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Synthesis and in Vitro Photodynamic Activities of Pegylated Distyryl Boron Dipyrromethene Derivatives

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Supporting Information

ABSTRACT: A series of pegylated distyryl boron dipyrromethenes have been prepared and characterized. Their in vitro photodynamic activities in Tween 80 emulsions have also been investigated against HT29 human colorectal carcinoma cells. The derivative having five triethylene glycol chains (compound 8) exhibits the highest photocytotoxicity with an IC₅₀ as low as 7 nM. It is also localized preferentially in the endoplasmic reticulum of the cells and can induce predominately apoptosis upon illumination.

INTRODUCTION

Boron dipyrromethene (BODIPY) derivatives are versatile functional dyes receiving much current attention.¹ Owing to the many favorable and tunable characteristics, these compounds have been widely studied as fluorescent sensors for various analytes,^{2,3} building blocks for artificial photosynthetic models,⁴ and components for molecular logic gates⁵ and other optoelectronic devices.^{6,7} The distyryl derivatives prepared by Knoevenagel condensation of 3,5-dimethyl BODIPYs with aryl aldehydes have a more extended π system and absorb at a longer-wavelength position in the red visible to near-infrared region,¹ making them particularly useful for biomedical applications as this allows a deeper light penetration into tissues. As part of our continued interest in the development of efficient photosensitizers for photodynamic therapy (PDT),⁸ we believed that distyryl BOD-IPYs with appropriate substituents can serve as promising photosensitizing agents. However, this area has been little studied. Akkaya et al. first reported several brominated distyryl BODIPYs and briefly examined the photocytotoxicity of one of these compounds toward K562 human erythroleukemia cells.⁹ The same research group subsequently reported another novel distyryl BODIPY that functions as a molecular AND logic gate with Na⁺ and H⁺ as the inputs and singlet oxygen as the output.¹⁰ Recently, Lee et al.¹¹ have extended the earlier work of Nagano et al.¹² to examine the in vitro and in vivo photodynamic activities of a series of BODIPY derivatives. Although these compounds do not absorb at the favorable red visible region (λ < 560 nm), some of them are highly potent with IC₅₀, defined as the dye concentration required to kill 50% of the cells, as low as 45 nM. We report herein the preparation and in vitro photodynamic activities of a series of diiododistyryl BODIPYs substituted with different number of triethylene or polyethylene glycol chains. These substituents greatly affect the cellular uptake, singlet oxygen generation efficiency, and eventually the photocytotoxicity.

RESULTS AND DISCUSSION

Synthesis and Characterization. We have recently reported that condensation of the diiodo BODIPY 1 with 4-methoxybenzaldehyde (2) gives the distyryl BODIPY 6 (Scheme 1), which can couple with two 4-(dimethylamino)phenylethynyl groups to afford a pH-responsive near-infrared fluorescent dye.¹³ This methodology was extended to prepare several pegylated analogues as shown in Scheme 1. Treatment of 1 with an excess of benzaldehydes 3-5 gave the corresponding distyryl BODIPYs 7-9. The triethylene or polyethylene glycol (with an average molecular weight of 550) chains were introduced to enhance the hydrophilicity, biocompatibility, and cellular uptake of the dyes.¹⁴ Two iodo groups were also incorporated at the 2 and 6 positions of the BODIPY core to promote the intersystem crossing and the singlet oxygen generation through the heavy atom effect. $^{9-12}$ All the new compounds were characterized with various spectroscopic methods and elemental analysis (except the polymeric 5 and 9). For these two polymeric compounds, the electrospray ionization (ESI) mass spectra (Figures S1 and S2 in Supporting Information) showed the molecular ion's signals as two overlapping envelopes of $[M + Na]^+$ (major) and $[M + K]^+$ (minor) isotopic clusters, each separated by 44 mass units, which corresponds to the molecular mass of the OCH₂CH₂ repeating unit.

