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Comparison between non-peripherally and peripherally tetra-substituted zinc (II) phthalocyanines as photosensitizers: Synthesis, spectroscopic, photochemical and photobiological properties

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ABSTRACT

A series of zinc phthalocyanines tetra-α-substituted with 4-(butoxycarbonyl) phenoxy groups (**1a**) or 4-carboxylphenoxy groups (**2a**) or 4-(2-carboxyl-ethyl)phenoxy groups (**3a**), and the corresponding tetra-β-substituted (**1**–**3b**) analogues, have been synthesized and characterized. The effects of the position of substituents at the phthalocyanine skeleton on their spectroscopic, photochemical and photobiological properties have been revealed. When compared with the tetra-β-substituted phthalocyanines, the corresponding tetra-α-substituted analogues exhibit a less aggregating trend in the cellular growth medium, a slightly higher singlet oxygen quantum yield and higher photo-stability in DMF, and a comparable cellular uptake. As a result, the tetra-α-substituted zinc phthalocyanines exhibit a higher photocytotoxicity toward MGC803 human gastric carcinoma cells than the tetra-β-substituted counterparts. Among all these compounds, phthalocyanine **2a** shows the highest photodynamic activity, which may mainly be due to its non-aggregated nature in cellular culture medium and high cellular uptake.

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1. Introduction

Photodynamic therapy (PDT) is an attractive minimal-invasive approach for the treatment of a variety of cancers by the combined action of a photosensitizing drug and low energy light [1,2]. The treatment involves the administration of the photosensitizer, which is preferentially retained by malignant tissues, followed by illumination with light of an appropriate wavelength and dose. The tumor-localized photosensitizer absorbs the light to produce intracellular reactive oxygen species, particularly singlet oxygen, and results in tumor destruction. For successful PDT, the performance of photosensitizers plays a crucial role. However, the main photosensitizer approved for systemic oncological treatment (e.g. Photofrin® and Foscan®) still have some deficiencies such as poor absorption of tissue-penetrating red light, low initial selectivity and long cutaneous photosensitivity [3,4]. As a result, a large numbers of studies have been devoted to develop new photosensitizers with improved properties over the past 20 years. Among the latter, phthalocyanines have been proposed as the second-generation photosensitizers because of their many advantages such as the intense absorption in the red visible region, high efficiency to generate singlet oxygen, ease of chemical modification, and low dark toxicity [3,5–7].

We hereby describe the synthesis, spectroscopic and photochemical properties, and in vitro photodynamic activities of novel zinc (II) phthalocyanines (ZnPcs) tetra-substituted with carboxyl derivatives at non-peripheral or peripheral positions (namely so called α- or β-positions) of the macrocycle ring. For comparison, this study also involves a tetra-β-substituted analogue (**2b**, Scheme 1), of which the synthesis has been recently reported [8,9], but its photochemical and biological activity remains to be examined.

Owing to having water-solubility, a beneficial factor for transportation in vivo, the phthalocyanines containing carboxyls have received much interest. For example, Ng and co-workers [10] developed a novel β-substituted hexadeca-carboxy ZnPc, which showed a high and selective photodynamic activity against J744 mammary tumor cells; the same compound and its effective photocytotoxicity against human HEp2 cells were also reported by Vicente and co-workers [11]. However, the related studies mostly focused on the β-substituted compounds [8–24]. The phthalocyanine with the carboxyl-containing groups at the α-positions are very rarely reported [25].

Even for the phthalocyanine-based photosensitizers modified by other types of functional groups, only a little attention has been

