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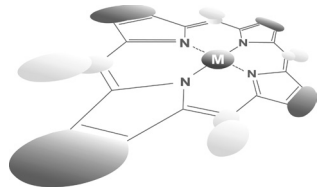

Enhanced *in vitro* photocytotoxicity of water-soluble dendritic pheophorbide-*a*

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ABSTRACT: Photosensitizers can produce highly reactive singlet oxygen with exposure to visible light and are used in photodynamic therapy to treat a variety of tumors. We report on the synthesis of triethylene glycol dendron-conjugated pheophorbide-*a* **5**, a novel photosensitizer. The characteristic absorption bands (Soret and Q-bands, λ_{max} = 405 and 670 nm, respectively) of **5** were appeared clearly in aqueous solution, due to the improved water-solubility of the dendron moiety. The value of singlet oxygen quantum yield of **5** (Φ_{Δ} = 0.22) was higher than free pheophorbide-*a* (Φ_{Δ} = 0.17) as reference in aqueous solution. Compound **5** also exhibited an enhanced *in vitro* phototoxicity than pheophorbide-*a* (PhA) in the concentration range of 1.0–5.0 μg/mL: cell viability in cells treated with **5** was reduced by ~20%, indicating a cell death rate of ~80%, while PhA treatment resulted in a cell death rate of only about 10%. These results indicate that **5** will likely be more efficient in PDT applications. Compared with free PhA, compound **5** showed highly enhanced singlet oxygen generation ability and *in vitro* photocytotoxicity.

KEYWORDS: pheophorbide-*a*, photosensitizer, photodynamic therapy, singlet oxygen, photocytotoxicity.

INTRODUCTION

Photodynamic therapy (PDT) is a photochemical treatment in which biological targets are destroyed with reactive oxygen species, primarily superoxide ions (O_2^-) and singlet oxygen ($^1O_2$), generated by a light-driven photosensitizing process [1]. Upon absorption of light, a photosensitizer (PS) generates excited triplet states *via* excited singlet states, which readily transfer excitation energy to the ground state of molecular oxygen ($^3O_2$) to produce singlet oxygen ($^1O_2$), which, in turn, readily reacts with biological tissues [2]. The inhibitory effects of PDT can be achieved by inducing apoptosis and systemic antitumor immunity through the activation of neutrophils and natural killer cells [3]. Various tetrapyrrolic compounds, including porphyrins [4], chlorins [5], bacteriochlorins [6], and phthalocyanines [7], are used widely as photosensitizers for both clinical and experimental applications of PDT [8]. The first PDT agent used in clinical practice was "Photofrin," a mixture of oligomers formed by ether and ester linkages of up to eight porphyrin units [9]. Porphyrin-based PS agents have, however, been shown to have some undesirable properties including poor absorption in the PDT optical window (>630 nm) and relatively long retention times in biological systems. To overcome these limitations, chlorine-based PS agents, such as *m*-tetrahydroxyphenyl chlorin and mono-aspartyl chlorin e_6, have been developed in the past decade [10, 11]. The chlorin derivative pheophorbide-*a* (PhA) is a promising PS, and has received attention because of its potential application in PDT [12]. Most conventional PS-conjugated drug delivery systems readily aggregate in aqueous media due to strong π–π interactions among PS molecules, resulting

