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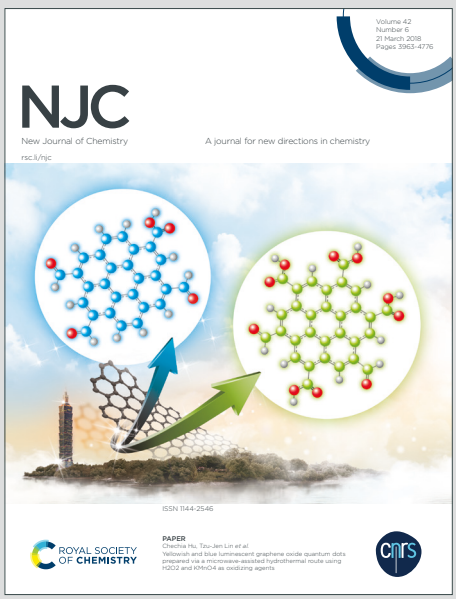

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Design of Novel Photosensitizers and Controlled Singlet Oxygen Generation for Photodynamic Therapy

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ABSTRACT

Photodynamic therapy (PDT) is a promising strategy in cancer treatment with its relatively lower side effect profile. Undoubtedly, the key component of PDT is the photosensitizers with a high ability to produce singlet oxygen. In this work, iodinated or brominated boron-dipyrromethene-bearing trimers (**5** and **6**) were prepared as photosensitizers and their molecular structures were characterized by mass spectrometry and NMR (^{1}H and ^{13}C) spectroscopy. Their photophysical and photochemical abilities were also evaluated in detail. The molar absorption coefficient (ε) of compound **5** was found to be 2.5 times higher than that of compound **6**. The photosensitizer (**5**), bearing iodinated-BODIPY units, exhibited almost non-fluorescent profile with 0.12 ns lifetime whereas the other photosensitizer (**6**) exhibited more moderate fluorescence character with 1.22 ns fluorescence lifetime. The singlet oxygen quantum yields of photosensitizers were determined to be 0.88 and 0.76, respectively by chemical trapping method. Moreover, the biological assessment of the novel compounds showed that these PSs internalized into the cells and triggered cell death in a light source-dependent manner, underlying the success of the compounds in PDT *in vitro*.

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1. Introduction

Cancer is a global health problem and one of the most fatal diseases. Although conventional methods such as surgical intervention, chemotherapy and radiation therapy are frequently preferred in cancer treatment, low success rates and negative effects of these methods on the quality of the patient life are widely reported disadvantages.[1] Photodynamic therapy (PDT), which has a lower side effect than the traditional methods, has emerged as a promising technique in cancer treatment.[2-5]

The success of PDT depends on the ability of the photosensitizer (PS), which is ineffective in the dark, to destroy the cancerous cells through controlled production of singlet oxygen ($^1O_2$) under light. [6-10] Photoactivatable PSs are generally designed from heteroatom-containing macrocycles, modified organic dyes, organic/inorganic nanostructures or metal-organic frameworks.[11-16]

Even though many PSs have been developed, the disadvantages such as low singlet oxygen production yield, high dark toxicity and low photostability are frequently reported to be overcome. [6,15,16] To improve photosensitizing ability of PS, one way is to increase its concentration, usually resulting in the aggregation effect.[17] Another way is to enhance the photosensitizing abilities of PS molecule by the suitable structural modification.[10-12,15]

Boron dipyrromethene (BODIPY) unit is considered as a remarkable candidate for PDT applications since it can easily be modified and structure-activity relationship can be improved.[6,11, 12, 15, 18] The heavy atoms such as iodine or bromine covalently linked to the BODIPY unit strongly promote the intersystem crossing (ISC) and increase the production of singlet oxygen.[19] Some researchers have focused on multiple BODIPY derivatives, such as orthogonal dimers and oligomers to improve the photosensitizing ability.[20-22] Cyanuric chloride (triazine), possessing three chloro atoms, is an ideal structure to construct complexes baring

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multiple BODIPY units. [18, 23,24] Also, triazine core is biocompatible and displays critical pharmacological profile. [18,25,26]

In this study, we focused on photodynamic activities of triazine rings bearing three BODIPY units (Fig 1). Novel triazine-based BODIPY trimers (**5** and **6**) were designed, synthesized and characterized. The singlet oxygen generation capacities of the trimers were determined using chemical and phosphorescence methods. The effects of different halogen atoms (I and Br) on the photophysical and photochemical properties of the compounds were evaluated. Moreover, the photodynamic therapy efficacy of the novel trimers were evaluated on human cervical cell line, HeLa, *in vitro*. Results point out that the novel trimers and also the precursors are the important candidates for *in vivo* photodynamic therapy applications.

