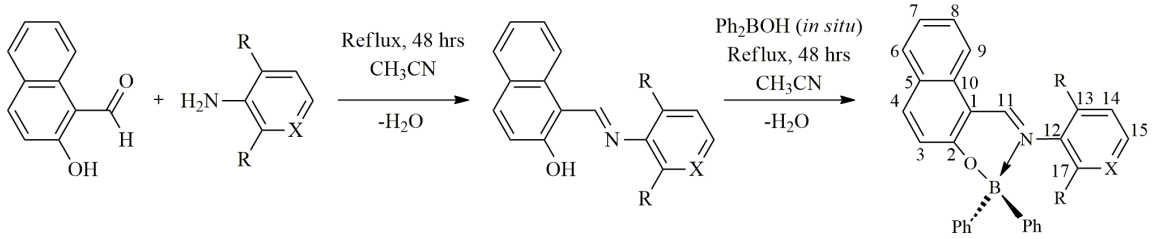

Transmitted Light
Ex: 405nm Em: 420-550nm
Ex: 488 nm Em: 500-600
DMSO
1a
2a
3a
A
B
C
D
E
F
G
H
I
J
K
M
Me
iPr
Ph
Ph
O
B
N

a)



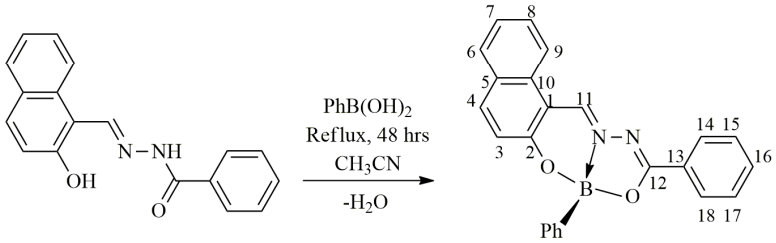
b)



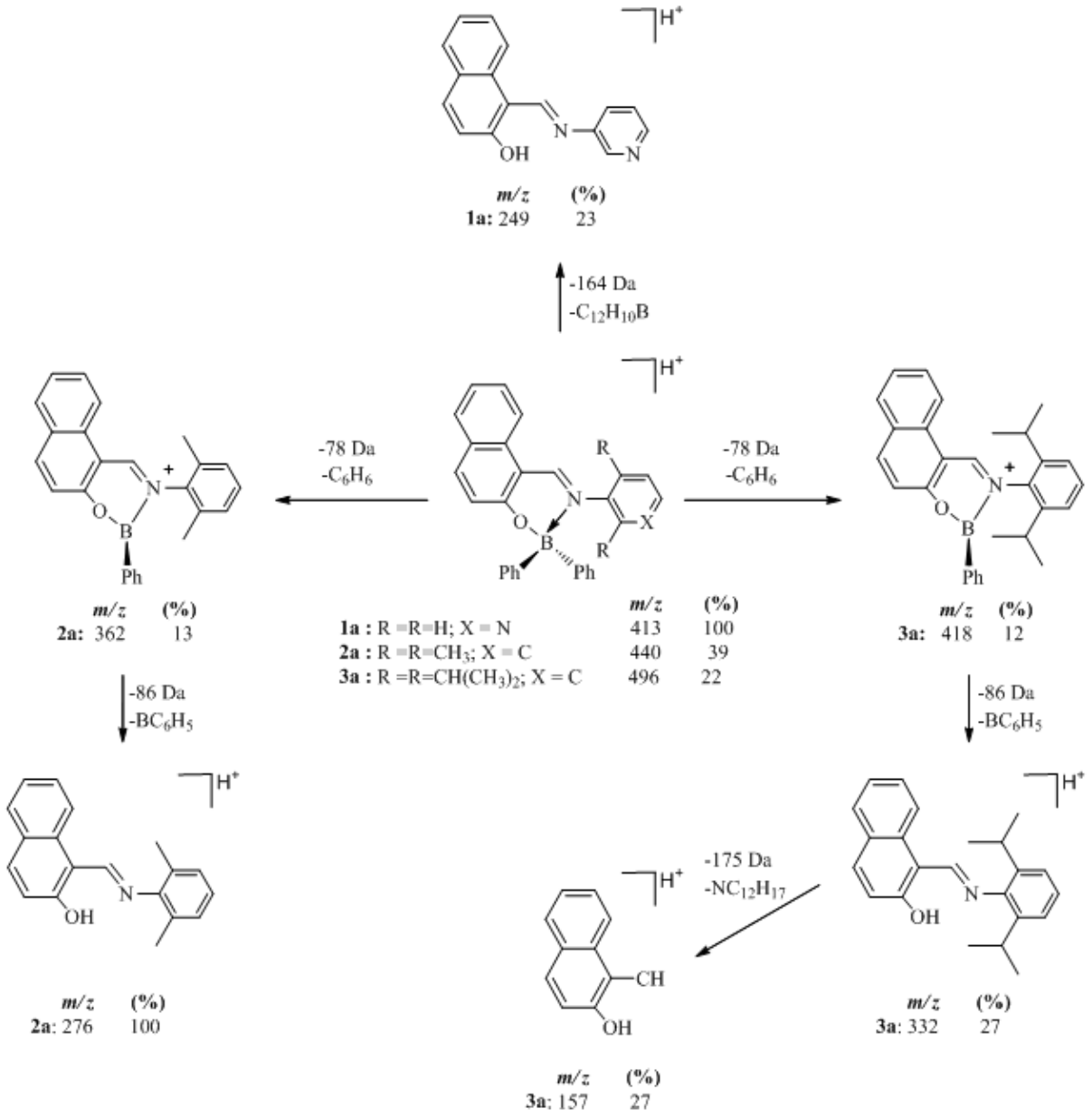


1 : R =R=H; X = N, (79%)
2 : R =R=CH_3; X = C, (75%)
3 : R =R=$CH(CH_3)_2$; X = C, (73%)

1a : R =R=H; X =N, (95%)
2a : R =R=CH_3; X =C, (92%)
3a : R =R=$CH(CH_3)_2$; X =C, (30%)



4a (74%)


H+
OH
N
N
m/z (%)
1a: 249 23
-164 Da
-C12H10B
-78 Da
-C6H6
-78 Da
-C6H6
O
B
Ph
Ph
R
X
R
m/z (%)
2a: 362 13
1a : R =R=H; X = N 413 100
2a : R =R=CH3; X = C 440 39
3a : R =R=CH(CH3)2; X = C 496 22
m/z (%)
3a: 418 12
-86 Da
-BC6H5
-86 Da
-BC6H5
m/z (%)
2a: 276 100
-175 Da
-NC12H17
CH
m/z (%)
3a: 157 27
m/z (%)
3a: 332 27

Highlights

- New fluorescent boron Schiff bases (BOSCHIBAs) showed their application as cytoplasm staining dyes *in vitro*.

- The BOSCHIBAs **2a**, and **3a** as cytoplasm staining dyes *in vitro* showed very low cytotoxicity, simple synthesis, high photo-stability, and specific staining for cytoplasm structure.

- The BOSCHIBAs syntheses are cheap, simple, fast, and reproducible with a high atomic economy and low E-factor values.