◊ SPP full member in good standing

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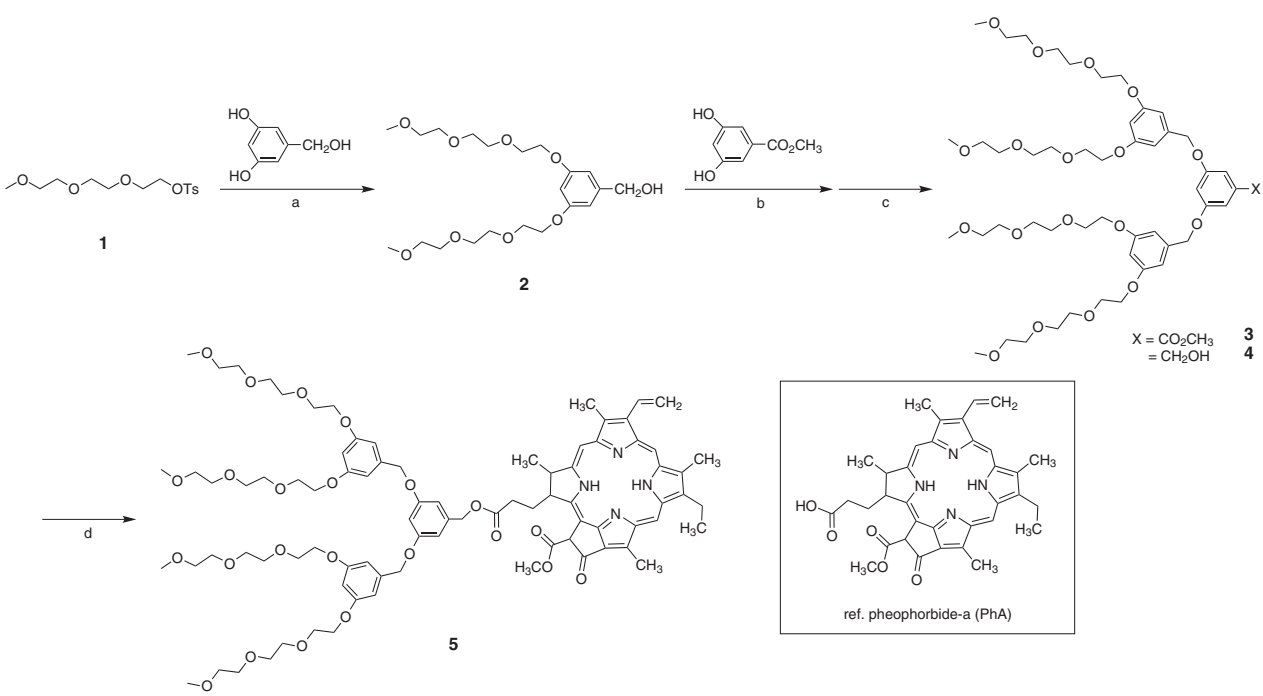
in the suppression of $^1O_2$ generation by self-quenching. Accordingly, PhA is used to chemically/physically bind to solubilizing PS-carriers, including human serum albumin (HSA) [13], Au nanoparticles [14], pullulans [15], PEGylated polymers [16], polymeric micelles [17], and PMMA dendrimers [18] for high therapeutic efficacy. Dendrimers in particular have been investigated widely as carriers due to the relatively facile size-tunability and surface-functionality they exhibit [19]. A dendrimer-encapsulated porphyrin sensitizer was, for example, shown to have strong photodynamic efficacy and singlet oxygen generation [20]. It is also well known that ethylene glycol moieties inhibit aggregate formation and enhance cytotoxicity and tumor-targeting efficacy [21]. It was thus of interest to incorporate hydrophilic dendron units for efficient photodynamic activity. In this study, a dendritic PhA derivative with tri(ethylene glycol) dendron (TEG-dendron) was designed and synthesized, which showed relatively enhanced *in vitro* photocytotoxicity.

RESULTS AND DISCUSSION

Synthesis and structural characterization

As shown in Scheme 1, the overall synthesis of **5** was carried out according to a previously described method with slight modifications [25]. Briefly, compound **1** was produced through tosylation of triethylene glycol monomethyl with *p*-toluenesulfonyl chloride, promoted by alkali conditions. Compound **2** was prepared from the coupling reaction of 3,5-dihydroxybenzyl alcohol with tosylated triethylene glycol monomethyl (**1**). Esterfication of **2** with 3,5-dihydroxybenzyl alcohol using DEAD/TPP was followed by the terminal ester-reduction to produce **4**. Finally, the synthesis of target compound **5** was carried out with **4** and PhA under DCC/DMAP coupling conditions. Compound **5** was purified by recycling GPC and characterized structurally by UV-vis absorption, fluorescence emission, ^{1}H NMR spectroscopy, and MALDI-TOF mass spectrometry. Size-exclusion chromatography (SEC) of **5** and PhA produced sharp elution peaks at retention time (t_R) = 16.8 and 22.3 min, respectively, with minor peaks from column impurities (Fig. S1). The structure of **5** was confirmed by ^{1}H NMR spectrum in $CDCl_3$, where the peripheral methyl group of TEG-dendron appeared at 3.36 ppm as a single peak, while the other TEG-dendron protons appeared over 3.54–4.14 ppm as multiple peaks. The protons from the PhA moiety appeared at 7.63, 6.91, 5.31, 2.18, 1.58 and 1.27 ppm as a single or multiple peaks (Fig. S2a). As can be seen from the MALDI-TOF mass spectrum of **5** (Fig. S2b), a most intense peak at *m/z* = 1543.82 was assigned to the molecular ion $[M + H]^+$ (calculated value: *m/z* = 1543.76) and a minor peak at *m/z* = 1566.79 was observed for the sodium-coupled molecular ion $[M + Na]^+$ (calculated value: *m/z* = 1565.75) where the sodium ion is thought to come from the brine used in the washing process. A partially defective structure (fragmentation) at *m/z* = 1033.54 was also observed with low intensity.



Scheme 1. Synthetic routes of compound **5**: (a) 18-crown-6, K_2CO_3, THF; (b) diethyl azodicarboxylate (DEAD, 40% in toluene), PPh_3, THF; (c) $LiAlH_4$, THF; (d) pheophorbide-*a*, DCC, DMAP, THF