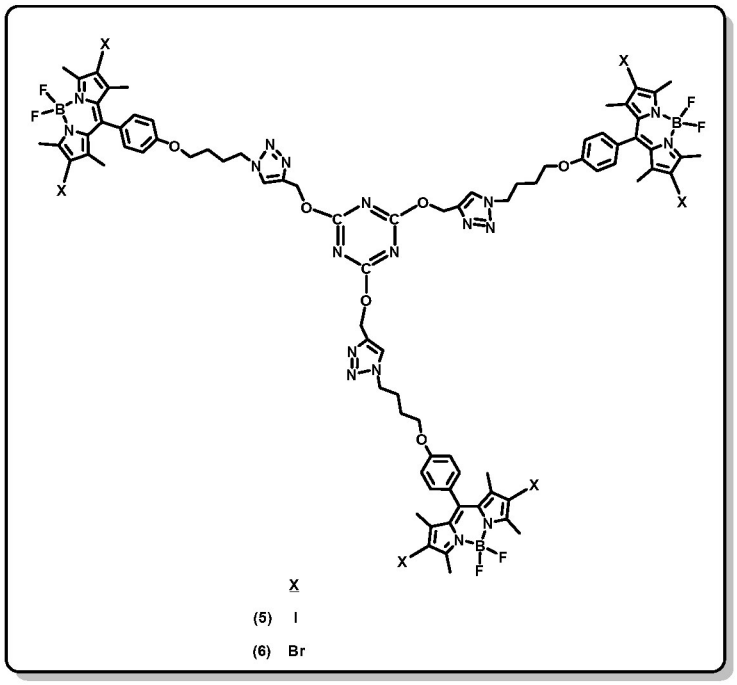

Figure 1. Chemical structure of triazine-based BODIPY trimers

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2. Experimental section

2.1. General methods

All chemicals and solvents were procured from commercial suppliers. Analytical thin-layer chromatography (TLC) was performed on silica gel plates (Merck, Kieselgel 60 Å, 0.25 mm thickness) with the F254 indicator. Column chromatography was performed on silica gel (Merck, Kieselgel 60 Å, 230-400 mesh). Mass spectra were recorded on a Bruker Daltonics Microflex mass spectrometer using dithranol as the MALDI matrix. All NMR spectra (^{1}H and ^{13}C NMR) were recorded on a Varian INOVA 500 MHz spectrometer. Electronic absorption spectra were analyzed with a Shimadzu 2101 UV spectrophotometer. Fluorescence excitation and emission spectra were obtained on a Varian Eclipse spectrofluorometer using 1.0 cm pathlength cuvettes at room temperature. The fluorescence lifetimes were recorded using Horiba- Jobin-Yvon-SPEX Fluorolog 3-2iHR instrument with Fluoro Hub-B Single Photon Counting Controller at an excitation wavelength of 390 nm for compounds. Signal acquisition was performed using a TCSPC module.

2.2. Synthesis

The meso-azido-substituted BODIPY derivative (**1**), di-iodinated BODIPY monomer (**2**), di-brominated BODIPY monomer (**3**), and tris-alkyne-carrying triazine core (**5**) were synthesized and purified as in the literature.[22, 27, 28]

2.2.1. Synthesis of compound 5

Compound **2** (100.0 mg, 0.145 mmol) and compound **4** (11.36 mg, 0.046 mmol) were dissolved in the solvent mixture (4.0 mL dichloromethane: 1.0 mL methanol: 1.0 mL water). Sodium ascorbate (7.5 mg; 0.03 mmol), $CuSO_4.5H_2O$ (6.0 mg, 0.03 mmol) and one drop of triethylamine were added to this reaction medium and the reaction mixture was stirred at room

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temperature for two days. The crude product was extracted with CH_2Cl_2: H_2O (50.0 mL : 50.0 mL) and the organic phase was dried over Na_2SO_4. The solvent was removed on the rotary evaporator. The reaction mixture was subjected to column chromatography on silica gel using MeOH-CH_2Cl_2 (4: 96) as the mobile phase. The pure product (**5**, 36.0 mg, 33 %) was obtained as red solid. MALDI TOF (m/z) Calc. 2310.51, Found: 2309.97 $[M]^+$, 2288.91 $[M+H-F]^+$ (Fig S1). ^1H NMR (500 MHz, $CDCl_3$) δ_H 7.8 (s, 3H), 7.1 (d, J = 8.1 Hz, 6H), 7.0 (d, J = 8.1 Hz, 6H), 5.6 (s, 6H), 4.5 (t, J = 6.4 Hz, 6H), 4.1 (m, 6H), 2.6 (s, 18H), 2.2-2.1 (m, 6H), 1.9-1.8 (m, 6H), 1.4 (s, 18H) ppm (Fig. S2). ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 172.78, 159.66, 156.58, 145.29, 142.25, 141.42, 131.68, 129.16, 126.87, 123.84, 115.28, 85.59, 67.07, 61.51, 50.11, 27.26, 26.21, 17.23, 16.02 ppm (Fig. S3).