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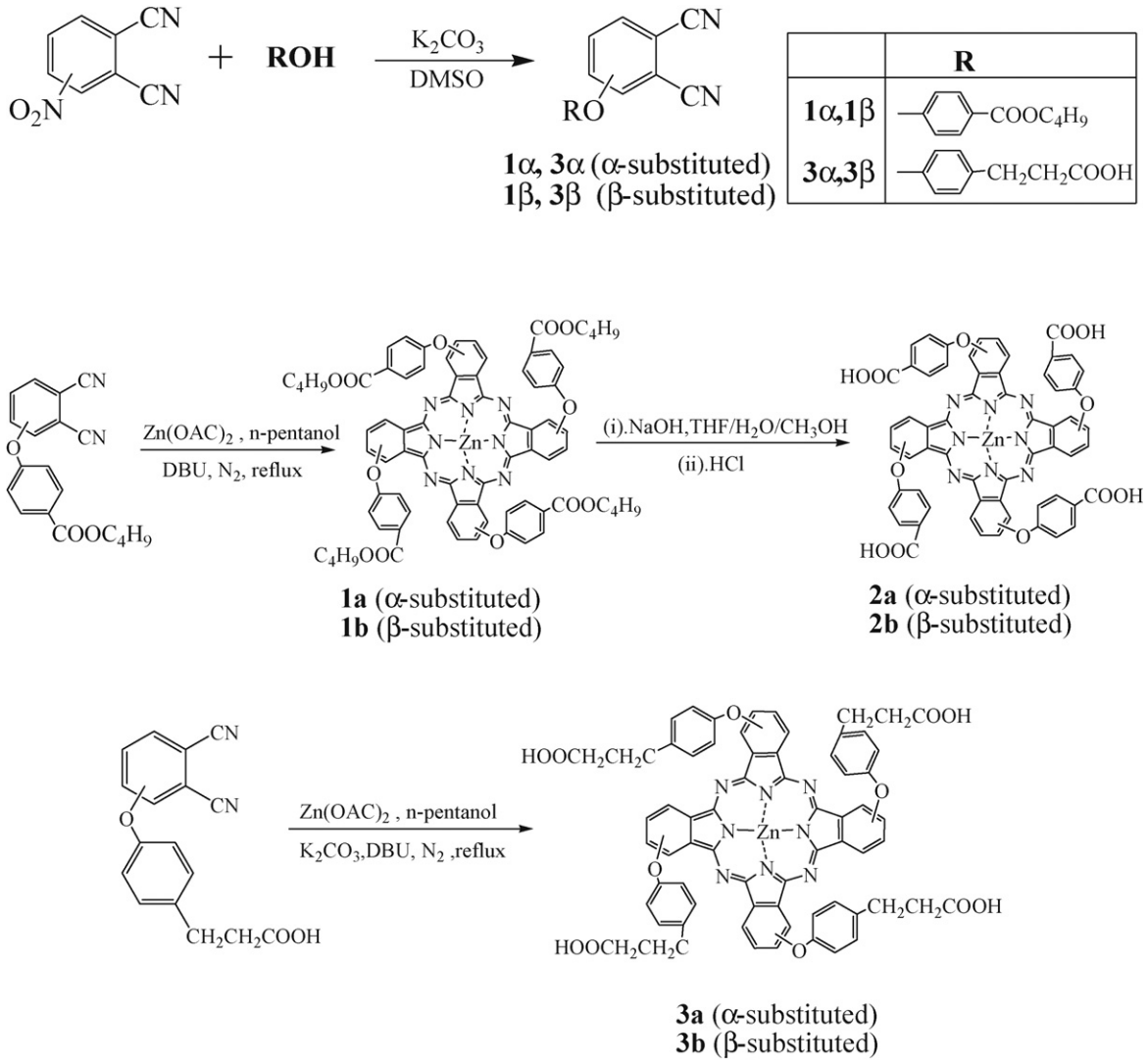

Scheme 1. Synthesis of phthalocyanine derivatives.

given to the substitution at the α-positions [26–32]. Furthermore, the difference in cellular photodynamic activity of non-peripherally and peripherally tetra-substituted phthalocyanines has not been revealed. Hence, the main aim of this work is to explore the influences of the position of substituents at the phthalocyanine skeleton on their photochemical and photobiological properties. In order to optimize PDT agents, it is necessary to carefully evaluate the structure–activity relationship of photosensitizers via variedly substituted phthalocyanines.

2. Experimental

2.1. Materials and equipment

Dimethyl sulfoxide (DMSO) and *n*-pentanol were dried over molecular sieves and further distilled before use. Potassium carbonate and zinc acetate were dried at 110 °C under normal pressure before use. All other solvents and reagents were used as received. Merck Silica Gel 60F254 plates were used for thin-layer chromatography (TLC) and the spots were detected under UV light (254 nm).

^{1}H NMR spectra were recorded on a Bruker DPX 300 spectrometer (300 MHz) in $CDCl_3$ or [D_6]DMSO. Chemical shifts were relative to internal $SiMe_4$ (δ = 0 ppm). Mass spectra were recorded on a Finnigan LCQ Deca xpMAX mass spectrometer. Elemental analyses were performed by Element Vario EL III equipment. IR spectra were recorded on a PerkinElmer SP2000 FT-IR spectrometer, using KBr disks. Electronic absorption spectra were measured on a PerkinElmer Lambda 900 UV–vis-NIR spectrophotometer. Fluorescence spectra were taken on an Edinburgh FL900/FS900 spectrofluorometer.

2.2. Synthesis

2.2.1. 3-[4-(Butoxycarbonyl)phenoxy]phthalonitrile (**1α**)

A mixture of 3-nitrophthalonitrile (0.87 g, 5 mmol), 4-(butoxycarbonyl)phenol (0.97 g, 5 mmol) and anhydrous K_2CO_3 (1.38 g, 10 mmol) in dry DMSO (20 ml) was stirred at room temperature for 15 h under nitrogen. The reaction mixture was poured into ice water (200 ml) to give white precipitate, which was collected by filtration, washed with water until pH 7 and dried in vacuum. The crude product was purified by recrystallisation with CH_2Cl_2/ethanol to afford white solid **1α** (1.33 g, 83%). R_f = 0.55 (CH_2Cl_2). IR (KBr, cm^{-1}): 2982.1 (CH_3); 2886.9, 2833.5 (CH_2); 2236.4 (C≡N); 1683.8 (C=O). MS (ESI): *m*/*z* 320.3 $[M]^-$. ^{1}H NMR ($CDCl_3$, ppm): 8.14 (d, *J* = 4.25 Hz, 2H, Ar—H); 7.63 (t, *J* = 5.33 Hz, 1H, Ar—H); 7.54 (d, *J* = 7.35 Hz, 1H, Ar—H); 7.13–7.18 (m, 3H, Ar—H); 4.34

(t, J = 4.33 Hz, 2H, O—CH_2); 1.77 (t, J = 4.67 Hz, 2H, CH_2); 1.48–1.51 (m, 2H, CH_2—Me); 0.99 (t, J = 4.83 Hz, 3H, CH_3). Anal. Calcd for $C_{19}H_{16}N_2O_3$: C, 71.24; H, 5.03; N, 8.74. Found: C, 71.20; H, 5.22; N, 8.76.