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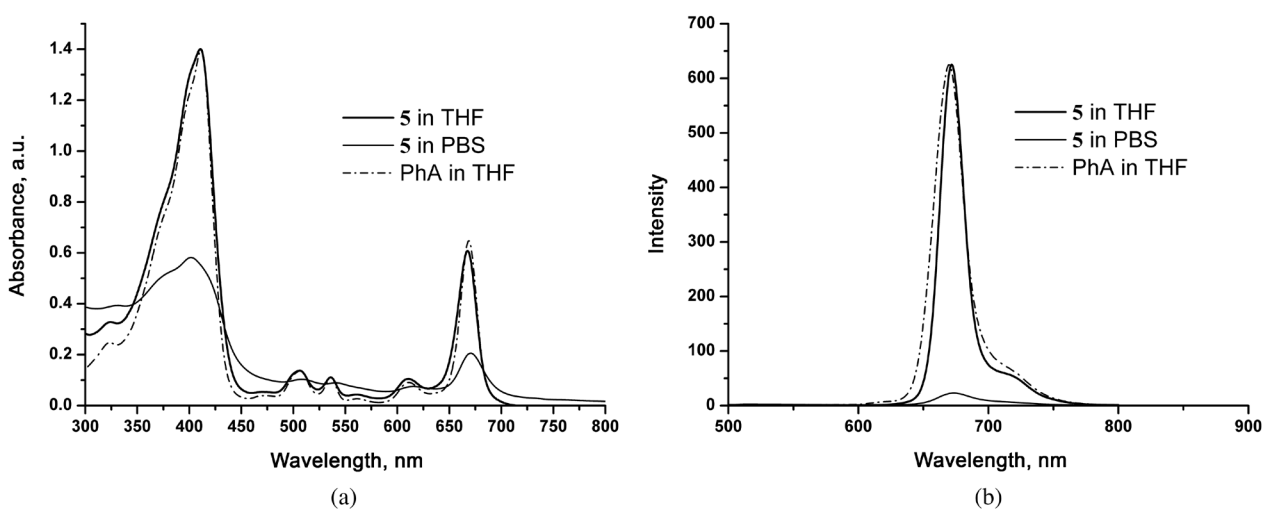
Absorption and emission properties

The absorption and emission spectra of **5** with some references are shown in Fig. 1, where the concentration of all samples was adjusted to 0.28 mM. As shown in Fig. 1a, the UV-vis absorption spectrum of **5** in THF showed an intense and broad Soret band at λ_{max} = 412 nm together with relatively weak Q-Bands at λ_{max} = 507, 536, 611 and 667 nm. These absorption characteristics of **5** correspond closely with those of a THF solution of PhA, indicating that there is a negligible/weak electronic interaction between the PhA moiety and the TEG-dendron moiety in **5**. In PBS buffer, free PhA does not show characteristic absorptions because it is not soluble at all. On the other hand, compound **5** showed the typical absorption features (Soret and Q-bands, λ_{max} = 405 and 670 nm, respectively) in the visible region due to the improved solubility of the TEG-dendron moiety, which is considerably different to a THF solution. The Soret band of **5** was significantly decreased and broadened compared with that of **5** in THF. These results indicate that stable colloidal aggregations of **5** were formed in aqueous medium due to strong hydrophobic and π–π interactions among PhA moieties, where the particle size was determined to be ~200 nm by dynamic light scattering measurements (Fig. S3). Particle sizes of >200 nm can be problematic in PDT applications due to the resulting insufficient circulation time in the bloodstream [26]. As shown in Fig. 1b, the emission characteristic of **5** (λ_{max} = 671 nm; λ_{ext} = 412 nm) in THF at room temperature were found to be very similar to those measured for PhA (λ_{max} = 671 nm). The fluorescence intensity of **5** in PBS was largely reduced, by 97%, compared with the intensity of **5** in THF, because fluorescence quenching occurs readily by collisions between molecular excited states in colloidal particles, which would lead to low cytotoxicity during circulation in the blood. The fluorescence of **5** may be recovered by decomposition of the colloidal particles in intracellular environment.

Singlet oxygen generation

The $^1O_2$ generation ability of **5** was determined by measuring the fluorescence intensity of 1,3-diphenylisobenzofuran (DPBF) with time (Fig. 2). In this process, photobleaching of the DPBF can be used to monitor singlet oxygen generation where DPBF reacts readily with $^1O_2$, causing a decrease in fluorescence intensity of DPBF in the range of 420–600 nm. In DMF solution (Fig. 2a), the DPBF fluorescence intensity was slightly decreased, by 10%, at an early irradiation time (120 s). At the middle stage of the irradiation (240–360 s), fluorescence was quenched by 60% of the initial intensity. Finally, the fluorescence intensity of DPBF was largely quenched, by 95%, at the final stage of the irradiation time (600 s), as compared with no irradiation. In contrast, a solution of **5** in PBS buffer (Fig. 2b) showed a relatively smaller decrease (~20%) in DPBF fluorescence with irradiation time (120–720 s) because particles that do not facilitate efficient triplet-triplet energy transfer from **5** to oxygen molecules ($^3O_2$) to give $^1O_2$ are formed. These results correlate with the change in fluorescence intensity measured for **5**, as described above. A solution of PhA in DMF and PBS buffer, as a reference, showed slightly higher and lower photobleaching behaviors in DPBF fluorescence compared to those of **5** (Fig. 2c), respectively. Thus, compound **5** was found to have an efficient ability to generate singlet oxygen, similar to PhA with improved water solubility. The singlet oxygen quantum yield (SOQ) was evaluated according to a previous report [27]. The value of SOQ of **5** (Φ_Δ = 0.49) was slightly lower than PhA (Φ_Δ = 0.52) in DMF solution.