Electronic Absorption and Photophysical Properties. The electronic absorption and fluorescence spectra of 6-9 were recorded in N,N-dimethylformamide (DMF) (Table 1). The absorption spectra of all these compounds are similar (Figure S3 in Supporting Information) showing an intense Q-band at 661-667 nm, which falls into the body's therapeutic window. Most of the absorptions of 8 and 9, which have two additional triethylene or polyethylene glycol chains at the styryl groups, are slightly red-shifted and attenuated in intensity compared with those of **6** and **7**. The Q-band of the polymeric **9** is particularly weak and broadened. This suggests that the compound is more aggregated than the analogues 6-8 as a result of the stronger dipole-dipole interactions due to the additional ethylene glycol units. In fact, for 6-8, the Q-band strictly follows the Lambert-Beer law, suggesting that they are not significantly aggregated in DMF [see the spectra of 8 in Supporting Information (Figure S4) as an example]. Upon excitation at 610 nm, these compounds

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Scheme 1. Synthesis of Distyryl BODIPYs 6-9



Table 1. Electronic Absorption and Fluorescence Data for6-9 in DMF

compd	λ_{\max} (nm) (log ε)	$\lambda_{em} (nm)^a$	$\Phi_{\mathrm{F}}{}^{b}$
6	319 (4.40), 378 (4.66), 447 (4.24), 661 (4.96)	689	0.19
7	320 (4.40), 379 (4.64), 447 (4.21), 662 (4.90)	690	0.18
8	331 (4.48), 387 (4.51), 460 (4.18), 667 (4.85)	703	0.09
9	331 (4.38), 386 (4.41), 457 (4.05), 667 (4.70)	701	0.04
a Excited at 610 nm. b Using ZnPc in DMF as the reference ($\Phi_{\rm F}$ = 0.28).			

show a fluorescence emission at 689–703 nm with a fluorescence quantum yield ($\Phi_{\rm F}$) of 0.04–0.19 relative to unsubstituted zinc(II) phthalocyanine (ZnPc) ($\Phi_{\rm F} = 0.28$).⁸ The relatively weak fluorescence emission of these compounds can be attributed to the presence of two iodo groups, which promote the intersystem crossing and reduce the chance of singlet-state depopulation by fluorescence emission. The particularly low fluorescence quantum yield of **9** may also be ascribed to its higher aggregation tendency.

The efficiencies of these compounds in generating singlet oxygen in DMF were also evaluated by monitoring the rates of decay of the singlet oxygen quencher 1,3-diphenylisobenzofuran (DPBF) induced by these compounds. As shown in Figure S5 (Supporting Information), all the compounds can induce photo-degradation of DPBF. The efficiencies of 6-8 are comparable with that of ZnPc. Compound 9 is significantly less efficient, which again may be ascribed to its higher aggregation tendency and the long polyethylene glycol chains that somewhat hinder the interaction between the fluorophore and the quencher.

In Vitro Studies. The in vitro photodynamic activities of distyryl BODIPYs 6–9 in Tween 80 emulsions were investigated against HT29 human colorectal carcinoma cells. The surfactant was added to increase the solubility of the dyes in aqueous media. Figure 1 shows the dose-dependent survival curves for these compounds. It can be seen that all of them are essentially noncytotoxic in the absence of light. However, upon illumination with red light ($\lambda > 610$ nm), all the compounds (except 9) become cytotoxic. The photocytotoxicity depends greatly on the



9: $R_1 = R_2 = O(CH_2CH_2O)_nCH_3$ (n~12) 7%

Figure 1. Comparison of the cytotoxic effects of **6** (squares), 7 (triangles), **8** (circles), and **9** (stars) on HT29 cells in the absence (closed symbol) and presence (open symbol) of light ($\lambda > 610$ nm, 40 mW cm⁻², 48 J cm⁻²). Data are expressed as the mean \pm standard error of the mean (SEM) of three independent experiments, each performed in quadruplicate.

substituents and follows the trend 8 (IC₅₀ = 7 nM) > 7 (IC₅₀ = 75 nM) > 6 (IC₅₀ = 3300 nM) > 9 (IC₅₀ > 8000 nM). Compound 8, which contains five triethylene glycol chains, is the most potent candidate. In fact, its photocytotoxicity is much higher than that of the classical photosensitizer porfimer sodium (IC₅₀ = 4600 ng mL⁻¹ under the same experimental conditions vs 11 ng mL⁻¹ for 8) and pheophorbide *a* (IC₅₀ = 500 nM)¹⁵ and is comparable with that of some highly potent phthalocyanine-based photosensitizers.^{8,16,17}

To account for the different photocytotoxicities of 6-9, their aggregation behavior in the Dulbecco's modified Eagle's medium (DMEM) was examined by absorption and fluorescence spectroscopic methods. It was found that the Q bands of 6-8 remain sharp and intense, while that of 9 is split into two weaker peaks



Figure 2. (a) Bright field (upper row) and intracellular fluorescence (lower row) images of HT29 cells after incubation with (i) 6, (ii) 7, (iii) 8, and (iv) 9 (all at $1 \mu M$) for 2 h. (b) Comparison of the intracellular fluorescence intensities of 6–9. Data are expressed as the mean \pm standard deviation (number of cells = 50).