*2.2.2. 4-[4-(Butoxycarbonyl)phenoxy]phthalonitrile (**1β**)*

According to the above procedure, 4-nitrophthalonitrile (0.87 g, 5 mmol) was treated with 4-(butoxycarbonyl)phenol (0.97 g, 5 mmol) and K_2CO_3 (1.38 g, 10 mmol) in DMSO (20 ml) to give white solid **1β** (1.20 g, 75%). R_f = 0.63 (CH_2Cl_2). IR (KBr, cm^{-1}): 2956.8 (CH_3); 2873.2 (CH_2); 2231.2 (C≡N); 1706.8 (C=O). MS (ESI): m/z 319.5 $[M-H]^-$. ^1H NMR ($CDCl_3$, ppm): 8.14–8.18 (m, 2H, Ar—H); 7.77 (d, J = 4.32 Hz, 1H, Ar—H); 7.35 (d, J = 1.22 Hz, 1H, Ar—H); 7.28–7.31 (m, 1H, Ar—H); 7.11–7.15 (m, 2H, Ar—H); 4.35 (t, J = 4.39 Hz, 2H, O—CH_2); 1.72–1.82 (m, 2H, CH_2); 1.43–1.55 (m, 2H, CH_2—Me); 0.99 (t, J = 4.91 Hz, 3H, CH_3). Anal. Calcd for $C_{19}H_{16}N_2O_3$: C, 71.24; H, 5.03; N, 8.74. Found: C, 71.16; H, 5.10; N, 8.85.

*2.2.3. 3-[4-(2-Carboxyl-ethyl)phenoxy]phthalonitrile (**3α**)*

A mixture of 3-nitrophthalonitrile (0.87 g, 5 mmol), 4-(2-carboxyl-ethyl)phenol (0.83 g, 5 mmol) and anhydrous K_2CO_3 (2.10 g, 15 mmol) in dry DMSO (35 ml) was stirred at 70 °C for 15 h under nitrogen. The reaction mixture was filtered, and then the filtrate was treated with ice water (200 ml) and acidified by 1 M HCl to induce precipitation. The brown precipitate was collected by filtration, and washed with water until pH 7, and dried in vacuum. The crude product was further purified by recrystallisation from acetone/water to afford the brown solid **3α** (0.93 g, 64 %). R_f = 0.58 (EtOH). IR (KBr, cm^{-1}): 3440.5 (O—H); 2934.5 (CH_2); 2232.8 (C≡N); 1709.5 (C=O). MS (ESI): m/z 292.3 $[M]^-$. ^1H NMR ([D_6]DMSO, ppm): 12.17 (s, 1H, OH); 7.78–7.85 (m, 2H, Ar—H); 7.36 (d, J = 4.20 Hz, 2H, Ar—H); 7.20–7.23 (m 1H, Ar—H); 7.15 (d, J = 4.20 Hz, 2H, Ar—H); 2.85 (t, J = 5.00 Hz, 2H, Ar—CH_2); 2.56 (t, J = 5.00 Hz, 2H, CH_2). Anal. Calcd for $C_{17}H_{12}N_2O_3$: C, 69.86; H, 4.14; N, 9.58. Found: C, 69.54; H, 4.26; N, 9.33.

*2.2.4. 4-[4-(2-Carboxyl-ethyl)phenoxy]phthalonitrile (**3β**)*

According to the above procedure for **3α**, 4-nitrophthalonitrile (0.87 g, 5 mmol) was treated with 4-(2-carboxyl-ethyl)phenol (0.83 g, 5 mmol) and K_2CO_3 (2.10 g, 15 mmol) in dry DMSO (35 ml) to give product **3β** (0.88 g, 60%). R_f = 0.67 (EtOH). IR (KBr, cm^{-1}): 3293.3 (O—H); 2918.2 (CH_2); 2231.4 (C≡N); 1740.7, 1702.3 (C=O). MS (ESI): m/z 291.5 $[M-H]^-$. ^1H NMR ([D_6]DMSO, ppm): 8.06 (q, J = 2.66 Hz, 1H, Ar—H); 7.73 (t, J = 1.11 Hz, 1H, Ar—H); 7.35–7.28 (m, 3H, Ar—H); 7.09 (t, J = 2.82 Hz, 2H, Ar—H); 2.83 (t, J = 5.04 Hz, 2H, Ar—CH_2); 2.54 (t, J = 5.03 Hz, 2H, CH_2—C=O). Anal. Calcd for $C_{17}H_{12}N_2O_3$: C, 69.86; H, 4.14; N, 9.58. Found: C, 69.80; H, 4.22; N, 9.63.