(a) (b)

Fig. 1. (a) Absorption and (b) emission spectra of **5** and PhA in THF or PBS

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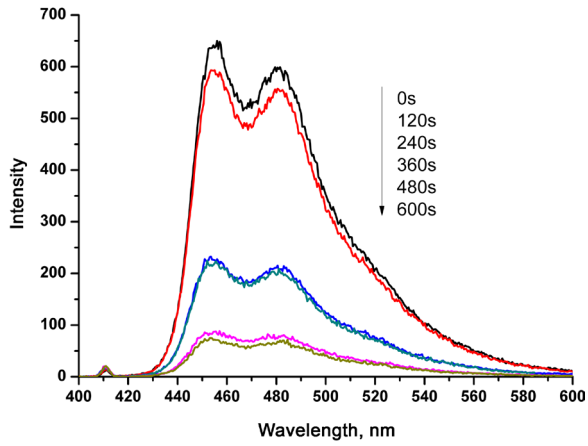

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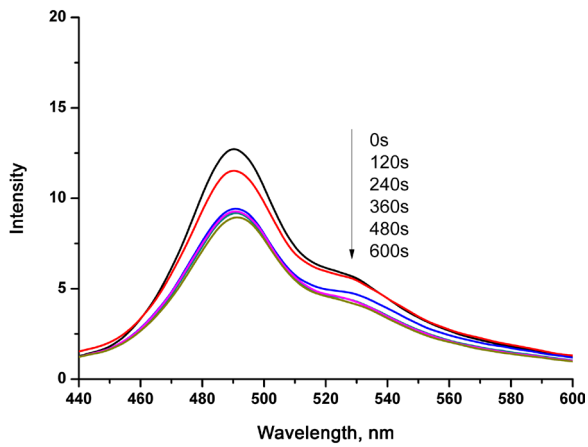

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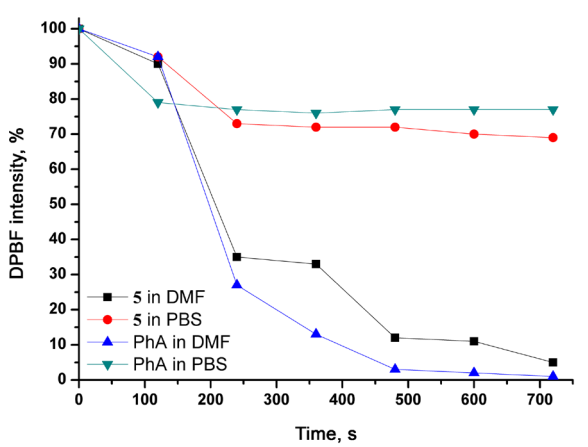
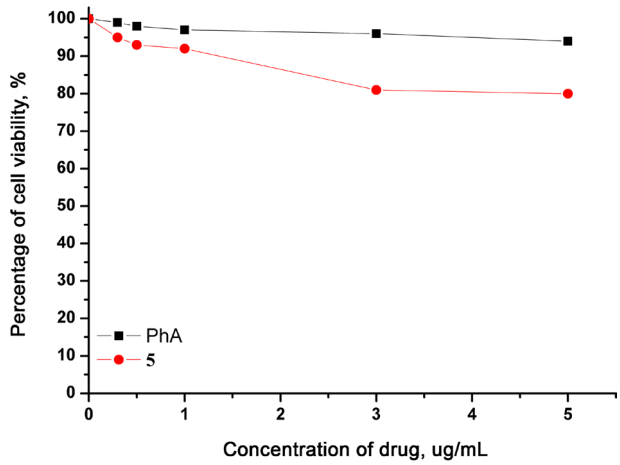


(c)

Fig. 2. Photobleaching of DPBF fluorescence (λ_{ext} = 360 nm) upon excitation of **5** with 670 nm laser for 0, 120, 240, 360, 480 and 600 s (a) in DMF (Φ_Δ = 0.49) (b) or in PBS buffer (Φ_Δ = 0.22) at room temperature. (c) Plot of change in DPBF fluorescence intensity of **5** *vs*. PhA in DMF and PBS

However, **5** (Φ_Δ = 0.22) in PBS showed an increase in SOQ value, by ~30% *vs*. that of PhA (Φ_Δ = 0.17). These results indicate that **5** will likely be more efficient in PDT applications.