2.2.2. Synthesis of compound 6

Compound **3** (100.0 mg, 0.17 mmol) and compound **4** (13.2 mg, 0.054 mmol) were dissolved in the solvent mixture (4.0 mL dichloromethane: 1.0 mL methanol: 1.0 mL water). Sodium ascorbate (7.5 mg; 0.03 mmol), $CuSO_4.5H_2O$ (6.0 mg, 0.03 mmol) and one drop of triethylamine were added to this reaction medium and the reaction mixture was stirred at room temperature for two days. The crude product was extracted with CH_2Cl_2: H_2O (50.0 mL : 50.0 mL) and the organic phase was dried over Na_2SO_4. The solvent was removed on the rotary evaporator. The reaction mixture was subjected to column chromatography on silica gel using MeOH-CH_2Cl_2 (4: 96) as the mobile phase. The pure product (**6**, 41.0 mg, 37 %) was obtained as red solid. MALDI TOF (m/z) Calc. 2028.50; found: 2028.76 $[M]^+$, 2007.82 $[M+H-F]^+$ (Fig. S4). ^1H NMR (500 MHz, $CDCl_3$) δ_H 7.8 (s, 3H), 7.2 (d, J = 8.3 Hz, 6H), 7.0 (d, J = 8.3 Hz, 6H), 5.6 (s, 6H), 4.5 (t, J = 6.9 Hz, 6H), 4.1 (t, J = 5.7 Hz, 6H), 2.6 (s, 18H), 2.2-2.1 (m, 6H), 1.9-1.8 (m, 6H), 1.4 (s, 18H) ppm (Fig. S5). ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 172.78, 159.66,

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153.76, 142.14, 140.56, 130.76, 129.17, 126.53, 123.81, 115.24, 111.66, 67.05, 61.52, 50.11, 27.27, 26.21, 13.95, 13.80 ppm (Fig. S6).

2.3. Photophysical parameters

The fluorescence quantum yield (Φ_F) was calculated in ethanol using the Rhodamine 6G (Φ_F = 0.90 in water) as a reference molecule.[29]

$$\Phi_F = \Phi_F(\mathrm{Std})\frac{F \cdot A_{Std} \cdot n^2}{F_{Std} \cdot A \cdot n_{Std}^2} \quad (1)$$

In the above equation, F is the area under the fluorescence emission curve of sample. A is the respective absorbance of the sample. η^2 is the refractive index of solvent.

2.4. Singlet Oxygen Sensitization

The singlet oxygen generation capacities (φ_Δ) of the trimers were evaluated by the comparative method. Rose Bengal (φ_Δ=0.68 in ethanol)[30] was employed as a reference compound and 1,3-diphenylisobenzofuran (**DPBF**) was used as a singlet oxygen scavenger. The trimers and Rose Bengal were irradiated with the light source (λ = 516 nm, 2.1 mWcm^{-2}) from a 6.0 cm distance. The following equation (2) was used to calculate the singlet oxygen quantum yield.[15]

$$\varphi_\Delta\,(\mathrm{samp}) = \varphi_\Delta\,(\mathrm{ref}) \times [m\,(\mathrm{samp})/m(\mathrm{ref})]\times[F(\mathrm{ref})/F(\mathrm{samp})] \quad (2)$$

In the above equation, m is the slope of difference in change in absorbance of DPBF (414 nm) with the irradiation time and F is the absorption correction factor, which is given by $F = 1 - 10^{-OD}$ (OD at the irradiation wavelength).

As the second method, singlet oxygen formation (Φ_Δ) were determined via the phosphorescence of $^1O_2$, using Horiba Jobin-Yvon Fluorimeter with Hamamatsu NIR PMT 5509.[31] The ability of singlet oxygen formation was obtained via according to the equation,

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$$\Phi_{\Delta} = \Phi_{\Delta}(\boldsymbol{std})\boldsymbol{x}\frac{\boldsymbol{I_{\Delta}\,(dyad)\,A(std)}}{\boldsymbol{I_{\Delta}\,(std)\,A(dyad)}} \quad (\mathbf{3})$$

where, Φ_{Δ} (std) is the singlet oxygen quantum yield of the Rose Bengal I_{Δ} is the area under the curve of peak of singlet oxygen at 1270 nm; and A is the absorbance which was set to 0.391, 0.362 and 0.366 for **5**, **6** and **RB**, respectively.

2.5. In vitro photodynamic therapy

Photodynamic therapy efficacy of the compounds were *in vitro* evaluated using a human cervical cancer cell line (HeLa). Briefly, 1×10^4 cells/well were seeded into the wells of 96-well plate and incubated for 24 h in RPMI 1640 medium (Biochrom AG, Germany) containing 10 % PBS (v/v; Biochrom AG, Germany) and 1 % gentamycin (v/v; Biological Industries, Israel) at 37°C and 5 % CO_2. Next, cells were washed with phosphate-buffered saline (PBS) twice and the medium was renewed. Cells were treated with compound **5** and **6** dissolved in dimethyl sulfoxide (DMSO) to minimize the solvent toxicity at a concentration of 0.0125-0.1 mg/mL as less than 1 % DMSO volume. The compounds **2-4** (each 0.1 mg/mL) which were also dissolved in DMSO were used to evaluate the PDT efficacy of the precursors and all compounds and DMSO were used as cell-free medium control to eliminate the background signal. These concentration range was the optimal one to see the stepwise cell death under these conditions. Cells were incubated at 37°C and 5 % CO_2 whether in dark or under green light source (λ= 516 nm, 2.1 $mWcm^{-2}$) for 5 h. Next, 10.0 µL of the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) solution (5.0 mg mL^{-1}; dissolved in PBS) was added onto cells and incubated for 4 h. Cells were disrupted with an SDS–HCl solution overnight and the microplates were read using a microplate spectrophotometer (Synergy H1, BioTek Instruments, USA) at 570 nm. Optical densities were converted to % viability by considering untreated cells

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to have 100 % viability and all the treatments were correlated with this group. All biological experiments were performed as three replica each of which including four technical replica. Data were analyzed by two-way ANOVA by GraphPad Prism 8.0 software (GraphPadInc, USA). The results were considered significant at the level of 0.05.