*2.2.5. Zinc (II) 1,8(11),15(18),22(25)-tetrakis [4-(butoxycarbonyl)phenoxy]phthalocyanine (**1a**)*

A mixture of dinitrile **1α** (0.64 g, 2 mmol) in dry n-pentanol (20 ml) was stirred at 70 °C under nitrogen for 10 min. Then zinc acetate (0.20 g, 1.1 mmol) and DBU (0.4 ml, 2.6 mmol) was added and the resulting mixture was stirred for 10 h at 130 °C. After removing the volatiles in vacuo, the residue was chromatographed (silica gel) with CH_2Cl_2/$CHCl_3$ (1:1) as eluent. A green band was collected and concentrated to give a crude product, which was further purified by chromatography using ethyl acetate/$CHCl_3$ (1:4) as eluent. The green band was collected and rotary-evaporated to give dark green solid **1a** (0.52 g, 78%). R_f = 0.28 (CH_2Cl_2). IR (KBr, cm^{-1}): 2956.7 (CH_3); 2931.2 (CH_2); 1716.2 (C=O); 1607, 1582.4, 1504.5, 1483.5 (C=C, C=N—); 1333.9 (Ar—O—Ar). MS (ESI): m/z 1347.1 $[M+3H]^+$. ^1H NMR ($CDCl_3$, ppm): 7.99–8.20 (m, 4H, Pc-H_α); 7.66–7.86 (m, 12H, Ar—H and Pc-H_β); 6.88–7.21 (m, 12H, Pc-H_β and Ar—H); 4.04–4.32 (m, 8H, CH_2—O); 1.74 (q, J = 5.18 Hz, 8H, CH_2); 1.26–1.30 (m, 8H, CH_2); 0.82–1.01 (m, 12H, CH_3). Anal. Calcd for $C_{76}H_{64}N_8O_{12}Zn$: C, 67.78; H, 4.79; N, 8.32. Found: C, 67.62; H, 4.89; N, 8.20.

*2.2.6. Zinc (II) 2,9(10),16(17),23(24)-tetrakis [4-(butoxycarbonyl)phenoxy]phthalocyanine (**1b**)*

According to the above procedure for **1a**, dinitrile **1β** (0.32 g, 1 mmol) was treated with zinc acetate (0.10 g, 0.56 mmol), and DBU (0.2 ml, 1.3 mmol) in 8 ml n-pentanol (8 ml) to give the product **1b** as a blue solid (0.11 g, 33%). R_f = 0.82 (ethyl acetate/n-hexane = 1:1). IR (KBr, cm^{-1}): 2958.1, 2930.5, 2872.1 (CH_3, CH_2); 1717.1 (C=O); 1600.5, 1504.4, 1487.0, 1470.5 (C=C, C=N—); 1393.1 (Ar—O—Ar). MS (ESI): m/z 1380.4 $[M+H+Cl]^-$. ^1H NMR ($CDCl_3$, ppm): 7.70 (s(br.); 12H, Ar—H and Pc-H_α); 7.35–7.50 (m, 4H, Pc-H_α); 6.62–6.83 (m, 12H, Pc-H_β, Ar—H); 4.06 (s(br.), 8H, CH_2-O); 1.65 (s(br.), 8H, CH_2); 1.37 (s(br.), 8H, CH_2); 0.91 (t, J = 4.60 Hz, 12H, CH_3). Anal. Calcd for $C_{76}H_{64}N_8O_{12}Zn$: C, 67.78; H, 4.79; N, 8.32. Found: C, 67.89; H, 4.65; N, 8.43.

*2.2.7. Zinc (II) 1, 8(11),15(18), 22(25)-tetrakis(4-carboxylphenoxy)phthalocyanine (**2a**)*

Phthalocyanine **1a** (0.50 g) was dissolved in THF (20 ml) and added slowly to a mixture of water (25 ml) and methanol (125 ml) which was previously saturated with NaOH. The mixture was stirred at 40 °C overnight. After removing the volatiles in vacuo, the residue was treated with water (200 ml), and filtered. The filtrate was then acidified by 1 M HCl to induce precipitation. The precipitate was collected by filtration, and washed with water, and dried in vacuum. Dark green solid **2a** (0.33 g, 79 %). R_f = 0.69 (CH_3OH). IR (KBr, cm^{-1}): 3279.8 (O—H); 1693.1 (C=O); 1603.0, 1583.8, 1482.0 (C=C); 1324.4 (Ar—O—Ar). MS (ESI): m/z 1158.1 $[M+K-H]^-$. ^1H NMR ([D_6]DMSO, ppm): 8.94–9.06 (m, 4H, Pc-H_α); 8.23–8.42 (m, 4H, Pc-H_β); 7.93–8.03 (m, 4H, Pc-H_β); 7.80 (d, J = 2.28 Hz, 8H, Ar—H); 7.16–7.34 (m, 8H, Ar—H). Anal. Calcd for $C_{60}H_{32}N_8O_{12}Zn{\cdot}2H_2O$: C, 62.21; H, 3.13; N, 9.67. Found: C, 62.11; H, 3.20; N, 9.58.