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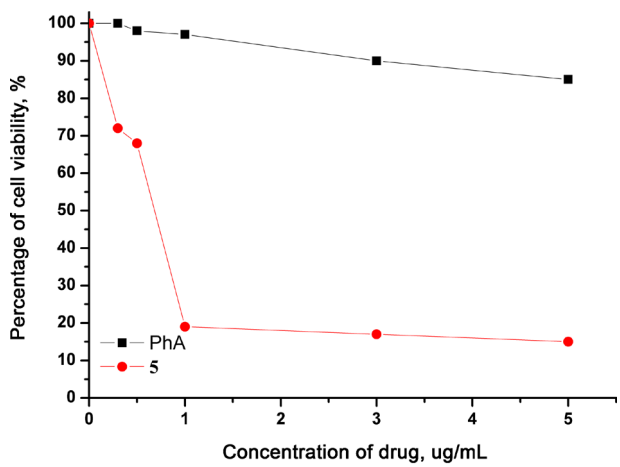

(b)

Fig. 3. Cell viability assessment of MCT-7 cells with **5** and PhA (a) without light irradiation and (b) with light irradiation

In vitro phototoxicity

The *in vitro* phototoxicity of **5** in breast cancer cells (MCF-7) was examined using the MTT assay, which is a colorimetric assay to assess cell viability. In the assay, a yellow tetrazole MTT is reduced to the optically detectable purple formazan by living cells [28]. Upon light irradiation with 100 mW/cm^2 at 670 nm, **5** displayed higher phototoxicity than PhA in the concentration range of 1.0–5.0 μg/mL: cell viability in cells treated with **5** was reduced by ~20%, indicating a cell death rate of about 80%, while PhA treatment resulted in a cell death rate of only ~10% (Fig. 3). In contrast, the dark cytotoxicity (without light treatment) was significantly lower, showing a cell death rate of ~10% for PhA and ~20% for **5**. There was no significant difference in the dark cytotoxicity between **5** and PhA because an acceptable cell viability above 80% was obtained in the concentration range of 1.0–5.0 μg/mL. The result of MTT assay indicated that **5** is more efficient in photodynamic theraphy than PhA due to improved water-solubility.

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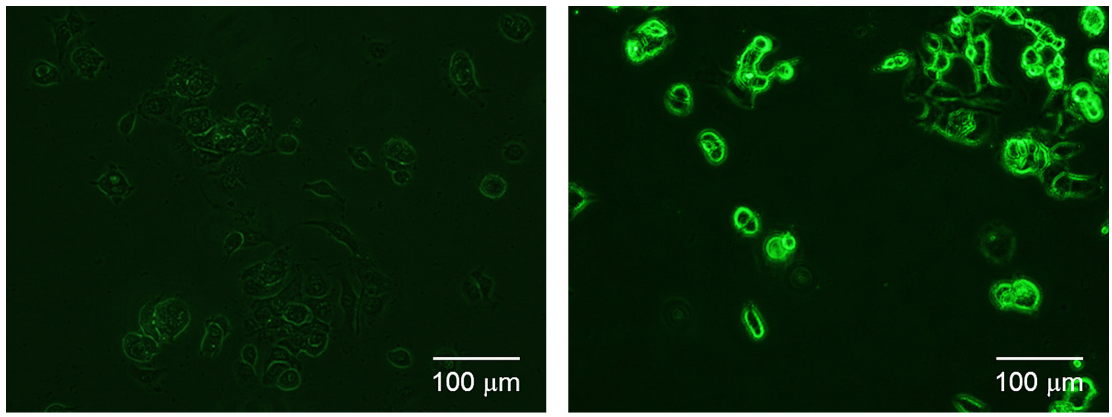

(a) (b)

Fig. 4. Apoptotic cell death study in MCF-7 cells treated with (a) PhA and (b) **5** using Annexin V-FITC

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The phototoxicity-induced apoptosis in cells treated with **5** was assessed by an Annexin V-FITC study. Apoptotic cells lose the asymmetry of membrane phospholipids, resulting in exposure of phosphatidylserine on the outer leaflet of the plasma membrane. Cells were thus stained with Annexin V-FITC, which binds with phosphatidylserine and gives a green emission at the outer membrane [29]. It is well known that PhA can cause apoptotic cell death. As shown in Fig. 6, a stronger fluorescent signal, indicative of apoptotic cells, was detected in irradiated MCF-7 cells treated with **5** (Fig. 4b) than in irradiated cells treated with PhA (Fig. 4a).

EXPERIMENTAL

Materials

4-Dimethyaminopyridine (DMAP), 3.5-dihydroxybenzyl alcohol, and methyl-3.5-dihydroxybenzoate were purchased from Tokyo Chemical Industry. 1,3-Dicyclohexylcarbodiimide (DCC), diethyl azodicarboxylate (DEAD, 40% toluene solution), diphenylisobenzofuran (DPBF), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and Annexin V-FITC were purchased from Sigma-Aldrich Co., (St. Louis, MO, USA). Pheophorbide *a* (PhA) was purchased from Frontier Scientific, Inc., (Salt Lake City, UT, USA). Human breast cancer cell line (MCF-7) was received from the Korean Cell Line Bank (KCLB, Korea). RPMI-1640, fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Gibco BRL (USA). Annexin V-fluorescent isothiocyanate (FITC) fluorescence microscopy kit was from BD Biosciences (USA). All chemicals were used without further purification. Tosylated triethylene glycol monomethyl ether (**1**) was prepared according to a previously described method [22]. Deionized water was purified using a U Pure Power deionization system.