2.6. Live cell imaging

To further characterize the novel compounds and their precursors biologically, we evaluated the cellular imaging properties and internalization patterns of the compounds. 1×10^5 cells/well were seeded into the wells of 12-well plate and the cells were incubated overnight. Next, the cells were washed by PBS twice and incubated with 0.1 mg/mL of the compounds **2**-**6** for 5 min at room temperature in PBS. Then, the cells were washed by PBS twice and treated with Trypan Blue (0.4 %) for 5 min at room temperature to block the extracellular fluorescent signal, and the cells were washed by PBS twice. Images were obtained by Zeiss Axio Observer (Carl Zeiss Microscopy GmbH, Germany) using the default settings and filters.

3. Results and Discussion

3.1. Synthesis and structural characterization of triazine-based BODIPY trimers

Molecular scaffolds that allow fine tuning for desired pharmacological properties are extremely important in drug or biomolecule discovery. The triazine, a heterocyclic ring, has frequently been preferred as molecular platforms in novel biomolecule designs due to its predictable pharmacological properties, biocompatibility and chemically stable structure. [25,32,33] In this study, we focused on preparing novel triazine-based BODIPY trimers and determining their PDT activities, inspired by excellent pharmacological properties of triazine core and photochemical character of BODIPY. Synthesis pathway for the triazine-based BODIPY

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trimers (**5** and **6**) are depicted in Scheme 1. The meso-azido-substituted BODIPY (**1**) was synthesized from the two-step reaction of 2,4-dimethyl pyrrole with 4-azidobenzaldehyde in dichloromethane (DCM) in the presence of trifluoro acetic acid, p-chloranil, Et_3N and boron trifluoride diethyl etherate. To promote the intersystem crossing, 2 and 6 positions of the compound **1** was iodinated with iodine and iodic acid in ethanol and di-iodinated monomer **(2**) was obtained.[27] Then, the compound **1** was brominated in the presence of *N*-boromosuccinimide (NBS) in DCM to get di-brominated monomer **(3**).[28] The nucleophilic substitution reaction of cyanuric chloride (triazine) with propargyl alcohol in the presence of NaH in THF was carried out and tris-alkyne-carrying triazine (**4**) formed.[23] The click reactions of triazine core (**4**) with BODIPY monomers (**2** and **3**) in the presence of copper sulfate pentahydrate and sodium ascorbate yielded triazine-based BODIPY trimers (**5** and **6**). All compounds were purified by column chromatography and then novel compounds (**5** and **6**) were characterized by mass spectrometry and ^1H, and ^{13}C NMR spectroscopy. The molecular ion peaks of the trimers (**5** and **6**) were marked as 2309.97 and 2028.76 Da by MALDI-TOF mass spectrometer (Fig. S1 and S4). Also, $[M+H-F]^+$ ion peaks were observed as 2288.91 and 2007.82 for compound **5** and **6**, respectively. The aromatic carbons of trimers were marked between 173.0 and 111.0 ppm in the ^{13}C NMR spectra (Fig. S3 and S6).The aliphatic carbon signals were seen between 85.0-13.0 ppm regions. The ^1H NMR spectra of the trimers (**5** and **6**; Table 1) were evaluated in comparison to that of the monomers (**2** and **3**).[27,28] The ^1H NMR spectra of the trimers exhibited doublet signals for meso aromatic protons around 7.0 ppm. When the ^1H NMR spectra of monomers and trimers were compared, it was seen that a new peak ($-NCH_2-$) was formed at around 4.0 ppm instead of $C\underline{H_2}N_3$ proton peak. It was also observed that two new peaks belonging to the triazole ring protons at 7.8 ppm and $-COC\underline{H_2}$ protons at 5.6 ppm for the trimers formed. While the $-OCH_2$ protons were marked as triplet at 4.5 ppm, the $-CH_3$ protons on the