Figure 3. Visualization of the intracellular fluorescence of HT29 by using filter sets specific for 8 (in red, column 2) and ER-Tracker, LysoTracker, or MitoTracker (in green, column 3). The corresponding superimposed images and the bright field images are given in column 4 and column 1, respectively.

(Figure S6 in Supporting Information). Upon excitation, **6** and 7 showed a strong fluorescence peak at 691 nm. The emission of **8** was slightly red-shifted (at 705 nm) and weaker, while that of **9** could hardly be observed. These observations indicate that **6**–**8** are relatively free from aggregation in the culture medium. However, the polymeric **9** is significantly aggregated under this condition, which hinders the formation of reactive oxygen species (ROS), such as singlet oxygen.

To further explain the photocytotoxicity results, fluorescence microscopic studies were also carried out to investigate the cellular uptake of these compounds. HT29 cells were incubated respectively with distyryl BODIPYs 6-9 (1 μ M) for 2 h. Upon

excitation at 633 nm, the bright field and fluorescence images of the cells were captured (Figure 2a), and the intracellular fluorescence intensities were also determined (Figure 2b). It can be seen clearly that **8** shows much stronger intracellular fluorescence throughout the cytoplasm than the other compounds, which suggests that it has higher cellular uptake and/or efficiency to emit fluorescence inside the cells. The enhanced uptake may be due to the increased number of triethylene glycol chain. Hence, the highest photocytotoxicity of **8** is believed to be attributed to its high cellular uptake and low aggregation tendency in the biological environment, which facilitates the ROS generation.



Figure 4. Flow cytometric analysis of the cell death mechanism induced by 8 upon PDT treatment ($\lambda > 610$ nm, 40 mW cm⁻², 48 J cm⁻²) on HT29 cells. Data are expressed as the mean \pm standard deviation of three independent experiments.

Owing to the high potency of 8, its subcellular localization was also investigated. The cells were stained with LysoTracker Green DND 26, MitoTracker Green FM, or ER-Tracker Green, which are specific fluorescent probes for lysosomes, mitochondria, and endoplasmic reticulum, respectively. As shown in Figure 3, the fluorescence caused by the ER-Tracker Green (excited at 488 nm, monitored at 510-560 nm) is well superimposed with the fluorescence caused by 8 (excited at 633 nm, monitored at 650-720 nm), indicating that endoplasmic reticulum is the preferential accumulation site of 8. This cell's organelle is known to play a central role in lipid and protein biosynthesis and serve as an intracellular calcium store. It has been reported that accumulation of photosensitizers in endoplasmic reticulum also results in efficient triggering of cell death upon illumination.^{18,19} By contrast, the fluorescence images of 8 cannot be superimposed with those of LysoTracker and MitoTracker, which indicates that 8 is not localized in the lysosomes and the mitochondria.

To study the mode of cell death induced by 8, a flow cytometric assay of annexin V-green fluorescent protein (GFP) and propidium iodide (PI) costaining was employed.²⁰ The cell populations at different phases of cell death, namely, viable (annexin V-GFP⁻/PI⁻), early apoptotic (annexin V-GFP⁺/PI⁻), and necrotic or late-stage apoptotic (annexin V-GFP⁺/PI⁺), were examined at different drug doses. As shown in Figure 4, most of the cells are negative for annexin V-GFP and PI after the treatment with 8 (32 nM) in the absence of light. This indicates that this compound is essentially noncytotoxic toward HT29 cells in darkness. However, upon illumination, the percentage of cells in the early apoptotic stage increases from 4% to 66% as the concentration of 8 increases from 0 to 32 nM. A significant amount of necrotic cells (less than 30%) was also observed (in annexin V-GFP⁺/PI⁺ region).