Characterization

Basic contamination in the reaction mixture was removed by column chromatography using silica gel 60 (Merck, 0.063–0.200 mm). Further purification was carried out on a recycling preparative HPLC (Japan Analytical Industry model LC-9201) equipped with column set consisting of JAIGEL-2H and JAIGEL-2.5H with THF as the eluent at a flow rate of 3.5 mL.min^{-1}. Analytical size-exclusion chromatography (SEC) was performed on a Young Lin Instrument (model YL 9100) with DMF as the eluent. UV-vis absorption spectra were recorded on a JASCO V-670 spectrometer and steady-state fluorescence spectra were measured with a Perkin Elmer LS 55 fluorescence spectrometer. ^{1}H NMR data were recorded on a Mercury Plus 300 MHz spectrometer at ambient temperature using an internal deuterium lock. MALDI-TOF mass spectrometry was performed on a Voyager-DETM STR Biospectrometry Workstation, equipped with a 337 nm N_2 laser producing 3 ns pulses at a repetition rate to 20 Hz in reflector mode. Particle size and distribution were measured by dynamic light scattering (DLS) using a K-ONE model scatteroscope.

Synthesis

Compound 1. A solution of NaOH (14.3 g, 0.357 mol) in water (62 mL) was mixed with a solution of triethylene glycol monoethyl ether (50.0 g, 0.243 mol) in dry THF (62 mL), and then a solution of 4-toluenesulfonyl chloride (55.5 g, 0.291 mol) in dry THF (62 mL) was slowly added drop-wise. After stirring for 18 h at room temperature, the reaction mixture was poured into water and extracted with CH_2Cl_2 (3 × 50 mL). Collected organic layers were washed with water and brine, and dried with $MgSO_4$. The solvent was evaporated and the residue was purified by column chromatography on silica gel using a solvent mixture of CH_2Cl_2/ethyl acetate (9:1) as eluent. The second band was collected and evaporated to dryness to give a colorless oil (yield 74%). ^{1}H NMR ($CDCl_3$, 300 MHz):

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δ, ppm 7.80 (d, *J* = 4.1 Hz, 2H), 7.35 (t, *J* = 0.3 Hz, 2H), 4.16 (t, *J* = 0.6 Hz, 2H), 3.69–3.53 (m, 10H), 3.37 (s, 3H).

Compound 2. A mixture of 3,5-dihydroxybenzyl-alcohol (7.7 g, 0.055 mol), K_2CO_3 (76 g, 0.55 mol), 18-crown-6 (5.8 g, 0.022 mol), and **1** (35 g, 0.11 mol) were added to dry THF (550 mL), and the solution was refluxed for 12 h. The reaction mixture was concentrated and worked up by adding an equal volume of CH_2Cl_2 and water. Collected organic layers were washed with water and brine, and dried with $MgSO_4$. The solvent was evaporated and the residue was purified by column chromatography on silica gel using a solvent mixture of CH_2Cl_2/methanol (10:1) as the eluent. The second band was collected and evaporated to dryness to give a colorless oil (yield 72%). ^{1}H NMR (300 MHz, $CDCl_3$): δ, ppm 6.53 (d, *J* = 2.3 Hz, 2H), 6.32 (d, *J* = 2.3, 1H), 4.52 (s, 2H), 4.18–3.47 (m, 12H), 3.31 (s, 6H), 1.98 (s, 1H).

Compound 3. A 40% toluene solution (11.56 mL) with diethyl azodicarboxylate (4.43 g, 25.43 mmol) was added to a THF solution (150 mL) of 3,5-dihydroxybenzoate (1.95 g, 11.56 mmol), **2** (10 g, 23.12 mmol) and triphenyl phosphine (6.67 g, 25.43 mol). The solution was stirred at room temperature for 24 h. The work up was carried out in a manner similar to that described above for **2**. The crude product was isolated by column chromatography on silica gel using a solvent mixture of ethyl acetate/methanol (10:1) as the eluent. The fourth band was collected and evaporated to dryness to give a colorless oil (yield 70%). ^{1}H NMR (300 MHz, $CDCl_3$): δ, ppm 7.26 (s, 2H), 6.76 (s, 1H), 6.59 (d, *J* = 2.3 Hz, 4H), 6.46 (d, *J* = 2.3 Hz, 2H), 4.99 (s, 4H), 3.91 (s, 3H), 4.13–3.54 (m, 48H), 3.37 (s, 12H).

Compound 4. A solution of **3** (4.2 g, 4.21 mmol) dispersed in THF (150 mL) was added to a THF solution (100 mL) of $LiAlH_4$ (0.24 g, 6.32 mol). The solution was stirred at 4 °C for 1 h. The workup was carried out in a manner similar to that described above for **2**. The crude product was isolated by column chromatography on silica gel using a solvent mixture of ethyl acetate/methanol (10:1) as the eluent. The first band was collected and evaporated to dryness to give a colorless oil (yield 61%). ^{1}H NMR (300 MHz, $CDCl_3$): δ, ppm 6.59 (d, *J* = 2.3 Hz, 2H), 6.58 (d, *J* = 2.3 Hz, 4H), 6.50 (s, 1H), 6.43 (d, *J* = 2.3 Hz, 2H), 4.96 (s, 4H), 4.62 (s, 2H), 4.13–3.54 (m, 48H), 3.37 (s, 12H).