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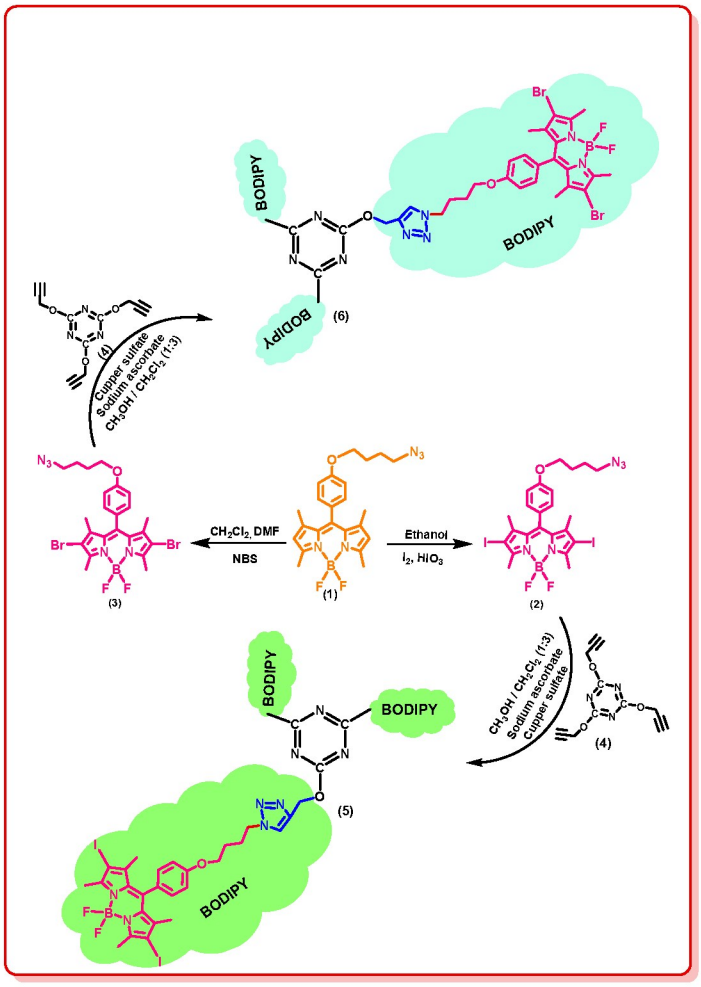
pyrrole ring were determined as sharp singlet peak at 2.6 and 1.5 ppm. The other aliphatic -CH_2- protons gave multiple signals in the range of 2.2-1.4 ppm.



Scheme 1. Synthesis pathway of BODIPY monomers (**2** and **3**) and triazine-based BODIPY trimers (**5** and **6**).

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Table 1. ^{1}H NMR parameters for monomers (**2**, **3**)[27,28] and trimers (**5**, **6**).

Compounds [δ_H (ppm)][a]				
	2	**3**	**5**	**6**
Aromatic-CH-	7.1 (d, *J* =7.6 Hz) 7.0 (d, *J* =7.6 Hz)	7.1 (d, *J* =8.4 Hz) 7.0 (d, *J* =8.4 Hz)	7.2(d, *J* =8.3 Hz) 7.0 (d, *J* =8.3 Hz)	7.1 (d, *J*=8.1 Hz) 7.0 (d, *J* =8.1 Hz)
-CH-N	-	-	7.8 (s)	7.8 (s)
-$COCH_2$-	-	-	5.6 (s)	5.6 (s)
-OCH_2-	4.1 (t, *J* = 6.0 Hz)	4.1 (t, *J* = 6.0 Hz)	4.5 (t, *J* = 6.9 Hz)	4.5 (t, *J* = 6.4 Hz)
N_3CH_2-	3.4 (t, *J* = 6.3 Hz)	3.4 (t, *J* = 6.6 Hz)	-	-
-NCH_2-	-	-	4.1 (t, *J* = 5.7 Hz)	4.1 (m)
Aliphatic -CH-	2.0-1.9 (m)	2.0-1.8 (m)	2.2-2.1 (m) 1.9-1.8 (m)	2.2-2.1 (m) 1.9-1.4 (m)
Ar-CH_3	2.6 (s) 1.4 (s)	2.6 (s) 1.4 (s)	2.6 (s) 1.4 (s)	2.6 (s) 1.4 (s)

[a]500 MHz ^{1}H NMR chemical shifts (ppm) in $CDCl_3$ solution. $^{3}J_{(HH)}$ [Hz], m: multiplet, s: singlet, d: doublet, t:triplet.

3.2. Photophysical properties of triazine-based BODIPY trimers

The optical properties of the triazine-based BODIPY trimers (**5** and **6**) were investigated in ethanol at room temperature (Fig.2). The basic photophysical data of trimers were given at Table 2. The absorption spectra of trimers exhibited major bands at 635 and 525 nm, which can be assigned to the S_0-S_1 transitions, for compound **5** and **6**, respectively. The absorption maximum of compound **5** was slightly red-shifted (10 nm) compared to compound **6**, which can be attributed to the minor contribution of halogen atom size to the molecular π-system. The molar absorption coefficient (ε) of the trimer **5** ($\varepsilon = 22.30 \times 10^4$ cm^{-1} M^{-1}) was determined to be 2.5 times higher than that of the trimer **6** (ε = 6.94 ×104 cm-1 M-1; Fig. S7 and S8). As shown in Fig. 2, the excitation of compound **5** at 520 nm gave a very weak emission band at 551 nm (Φ_F=0.05). This high emission quenching can be explained by the effect of the heavy atom.[34] The trimer **6** showed emission peak at 543 nm when excited at 500 nm and florescence quantum yield was calculated as 0.11. Fluorescence lifetimes (τ_F) of the trimers were measured with mono-exponential calculation (Fig. S9). The lifetime values were found to be 0.12 and 1.22 ns for trimers **5** and **6**, respectively. The difference in fluorescence lifetime values is compatible with the fluorescence quantum yields. The trimer, bearing iodinated- BODIPY units (**5**)