CONCLUSIONS

We have prepared and characterized a series of pegylated distyryl BODIPY derivatives. The photodynamic activities of these compounds depend greatly on the substituents. Compound 8, having five triethylene glycol chains, exhibits the highest photocytotoxicity with an IC₅₀ of 7 nM for HT29 cells, which can be ascribed to its high cellular uptake and low aggregation tendency in the biological media. It also shows a high localization in the endoplasmic reticulum of the cells and can induce predominately apoptosis upon illumination. The results suggest that distyryl BODIPYs with appropriate substituents can behave as highly efficient photosensitizers for PDT.

EXPERIMENTAL SECTION

Experimental details regarding the purification of solvents, spectroscopic and photophysical measurements, and in vitro studies are described elsewhere unless otherwise stated.⁸ Compounds 1 and 6 were prepared by our previously described procedure.¹³ Benzaldehydes 3^{21} and 4^{22} were prepared as described. The purity of distyryl BODIPYs 7–9 was determined by elemental analysis (or ¹H NMR spectroscopy for 9) and was found to be \geq 95%.

Benzaldehyde 5. A mixture of 3,4-dihydroxybenzaldehyde (0.43 g, 3.1 mmol), tosylated polyethylene glycol monomethyl ether (with an average molecular weight of 550)²³ (5.05 g, \sim 7 mmol), and K₂CO₃ (2.47 g, 17.9 mmol) in DMF (40 mL) was stirred at 100 °C for 4 days. The mixture was filtered through a Celite pad, and the residue was washed with CH₂Cl₂ (150 mL). After removal of the volatiles in vacuo, the residue was purified by silica gel column chromatography using CH₂Cl₂/acetone (10:1, v/v) as the eluent to give a colorless oil (1.88 g, \sim 50%). ¹H NMR (400 MHz, CDCl₃): δ 9.84 (s, 1 H, CHO), 7.43–7.46 (m, 2 H, ArH), 7.01 (d, *J* = 8.0 Hz, 1 H, ArH), 4.21–4.27 (m, 4 H, OCH₂), 3.88–3.92 (m, 4 H, OCH₂), 3.73–3.75 (m, 4 H, OCH₂), 3.64–3.66 (m, \sim 90 H, OCH₂), 3.54–3.56 (m, 4 H, OCH₂), 3.38 (s, 6 H, OCH₃). MS (ESI): an envelope of isotopic clusters of [M + Na]⁺ at *m*/z 1158 (90%, for *n* = 11), 1246 (69%, for *n* = 12), 1334 (36%, for *n* = 13), etc.

Distyryl BODIPY 7. A mixture of iodinated BODIPY 1 (51 mg, 69 µmol), benzaldehyde 3 (56 mg, 0.2 mmol), glacial acetic acid (0.1 mL, 1.7 mmol), piperidine (0.46 mL, 4.7 mmol), and a small amount of $Mg(ClO_4)_2$ in toluene (20 mL) was refluxed for 2 h. The water formed during the reaction was removed azeotropically with a Dean-Stark apparatus. The mixture was concentrated under reduced pressure. Then the residue was purified by silica gel column chromatography using CHCl3 as the eluent, followed by size exclusion chromatography with Bio-beads S-X1 beads using THF as the eluent. The green fraction was collected and rotary evaporated to afford the desired product (30 mg, 35%). ¹H NMR (400 MHz, CDCl₃): δ 8.13 (d, J = 16.4 Hz, 2 H, CH=CH), 7.60 (d, J = 8.8 Hz, 4 H, ArH), 7.58 (d, J = 16.4 Hz, 2 H, CH=CH), 7.16 (d, J = 8.4 Hz, 2 H, ArH), 7.06 (d, J = 8.4 Hz, 2 H, ArH), 6.96 (d, J = 8.8 Hz, 4 H, ArH), 4.18-4.23 (m, 6 H, OCH₂), 3.94 (virtual t, J = 4.8 Hz, 2 H, OCH₂), 3.90 (virtual t, J = 4.8 Hz, 4 H, OCH₂), 3.66-3.81 (m, 18 H, OCH₂), 3.55-3.60 (m, 6 H, OCH₂), 3.40 (s, 3 H, OCH_3 , 3.39 (s, 6 H, OCH_3), 1.50 (s, 6 H, CH_3). ¹³C{¹H} NMR (100.6 MHz, CDCl₃): δ 160.1, 159.9, 150.5, 145.9, 139.1, 138.7, 133.4, 129.8, 129.7, 129.4, 127.6, 116.9, 115.6, 115.1, 82.8, 72.1, 71.0, 70.8, 70.7, 69.8, 67.7, 59.2, 17.9 (some of the OCH₂ signals are overlapped). MS (ESI): an isotopic cluster peaking at m/z 1262 {100%, $[M + Na]^+$ }. HRMS (ESI): m/z calcd for C₅₄H₆₇BF₂I₂N₂NaO₁₂ [M + Na]⁺, 1261.2737; found, 1261.2723. Anal. Calcd for C54H67BF2I2N2O12: C, 52.36; H, 5.45; N, 2.26. Found: C, 52.44; H, 5.63; N, 2.13.