Compound 5. PhA (0.2 g, 0.34 mmol) was added to a THF solution (100 mL) of **4** (0.39 g, 0.405 mmol), DCC (0.1 g, 0.51 mmol) and DMAP (0.06 g, 0.51 mol). The solution was stirred at room temperature for 24 h. The workup was carried out in a manner similar to that described above. The crude product was isolated by column chromatography on silica gel using a solvent mixture of ethyl acetate/methanol (10:1) as the eluent. The second band was collected and evaporated to dryness to give a greenish oil (yield 24%). ^{1}H NMR (300 MHz, $CDCl_3$): δ, ppm 7.63 (s, 1H), 6.91 (s, 1H), 6.59–6.58 (m, 8H), 6.44–6.42 (m, 2H), 5.31 (s, 1H), 5.04 (s, 4H), 4.95 (s, 4H), 4.14–4.09 (m, 12H), 3.87–3.83 (m, 9H), 3.76–3.65 (m, 24H), 3.57–3.54 (m, 10H), 3.36 (s, 12H), 2.19–2.17 (m, 16H), 1.58–1.57 (m, 3H), 1.29–1.25 (m, 3H). MS (MALDI-TOF): *m/z* 1543.82 (cacld. for $[M + H]^+$ 1543.76).

Evaluation of singlet oxygen generation

1,4-Diphenyl-2,3-benzofuran (DPBF) was used as singlet oxygen ($^1O_2$) chemical quencher to evaluate the capability of **5** to generate $^1O_2$ for PDT applications [23]. To prepare solution samples, a DMF solution of **5** (0.97 mM) containing DPBF (20 mM) was 1000-fold diluted with DMF or PBS buffer (pH 7.4). Sample solutions in DMF and PBS were continually irradiated by a 670 nm laser at room temperature while the DPBF fluorescence emission (λ_{ext} = 360 nm) was recorded with time (0–600 s). The singlet oxygen quantum (SOQ) yield was determined using the equation described in a previous report [15].

Cell culture and incubation conditions

A human breast cancer cell line (MCF-7) was used for all cell culture experiments. MCF-7 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. When the cells reached 80% confluence, they were harvested by 0.25% trypsin-EDTA and seeded into new tissue culture plates for subculture. Compound **5** and free PhA were dispersed in serum-free medium. Irradiated untreated cells and untreated cells kept in the dark were used as controls.

Cell phototoxicity assay

To determine cell viability under dark conditions, MCF-7 cells (1×10^4 cells/well) were seeded in 96-well plates and incubated for 24 h at 37 °C. After cell stabilization, the culture medium was replaced with 200 μL serum-free culture medium containing **5** or PhA (0–5 μg/mL), followed by incubation for 4 h. The cells were then washed twice with serum-free medium and cell viability was evaluated after 24 h using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. To determine *in vitro* phototoxicity of **5** and PhA after laser irradiation, MCF-7 cells (1×10^4 cells/well) were seeded in 96-well plates and incubated for 24 h at 37 °C. These cells were then treated with serum-free medium containing **5** or free PhA (0–5 μg/mL). After a 4-h incubation, the cells were washed twice with serum-free medium and irradiated with a 670 nm diode laser (100 mW/cm^2) for 3 min. To avoid any photothermal-induced cell damage, the irradiation time was limited to below 9 min [24]. The cell viability of irradiated cells was evaluated by the MTT assay after 24 h incubation using a microplate reader (OPSYS-MR, Dynex Technology Inc., USA). The mechanism of this

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assay is that the metabollically active cells react with tetrazolium salt in the MTT reagent to produce water-insoluble formazan dye that can be observed at 570 nm.

Apoptosis analysis

Cellular apoptosis was visualized using an Annexin V-FITC fluorescence microscopy kit. MCF-7 cells (1×10^4 cells/well) were seeded on 8-well chamber slides for 24 h before being treated with **5** or free PhA (0–5 μg/mL) for 4 h. Cells were then rinsed twice with Dulbecco's phosphate-buffered saline (DPBS) and illuminated with a 670 nm diode laser (100 mW/cm^2) for 3 min. The cells were stained with l mL Annexin V-FITC (10% w/w) for 15 min at room temperature, after which they were washed twice with DPBS and fixed using 4% paraformaldehyde in DPBS for 30 min. A cover slip was mounted on a microscope slide with a drop of anti-fade mounting solution to reduce fluorescence photobleaching. Cells were observed with an inverted fluorescence microscope (Eclipse TS100, Nikon, Japan) using a filter set for FITC visualization.

CONCLUSION

In this study, a novel photosensitizer **5** with improved water solubility was developed. Light irradiation showed that **5** exhibited efficient $^1O_2$ generation, and was found to form particles of a suitable size (<200 nm in PBS) for PDT applications. As determined by an MTT cell viability assay, **5** exhibits a higher phototoxicity than PhA at concentrations of 1.0–5.0 μg/mL, while no significant difference was measured in the dark toxicities of the drugs. An Annexin V-FITC assessment clearly demonstrated that **5** was capable of inducing more apoptotic cell death than PhA. Further work is in progress to optimize **5** towards reducing *in vivo* cytotoxicity for clinical applications.