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exhibited almost non-fluorescent profile whereas trimer **6** showed more moderate fluorescence characteristics. The lower fluorescence profile of trimer **5** compared to **6** can be attributed to promotion of more intersystem crossing (ISC) in the molecule by iodine atoms.[19,34,35]

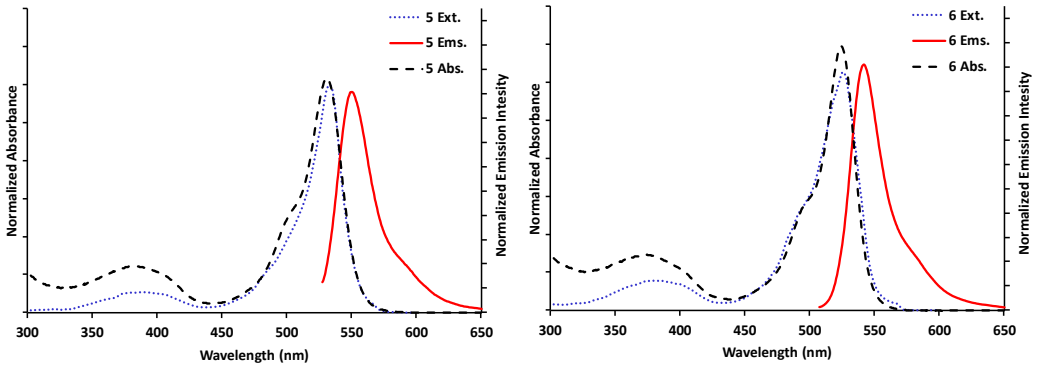

Figure 2. Absorbance, excitation and emission spectra of **5** (λ_{ex}= 520 nm) and **6** (λ_{ex}= 500 nm; 2.0 μM) in ethanol.

Table 2. Photophysical and photochemical properties of triazine-based BODIPY trimers (**5** and **6**)[a]

Compound	λ_{ab}, nm	λ_{em}, nm	ϵ^b, 10^4 M^{-1} cm^{-1}	Δ_{Stokes}, (nm)	τ_F (ns)[c]	Φ_F[d]	φ_Δ[e]
5	535	551	22.30	16	0.12 (CHISQ=0.89)	0.05	0.88
6	525	543	8.94	18	1.22 (CHISQ=0.86)	0.11	0.76

[a] Ethanol. [b] Molar extinction coefficients. [c] Lifetime, [d] Fluorescence quantum yield, [e] Singlet oxygen quantum yield.

3.3. Photosensitizing properties of triazine-based BODIPY trimers

The controlled singlet oxygen generation from the photosensitizer is essential for PDT applications.[16] In this study, the photosensitizing abilities of triazine-based BODIPY trimers (**5** and **6**) under controlled light were determined by two different techniques: chemical comparative method and singlet-oxygen phosphorescence peaks at 1270 nm. In the chemical method, 1,3- diphenylisobenzofuran (DPBF), a common singlet oxygen scavenger agent, was used to monitor the singlet oxygen production. Before starting the measurements, the solutions

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containing the trimer (**5** and **6**) and DPBF were kept in the dark for 30 min and it was determined that the trimers was inactive in the dark. Upon irradiation of solutions with a LED light (λ= 516 nm, 2.1 $mWcm^{-2}$), gradual decrease in the absorbance of DPBF at 414 nm was detected at time interval between 0 and 35 s (Fig. 3). The same measurement was repeated under the same conditions for Rose Bengal (Fig. S10). The singlet oxygen quantum yields (Φ_Δ) were determined as 0.88 and 0.76 for trimers bearing iodinated BODIPY units (**5**) and brominated BODIPY units (**6**), respectively. The iodine-substituted trimer exhibited the highest singlet-oxygen generation capacity, followed by the bromine-substituted trimer and finally RB (Φ_Δ=0.68). As the second method, singlet oxygen generation capacities of the trimers were determined via monitoring the phosphorescence signal of singlet oxygen at 1270 nm. The photosensitizers (**5** and **6**) were triggered by using a xenon lamp at their regarding absorption maxima and monitored with a near-IR detector (Fig.4). Based on the $^1O_2$ phosphorescence emission signal, the singlet oxygen quantum yields (Φ_Δ) were calculated as 0.85 and 0.82 for compound **5** and **6**, respectively. In this study, the stability of trimers under light was also tested. The solution of compound **5** and **6** without DPBF were stimulated with LED light (λ= 516 nm, 2.1 $mWcm^{-2}$) for 30 min and no significant mobility was observed in the absorbance intensities. The photophysical and photochemical results showed that compound **5** had the highest ability to generate singlet oxygen and had a rather low fluorescence profile. The differences in fluorescence characteristics and $^1O_2$ productions of the trimers can be explained by halogen atom size, known as the most popular method to facilitate the ISC of the molecule. To demonstrate the advantage of trimer structures on the singlet oxygen generation capacity compared to their monomer precursors, the ability of monomers (**2** and **3**) to produce singlet oxygen was also determined by the chemical method. Based on the oxidation data of the trap molecule in the presence of $^1O_2$, the values of Φ_Δ were calculated to be 0.83 and 0.65 for monomer **2** and **3**, respectively (Fig. S13 and S14). Both trimers but drastically brominated one