*2.2.8. Zinc (II) 2, 9(10), 16(17), 23(24)-tetrakis(4-carboxylphenoxy)phthalocyanine (**2b**)*

By using the above procedure described for **2a**, compound **1b** (0.05 g) was hydrolyzed to give blue solid **2b** (0.03 g, 72%). R_f = 0.74 (CH_3OH). IR (KBr, cm^{-1}): 3172.6 (O—H); 1693.0 (C=O); 1599.3, 1505.0, 1471.0 (C=C); 1333.3 (Ar—O—Ar). MS (ESI): m/z 1121.2 $[M+H]^-$. ^1H NMR ([D_6]DMSO, ppm): 12.94 (s, 4H, OH); 8.82–8.86 (m, 4H, Pc-H_α); 8.43 (d, J = 6.02 Hz, 4H, Pc-H_α); 8.09–8.23 (m, 8H, Ar—H); 7.81 (d, J = 4.50 Hz, 4H, Pc-H_β); 7.46–7.62 (m, 8H, Ar—H). Anal. Calcd for $C_{60}H_{32}N_8O_{12}Zn{\cdot}H_2O$: C, 63.19; H, 3.01; N, 9.83. Found: C, 63.23; H, 2.88; N, 9.57.

*2.2.9. Zinc (II) 1,8(11),15(18),22(25)-tetrakis [4-(2-carboxyl-ethyl)phenoxy]phthalocyanine (**3a**)*

A mixture of dinitrile **3α** (0.29 g, 1 mmol) and anhydrous K_2CO_3 (0.14 g, 1 mmol) in dry n-pentanol (15 ml) was stirred at 70 °C under nitrogen for 10 min. Then zinc acetate (0.10 g, 0.55 mmol) and DBU (0.2 ml, 1.3 mmol) was added and the mixture was stirred for 10 h at 130 °C. After removing the volatiles in vacuo, the residue was treated with water, which was acidified with 1 M HCl to induce precipitation. The precipitate was collected by filtration, and washed with water until pH 7, and dried in vacuum. The crude product was chromatographed (silica gel) with CH_3OH, following DMF as eluent. The green band was collected and concentrated, then treated with water, filtered, dried in vacuum to give dark green solid **3a** (0.12 g, 40%). R_f = 0.68 (CH_3OH). IR (KBr, cm^{-1}): 3276.6 (O—H); 2924.4 (CH_2); 1721.4 (C=O); 1586.4 1505.4, 1479.3 (C=C); 1328.3 (Ar—O—Ar). MS (ESI): m/z 1233.9 $[M+H]^-$. ^1H NMR ([D_6]DMSO,

ppm): 12.15 (s, 4H, OH); 8.53–9.09 (m, 4H, Pc-H_α); 7.90–8.09 (m, 4H, Pc-H_β); 7.60–7.63 (m, 4H, Pc-H_β); 7.32–7.41 (m, 8H, Ar—H); 7.08–7.23 (m, 8H, Ar—H); 2.77–2.89 (m, 8H, Ar—CH_2); 2.60–2.65 (m, 8H, CH_2—CO). Anal. Calcd for $C_{68}H_{48}N_8O_{12}Zn{\cdot}H_2O$: C, 65.20; H, 4.02; N, 8.95. Found: C, 65.14; H, 4.16; N, 8.73.

2.2.10. Zinc (II) 2,9(10),16(17),23(24)-tetrakis [4-(2-carboxyl-ethyl)phenoxy]phthalocyanine (**3b**)

According to the above procedure described for **3a**, dinitrile **3β** (0.44 g, 1.5 mmol) was treated with anhydrous K_2CO_3 (0.21 g, 1.5 mmol), zinc acetate (0.15 g, 0.82 mmol) and DBU (0.3 ml, 2.0 mmol) in *n*-pentanol (10 ml) to give blue solid **3b** (0.18 g, 19%). R_f = 0.67 (CH_3OH). IR (KBr, cm^{-1}): 3386.1 (O—H); 2923.7, 2853.5 (CH_2), 1716.8 (C=O); 1603.0, 1505.8, 1486.8 (C=C); 1393.1 (Ar—O—Ar). MS (ESI): *m*/*z* 1233.9 $[M+H]^-$. ^1H NMR ([D_6]DMSO, ppm): 12.17 (s, 4H, OH), 8.83 (s(br.); 4H, Pc-H_α); 8.40 (s(br.), 4H, Pc-H_α); 7.67 (s(br.), 4H, Pc-H_β); 7.29–7.52 (m, 16H, Ar—H); 2.95 (d, *J* = 3.75 Hz, 8H, Ar—CH_2); 2.66 (q, *J* = 6.15 Hz, 8H, CH_2—CO). Anal. Calcd for $C_{68}H_{48}N_8O_{12}Zn{\cdot}2H_2O$: C, 64.28; H, 4.13; N, 8.82. Found: C, 64.23; H, 4.25; N, 8.90.

2.3. Photophysical and photochemical properties

2.3.1. Fluorescence quantum yields

The fluorescence quantum yields (Φ_F) were determined are measured in the same solvent (DMF) by the equation: $\Phi_{F(\text{sample})} = (F_{\text{sample}}/F_{\text{ref}}){\cdot}(A_{\text{ref}}/A_{\text{sample}}){\cdot}\Phi_{F(\text{ref})}$, where *F*, and *A* are the measured fluorescence (area under the fluorescence spectra) and the absorbance at the excitation position (610 nm), respectively. The unsubstituted zinc (II) phthalocyanine (ZnPc) in DMF was used as the reference [$\Phi_{F(\text{ref})} = 0.28$] [33].