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Supporting information

Figures S1–S3 are given in the supplementary material. This material is available free of charge *via* the Internet at http://www.worldscinet.com/jpp/jpp.shtml.

REFERENCES

1. Celli JP, Spring BQ, Rizvi I, Evans CL, Samkoe KS, Verma S, Pogue BW and Hansan T. *Chem. Rev.* 2010; **110**: 2795.
2. *The Porphyrin Handbook; Synthesis of Meso Substituted Porphyrins*, Vol. 3, Kadish KM, Smith KM and Guilard R. (Eds.) Academic Press: Boston, 2002; pp 48–118.
3. Kousis PC, Henderson BW, Maier PG and Gollnick SO. *Cancer. Res*. 2007; **67**: 10501.
4. Garcia G, Hammerer F, Poyer F, Achelle S, Teulade-Fichou MP and Maillard P. *Bioorg. Med. Chem.* 2013; **21**: 153.
5. Bae BC, Li F, Ling D and Na K. *J. Porphyrins Phthalocyanines*. 2010; **14**: 851.
6. Chen CY, Sun E, Fan D, Taniguchi M, McDowell BE, Yang E, Diers JR, Bocian DF, Holten D, and Lindsey JS. *Inog. Chem*. 2012; **51**: 9443.
7. Goslinski T, Osmalek T, Konopka K, Wierzchowski M, Fita P and Mielcarek J. *Polyhedron.* 2011; **30**: 1538.
8. MacDonald IJ and Dougherty TJ. *J. Porphyrins Phthalocyanines* 2001; **5**: 105.
9. Lipson RL, Baldes EJ and Olsen AM. *J. Nat. Cancer. Inst*. 1961; **26**: 1.
10. Oleinick NL, Morris RL and Belichenko I. *Photochem Photobiol. Sci*. 2002; **1**: 1.
11. Pawel M, Sulbha KS, Timur Z, Ying-Ying H and Michael R. *Drug Delivery in Oncology*, WILEY-VCH: Weinheim, Germany, 2012; pp 1569–1603.
12. Chan JY, Tang PM, Hon PM, Au SW, Tsui SK, Waye MM, Kong SK, Mak TC and Fung KP. *Planta. Med*. 2006; **72**: 28.
13. Chen K, Preuß A, Hackbarth S, Wacker M, Langer K and Roder B. *J. Photochem. Photobiol., B* 2009; **96**: 66.
14. Li L, Nurunnabi M, Nafiujjaman M, Lee Y-K and Huh KM. *J. Controled Release* 2013; **171**: 241.
15. Bae BC and Na K. *Biomaterials* 2010; **31**: 6325.
16. Hamblin MR, Miller JL, Rizvi I, Ortel B, Maytin EV and Hasan T. *Cancer Res.* 2001; **61**: 7155.
17. Knop K, Mingotaud AF, El-Akra N, Violleau F and Souchard JP. *Photochem. Photobiol. Sci*. 2009; **8**: 396.
18. Hackbarth S, Horneffer V, Wiehe A, Hillenkamp F and Roder B. *Chem. Phys*. 2001; **269**: 339.
19. Malik N, Evagorou EG and Duncan R. *AntiCancer Drugs* 1999; **10**: 767.
20. Nishiyama N, Stapert HR, Zhang GD, Takasu D, Jiang DL, Nagano T, Aida T and Kataoka K. *Bioconjugate Chem*. 2003; **14**: 58.
21. Kimani S, Ghosh G, Ghogare A, Rudshteyn B, Bartusik D, Hasan T and Greer A. *J. Org. Chem*. 2012; **77**: 10638.
22. Hao J, Servello J, Sista P, Biewer MC and Stefan MC. *J. Materials. Chem*. 2011; **21**: 10623.
23. Gomes A, Fernandes E and Lima J. *J. Biochem. Biophys. Methods* 2005; **65**: 45.
24. Wang X, Yang CX, Chen JT and Yan XP. *Anal. Chem*. 2014; **86**: 3263.
25. (a) Yuan Z, Chen J, Zeng Y, Li YY, Han Y and Li Y. *Org. Biomol. Chem*. 2011; **9**: 6256. (b) But TYS and Toy PH. *J. Am. Chem. Soc*. 2006; **128**: 9636.

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(c) Neises B and Steglich W. *Angew. Chem*. 1978; **17**: 522. (d) Oar MA, Serin JM, Dichtel WR and Frechet JMJ. *Chem. Mater*. 2005; **17**: 2267.
26. Song JY, Kong HJ and Choi MS. *J. Porphyrins Phthalocyanines* 2012; **16**: 1196.
27. Hoebeke M and Damoiseau X. *Photochem. Photobiol. Sci*. 2002; **1**: 283.
28. Berridge MV, Herst PM and Tan AS. *Biotechnol. Annu. Rev*. 2005; **11**: 127.
29. Thomas AP, Saneesh Babu PS, Asha Nair S, Ramakrishnan S, Ramaiah D, Chandrashekar TK. Srinivasan A and Pillai MR. *J. Med. Chem*. 2012; **55**: 511.

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