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displayed increased singlet oxygen production capacity compared to their precursors, which was parallel to previously reported compounds.[5,19]

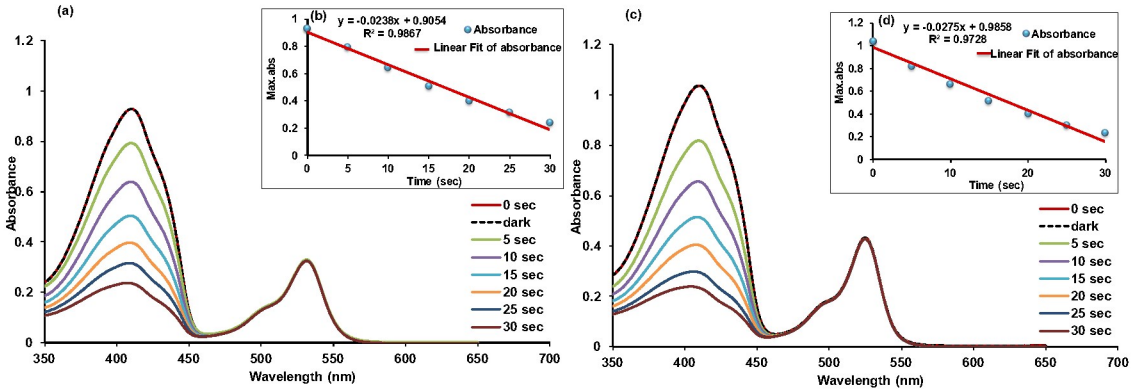

Figure 3. (a) Decrease in absorbance spectrum of DPBF in the presence of compound **5** (2.0 μM) in ethanol. (b) Absorbance decrease of DPBF at 414 nm by time in the presence of **5**. (c) Decrease in absorbance spectrum of DPBF in the presence of compound **6** (2.0 μM) in ethanol. (d) Absorbance decrease of DPBF at 414 nm by time in the presence of **6**.

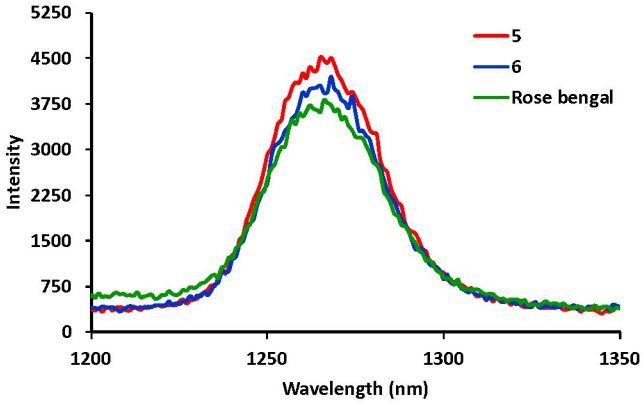

Figure 4. Singlet oxygen phosphorescence with sensitization from compound **5**, **6** and **Rose Bengal** in ethanol

3.4 In vitro photodynamic therapy

To investigate the possible *in vitro* photodynamic therapy efficacy of the compounds, we used human cervical cancer cell line, HeLa. At high concentrations which were aimed to use in the biological evaluations, ethanol did not totally dissolved the compounds; thus, DMSO was used for biological studies. We treated cells with **5** and **6** with diverse concentrations (0.0125-0.1 mg/mL) in the presence of untreated control (UT), solvent control (DMSO), and the precursors

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(**2**-**4**) with maximum concentration alone (0.1 mg/mL) in dark or green light source for 5 h and MTT cell viability assay was performed. Our results showed that the compounds were not toxic in dark but green light source was needed to induce the cell death. The minimum concentration to induce cell death was 0.025 mg/mL and 0.05 mg/mL was the optimal concentration to trigger the death of about all cells for both trimers (**5** and **6**; Fig. 5). Importantly, with the maximum concentration (0.1 mg/mL) used in the study, BODIPY precursors (**2** and **3**) was also effective to induce cell death as the novel compounds, **5** and **6**. In the previous studies, iodine- and bromide-containing BODIPY were proposed as well photosensitizers.[5] Thus, the results obtained in the present study correlated with the literature. As expected, triazine core alone (**4**) was not able to induce cell death even under green light. However, the ability of triazine complexes to be able to target some biological entities specifically[36] makes the triazine-based complexes instead of BODIPY alone units favorable in the PDT studies and those studies are under investigation by our research group.