2.3.2. Singlet oxygen yields

The singlet oxygen quantum yields (Φ_Δ) were determined by a steady-state method using DPBF (1,3-diphenylisobenzofuran) as the scavenger in DMF [34,35]. The DMF solution of phthalocyanine (ca. 4 μM) containing DPBF (35 μM) were prepared in the dark and irradiated with red light, then DPBF degradation at 413 nm was monitored along with irradiated time. The singlet oxygen quantum yields (Φ_Δ) is calculated by the equation: $\Phi_{\Delta(\text{sample})} = (k_{\text{sample}}/k_{\text{ref}}){\cdot}(A_{\text{ref}}/A_{\text{sample}}){\cdot}\Phi_{\Delta(\text{ref}),}$ where $\Phi_{\Delta(\text{ref})}$ is the singlet oxygen quantum yield for the reference (un-substituted ZnPc) in DMF ($\Phi_{\Delta(\text{ref})} = 0.56$) [34], k_{sample} and k_{ref} are the DPBF photobleaching rates in the presence of the samples and reference, respectively; A_{ref} and A_{sample} are the absorbance at Q band (area under the absorption spectra in 610–750 nm) of the samples and reference, respectively. The light source consisted of a 150 W halogen lamp, a water tank for cooling and a color glass filter cut-on 610 nm. The fluence rate (λ > 610 nm) was 2.8 mW cm^{-2}.

2.3.3. Photo-stabilities

The photo-stabilities of ZnPcs were determined by the decay of the intensity of the Q band after exposure to red light [24]. The light source is similar to that described above, but the fluence rate was adjusted to 20 mW cm^{-2}. Measurements were carried out under air in DMF. The photodegradation rate constants *k* were calculated by the equation: $\ln A_0/A_t = kt$, where *t*, A_0, A_t are the irradiation time, absorbance at $t = 0$, absorbance at different time, respectively.

2.4. In vitro studies and cell uptake

2.4.1. In vitro studies

Phthalocyanines were first dissolved in DMF (1.0 mM) and the solutions were diluted to 80 μM with 0.5% (wt) aqueous solution of Cremophor EL (Sigma, 0.5 g in 100 ml of water). The solutions were clarified with 0.45 μm filter, and then diluted with the cellular culture medium (as described below) to appropriate concentrations.

The MGC803 human gastric carcinoma cells (from ATCC) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 μg/ml). About 2×10^4 cells per well in this medium were incubated in 96-well plate and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. The cells were then incubated with 200 μl of the above phthalocyanine solutions for 2 h under the same conditions. After that, the cells were rinsed with PBS and re-fed with 100 μl of the culture medium before being illuminated at ambient temperature. The light source consisted of a 300 W halogen lamp, a water tank for cooling and a color glass filter cut-on 610 nm. The fluence rate (λ > 610 nm) was 50 mW cm^{-2}. An illumination of 20 min led to a total fluence of 60 J cm^{-2}.

Cell viability was determined by the colorimetric 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [36]. After illumination, the cells were incubated at 37 °C under 5% CO_2 overnight. An MTT (Sigma) solution in PBS (25 μl, 5 mg ml^{-1}) was added to each well followed by incubation for 4 h under the same environment. A 200 μl of DMSO was then added to each well. The 96-well plate was agitated on a ThermoLabsystems microplate reader (Multishan MK3) at ambient temperature for 20 s before the absorbance at 490 nm at each well was taken. The average absorbance of the blank wells, which did not contain the cells, was subtracted from the readings of the other wells. The cell viability was then determined by the equation: Cell Viability (%) = $[\sum(A_i/\bar{A}_{\text{control}} \times 100)]/n$, where A_i is the absorbance of the *i*th data ($i = 1, 2, \ldots, n$), $\bar{A}_{\text{control}}$ is the average absorbance of the control wells, in which the phthalocyanine was absent, and n (≥3) is the number of the data points.

2.4.2. Cell uptake

MGC803 cells were plated at a density of 7.5×10^5 cells per well in 12-multiwell plates in the growth medium (0.75 ml in each well). Following an overnight incubation, the medium was removed and the cells were rinsed with PBS, and then incubated with 1.5 ml of a 0.5 μM phthalocyanine dilution in the medium for 2 h. The cells were then rinsed with PBS and detached with 300 μl of 0.25% trypsin-EDTA solution (Invitrogen). Cells were collected by centrifugation in 1.5 ml plastic tubes (5 min, 3000 rpm) and washed with PBS. The cell pellets were re-suspended in 1 ml DMF, incubated overnight at 4 °C and sonicated for 30 min and centrifuged at 9000 rpm for 10 min. The fluorescence spectrum of the supernatant was recorded in an Edinburgh FL900/FS920 spectrofluorometer (excited at 610 nm and monitored emission at 650–800 nm). From the area of emission peak, the concentration of phthalocyanine in the solution was estimated by comparison with a calibration curve obtained with standard solutions of the sensitizer in the same solution. Each experiment was repeated three times.