Distyryl BODIPY 8. According to the procedure described for 7, BODIPY 1 (0.29 g, 0.4 mmol) was treated with benzaldehyde 4 (0.52 g, 1.2 mmol), glacial acetic acid (0.8 mL, 14.0 mmol), and piperidine (1.0 mL, 10.1 mmol) in toluene (80 mL) to give 8, which was purified by silica gel column chromatography using CHCl₃/MeOH (100:3, v/v) as the eluent, followed by size exclusion chromatography using THF as the eluent. The product was collected as a green solid (96 mg, 16%). ¹H NMR (400 MHz, CD₂Cl₂): δ 8.08 (d, *J* = 16.4 Hz, 2 H, CH=CH), 7.52 (d, *J* = 16.4 Hz, 2 H, CH=CH), 7.17 (d, *J* = 1.6 Hz, 2 H, ArH), 7.10 (d, *J* = 8.4 Hz, 2 H, ArH), 6.97 (d, *J* = 8.4 Hz, 2 H, ArH), 4.20–4.24 (m, 10 H,

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OCH₂), 3.85–3.89 (m, 10 H, OCH₂), 3.69–3.72 (m, 10 H, OCH₂), 3.56–3.66 (m, 20 H, OCH₂), 3.47–3.54 (m, 10 H, OCH₂), 3.35 (s, 3 H, OCH₃), 3.33 (s, 6 H, OCH₃), 3.31 (s, 6 H, OCH₃), 1.53 (s, 6 H, CH₃). ¹³C{¹H} NMR (100.6 MHz, CDCl₃): δ 159.8, 150.4, 148.9, 145.8, 139.2, 138.8, 133.4, 130.4, 129.6, 127.4, 121.6, 117.2, 115.5, 114.3, 114.2, 83.1, 71.9, 70.9, 70.7, 70.6, 70.5, 69.8, 69.7, 69.1, 68.7, 67.6, 59.1, 59.0, 17.8 (some of the OCH₂ signals are overlapped). MS (ESI): an isotopic cluster peaking at *m*/*z* 1585 {100%, [M + Na]⁺}. HRMS (ESI): *m*/*z* calcd for C₆₈H₉₅BF₂I₂N₂NaO₂₀ [M + Na]⁺, 1585.4521; found, 1585.4557. Anal. Calcd for C₆₈H₉₅BF₂I₂N₂O₂₀: C, 52.25; H, 6.13; N, 1.79. Found: C, 51.97; H, 6.27; N, 1.64.

Distyryl BODIPY 9. According to the procedure described for 7, BODIPY 1 (0.29 g, 0.4 mmol) was treated with benzaldehyde 5 (1.42 g, \sim 1.2 mmol), glacial acetic acid (0.8 mL, 14.0 mmol), and piperidine (1.0 mL, 10.1 mmol) in toluene (80 mL) to give 9, which was purified by silica gel column chromatography using CHCl $_3$ /MeOH (10:1, v/v) as the eluent, followed by size exclusion chromatography using THF as the eluent. The product was obtained as an oily green solid (92 mg, 7%). ¹H NMR (400 MHz, CD_2Cl_2): δ 8.08 (d, J = 16.4 Hz, 2 H, CH=CH), 7.52 (d, J = 16.4 Hz, 2 H, CH=CH), 7.30 (d, J = 8.4 Hz, 2 H, ArH), 7.22 (d, *J* = 8.4 Hz, 2 H, ArH), 7.16 (s, 2 H, ArH), 7.10 (d, *J* = 8.4 Hz, 2 H, ArH), 6.98 (d, J = 8.4 Hz, 2 H, ArH), 4.20-4.25 (m, 10 H, OCH₂), 3.87-3.89 (m, 10 H, OCH₂), 3.70-3.72 (m, 10 H, OCH₂), 3.54-3.67 (m, ~170 H, OCH₂), 3.49-3.51 (m, 10 H, OCH₂), 3.35 (s, 3 H, OCH₃), 3.33 (s, 12 H, OCH₃), 1.53 (s, 6 H, CH₃). MS (ESI): an envelope of isotopic clusters of $[M + Na]^+$ at m/z 2995 (37%, for n = 11), 3173 (21%, for n =12), etc.