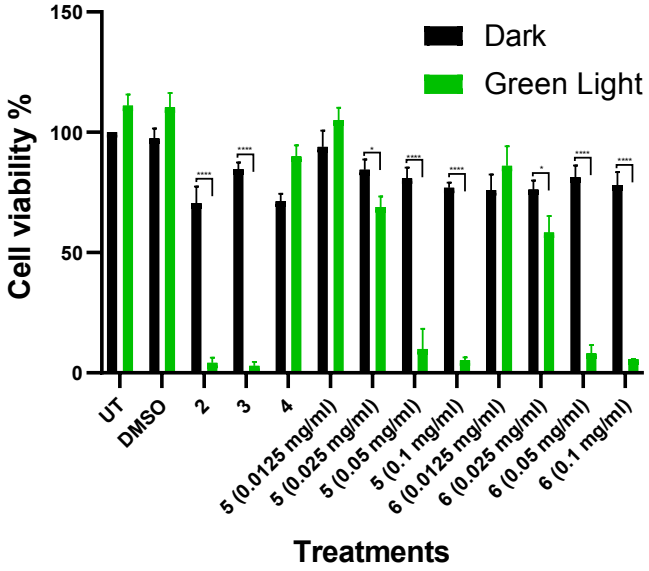

Figure 5. Effects of compounds on viabilities of HeLa cells in dark or under green light.*p<0.05; ****p<0.0001.

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To further characterize the compounds biologically, we determined the internalization pattern of the compounds. We treated the cells with 0.1 mg/mL of the compounds **2**, **3**, **5** and **6** and applied live cell imaging in the presence of Trypan Blue which blocks the extracellular signal using a fluorescence microscope with a maximal excitation wavelength of 475 nm and a maximal emission wavelength of 547 nm. Our results pointed that all compounds internalized into cells and stained the all detectable compartments of the cells (Fig. 6). Intracellular and/or extracellular singlet oxygen has been shown to trigger cell death.[37] In the present study, we propose that the cell death was performed by internalization of the compounds and intracellular production of the singlet oxygen. Moreover, according to the results, the novel compounds can also be evaluated as live cell imaging agents. Finally, the results of *in vitro* photodynamic therapy efficacies of the novel compounds make them well candidates for *in vivo* photodynamic therapy in further studies.

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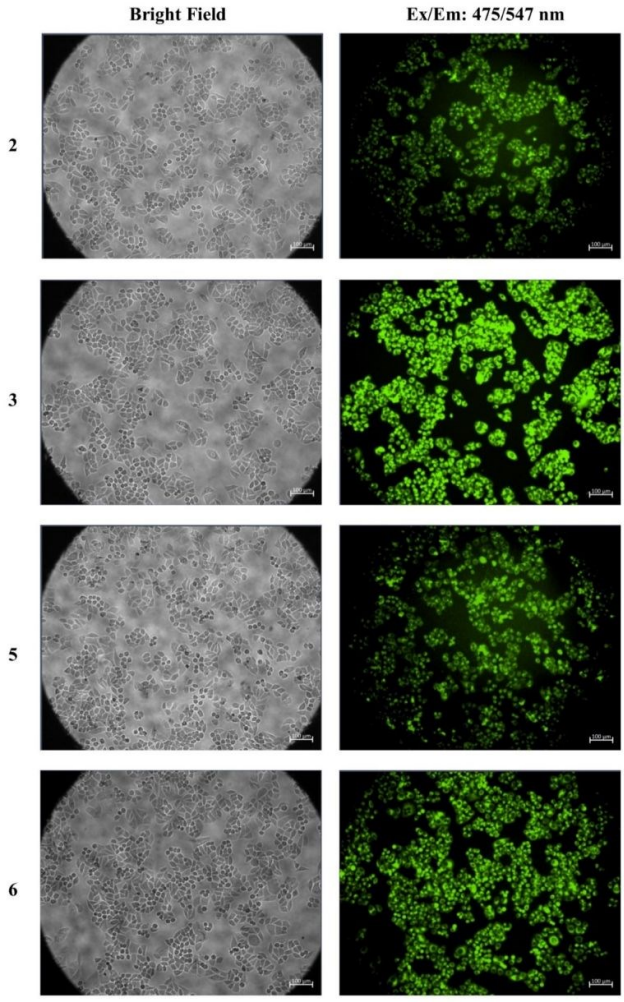

Figure 6. Cellular imaging properties of the compounds. Ex: 475 nm and Em: 547nm.

4. Conclusions

In this study, we described the molecular design, synthesis and characterization of novel triazine-based BODIPY trimers (**5** and **6)** comprising the triazine platform and iodinated/ brominated boron-dipyrromethene branch. Also, the photophysical, and photochemical characteristics and PDT activities of the photosensitizers were evaluated. The photosensitizers that were chemically stable under light excitation exhibited strong absorption bands at about 530 nm. These compounds, which were inactive in the dark, were able to produce highly efficient singlet oxygen when excited by light source. The singlet oxygen quantum yields of the

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compounds determined by chemical and phosphorescence methods have shown that these materials are very suitable designs and convenient for PDT applications. Especially, compound **5** exhibited a fluorescence quantum yield of 0.05 with very high chemical and phosphorescence singlet oxygen quantum yields of 0.88 and 0.85, respectively. Undoubtedly, our results provided critical candidate agents for *in vitro* and *in vivo*, as further studies, PDT applications. Moreover, triazine-based cellular targeting strategies may also combined to PDT studies to effectively and locally destroy the cancer cells, which is now under investigation by our laboratory. Still, the novel compounds are proved to be *in vitro* PDT agents with desirable photochemical and photophysical properties.

Electronic supplementary information

The mass and NMR spectra of the compounds and details of the photophysical studies were given in SI file.

Conflicts of interest

The authors declare no competing financial interest.

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