3. Results and discussion

3.1. Synthesis

Scheme 1 shows the synthetic pathway to the targeted zinc (II) phthalocyanines (ZnPcs). The phthalonitrile derivatives **1α** and **1β** were prepared by nucleophilic substitution reaction

Table 1
Photophysical data for phthalocyanines in DMF.

Compound	λ_{max} (nm) at Q-band	λ_{em} (nm)[a]	$\varepsilon \times 10^5$ (M^{-1} cm^{-1})	Φ_F[b]	τ_F (ns)[c]
1a	687	696	2.67	0.24	2.74
1b	675	685	2.24	0.31	3.43
2a	686	698	1.79	0.14	3.10
2b	674	685	1.94	0.25	3.38
3a	695	708	2.05	0.16	3.37
3b	679	686	1.82	0.23	3.51

[a] Excited at 610 nm.
[b] Using unsubstituted zinc (II) phthalocyanine (ZnPc) in DMF as the reference (Φ_F = 0.28) [33].
[c] Determined by the singlet photo account method.

between 3-nitrophthalonitrile or 4-nitrophthalonitrile and 4-(butoxycarbonyl)phenol in the presence of K_2CO_3 in dry DMSO. Under the similar condition, treatment of 3-nitrophthalo-nitrile or 4-nitrophthalonitrile with 4-(2-carboxyl-ethyl)phenol gave the corresponding substituted products **3α** and **3β**. All the phthalonitrile derivatives were obtained with high yields (60–83%). Cyclotetramerization of α-substituted precursor **1α** or **3α** in dry *n*-pentanol with zinc (II) acetate and a catalytic amount of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), followed by chromatographic separation and purification on silica gel column, resulted in the corresponding tetra-α-substituted ZnPcs **1a** and **3a** with 78% and 40% yield, respectively. Replacing α-substituted precursors with β-substituted analogues **1β** or **3β** for tetramerization led to the tetra-β-substituted ZnPcs **1b** and **3b** in 33% and 19% yield, respectively. During cyclotetramerization of phthalonitriles substituted with carboxyl-containing groups (**3α** and **3β**), the molar equivalent anhydrous K_2CO_3 was added to inhibit the possible esterification between the carboxylic groups and the solvent *n*-pentanol.

Hydrolysis of the ester groups of compounds **1a** or **1b** gave the corresponding products **2a** and **2b** with good yields (72–79%). The treatments were carried out by using NaOH as catalyst in a mixed solvent system (THF/MeOH/H_2O) and followed by acidification with HCl, according to the method described by Ng et al. [14].

3.2. Spectroscopic and photophysical properties

The electronic absorption and photophysical data of all these phthalocyanines have been measured in N, N-dimethylformamide (DMF), and the data are summarized in Table 1. Figs. 1 and 2 show the absorption and emission spectra of **3a** and 3**b** for illustration.

The absorption spectra of all compounds in DMF are typical for non-aggregated zinc phthalocyanines, exhibiting an intense and sharp Q band at 674–695 nm. The absorption position of the Q band is red-shifted 12–16 nm for the α-substituted phthalocyanines compared with the β-substituted counterparts, as can be seen in Table 1 and Fig. 1 (687 nm *vs.* 675 nm for **1a** *vs.***1b**; 686 nm *vs.* 674 nm for **2a** *vs.* **2b**; 695 nm *vs.* 679 nm for **3a** *vs.* **3b**). This observed red spectral shift is consistent with previous reports for substituted ZnPcs with electron-releasing groups [27–31,37], which can be reasonably explained by considering the magnitude of the atomic orbital coefficients of the carbon atoms derived from molecular orbital calculations [37]. Since the coefficients of α carbon atoms are larger than those of β carbon atoms in the HOMO, the extent of destabilization of this orbital by introducing electron-donating groups is larger when they are linked to the α-positions, which makes the HOMO–LUMO gap smaller and thereby results in the Q-band shifting to longer wavelength [37].

Furthermore, comparison of the spectra of ZnPcs with different substituents at the same positions, suggests that substitution with 4-carboxylphenoxy groups results in the Q-band absorption of phthalocyanine blue-shifted 5–8 nm compared with 4-(2-carboxyl-ethyl)phenoxy groups, while does not lead to obvious difference when compared with 4-(butoxycarbonyl)phenoxy groups.

Upon excitation at 610 nm, all the compounds show a fluorescence emission at 685–708 nm in DMF, which is essentially a mirror image of the absorption spectra in Q band with a small Stokes shift (ca. 10 nm). The influence of the position and species of substituents on the emission position of fluorescence is similar to that on the Q-band absorption. As shown in Table 1, these phthalocyanines are found to have a fluorescence quantum yield (Φ_F) of 0.14–0.31 and a fluorescence lifetime (τ_F) of 2.74–3.51 ns. The values of Φ_F, as well as τ_F, are slightly higher for the β-substituted compounds compared with the α-substituted counterparts. It seems that ZnPcs

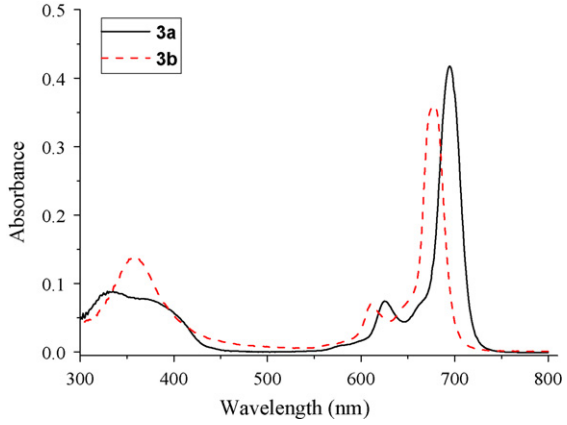

Fig. 1. Electronic absorption spectra of 2 μM of **3a–b** in DMF.