Photocytotoxicity Assay. Distyryl BODIPYs **6**–**9** were first dissolved in DMF to give 1.6 mM solutions, which were diluted to 80 μ M with an aqueous solution of Tween 80 (Arcos, 0.5% by volume in these 80 μ M solutions). The solutions were filtered with a 0.22 μ m filter, then diluted with the culture medium to appropriate concentrations. The remaining steps were the same as described previously.⁸

Intracellular Fluorescence Studies. About 1.2×10^5 HT29 cells in the culture medium (2 mL) were seeded on a coverslip and incubated overnight at 37 °C under 5% CO₂. The medium was removed. Then the cells were incubated with the solutions of distyryl BODIPYs **6**–**9** in the medium (1 μ M, 2 mL) for 2 h under the same conditions. The cells were rinsed with phosphate buffered saline (PBS) and then viewed with a Leica SP5 confocal microscope equipped with a 633 helium neon laser. Emission signals from 650 to 720 nm (gain of 750 V) were collected, and the images were digitized and analyzed by Leica Application Suite Advanced Fluorescence. The intracellular fluorescence intensities (a total of 50 cells for each sample) were also determined.

Subcellular Localization Studies. About 1.2×10^5 HT29 cells in the culture medium (2 mL) were seeded on a coverslip and incubated overnight at 37 °C under 5% CO₂. The medium was then removed. The cells were incubated with a solution of 8 in the medium $(1 \mu M, 2 mL)$ for 2 h under the same conditions. For the study using ER-Tracker, the cells were incubated with ER-Tracker Green (Molecular Probe, 1 µM in PBS) under the same conditions for a further 30 min. For the study using LysoTracker and MitoTracker, the cells were incubated with LysoTracker Green DND 26 (Molecular Probe, 0.2 μ M in the medium) or MitoTracker Green FM (Molecular Probe, 0.1 μ M in the medium) for a further 10 min. For all the cases, the cells were then rinsed with PBS and viewed with a Leica SP5 confocal microscope equipped with a 488 nm argon laser and a 633 nm helium neon laser. All the Trackers were excited at 488 nm and monitored at 510-560 nm, while 8 was excited at 633 nm and monitored at 650-720 nm. The images were digitized and analyzed using Leica Application Suite Advanced Fluorescence. The subcellular localization of 8 was revealed by comparing the intracellular fluorescence images caused by the ER-Tracker, LysoTracker, or MitoTracker and this dye.

ASSOCIATED CONTENT

Supporting Information. ESI mass spectra of 5 and 9; absorption spectra of 6-9 in DMF; absorption spectra of 8 at various concentrations; comparison of the rates of decay of DPBF in DMF using 6-9 and ZnPc as the photosensitizers; absorption and fluorescence spectra of 6-9 in DMEM; ¹H NMR spectra of 5 and 7-9; and ¹³C{¹H} NMR spectra of 7 and 8. This material is available free of charge via the Internet at http:// pubs.acs.org.

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ABBREVIATIONS USED

BODIPY, boron dipyrromethene; DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; DPBF, 1,3-diphenylisobenzofuran; ESI, electrospray ionization; GFP, green fluorescent protein; PBS, phosphate buffered saline; PDT, photodynamic therapy; PI, propidium iodide; ROS, reactive oxygen species; SEM, standard error of the mean; ZnPc, zinc(II) phthalocyanine; Φ_F , fluorescence quantum yield; IC₅₀, dye concentration required to kill 50% of the cells

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