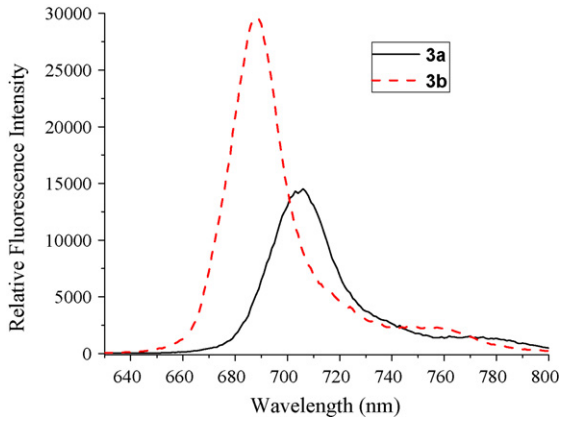

Fig. 2. Fluorescence emission spectra of 2 μM of **3a–b** in DMF.

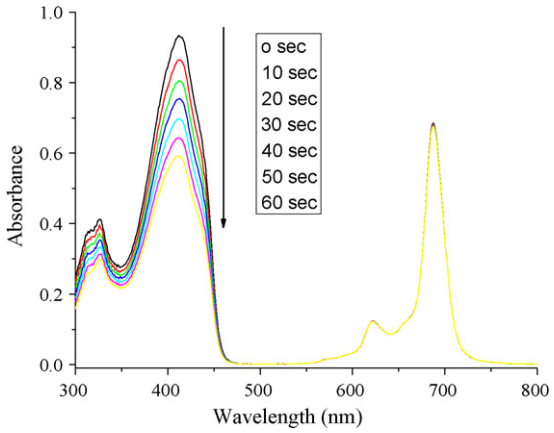

Fig. 3. The typical electronic absorption spectra for the measurement of singlet oxygen quantum yield in DMF. This measurement was for compound **2a** at a concentration of 4 μM.

showing emission peaks at shorter wavelength have higher quantum yields and longer lifetimes.

3.3. Photochemical properties

To evaluate the photosensitizing efficiency of these phthalocyanines, their singlet oxygen quantum yields (Φ_Δ) were determined using DPBF as chemical quencher. The disappearance of DPBF was monitored by electronic absorption spectra. During the determination of Φ_Δ, the photodegradation of phthalocyanines was not observed as shown by Fig. 3. There was no decrease in the Q band or formation of new bands. As shown in Table 2, all the prepared ZnPcs are excellent singlet oxygen generators with the high Φ_Δ of 0.56–0.76. This is consistent with a conclusion that substituted ZnPcs bearing electron-donating substituents have higher Φ_Δ values, which was reported by Wöhrle and co-workers [24]. The non-peripherally tetra-substituted ZnPcs(**1a**, **2a**, **3a**) show a slightly higher Φ_Δ value than the corresponding peripherally tetra-substituted ZnPcs (**1b**, **2b**, **3b**). It is worthwhile to further explore whether this observed trend has universality for the most of substituents.

The photo-stabilities of the synthesized ZnPcs and unsubstituted ZnPc were analyzed in DMF by measuring the decrease of the intensity of the Q-band over time by irradiation with red light under air. The spectral changes observed for the ZnPcs are shown in Fig. 4 (using **3b** as an example), which suggests that photodegradation occurred without phototransformation because the decrease in the Q-bands were not followed by the formation of a new band in this region [30]. The time decay of the absorbance maxima of the Q band for all the compounds obeyed first-order kinetics as shown in Fig. 5. The corresponding photodegradation first-order constants k are listed in Table 2. Smaller values of k mean a higher photooxidative stability. The α-substituted with 4-carboxylphenoxy groups phthalocyanine **2a** presents the highest stability in DMF, which is more stable than non-substituted ZnPc. For all there kinds of substituents, the tetra-α-substituted phthalocyanines are more stable compared with the tetra-β-substituted counterparts. A similar trend has also been reported previously for other substitutents (13,17-dioxanonacosane-15-hydroxys) [30]. Under irradiation in the presence of oxygen the formed singlet oxygen is known to be responsible for the decomposition of phthalocyanine [24,38]. However, in this work, the tetra-α-substituted compounds exhibit the higher singlet oxygen quantum yields and also show more photostabilities than the tetra-β-substituted analogues. The observed facts suggest that the α-substituted compounds have a higher inherent stability than the β-substituted counterparts towards the reaction with singlet oxygen.

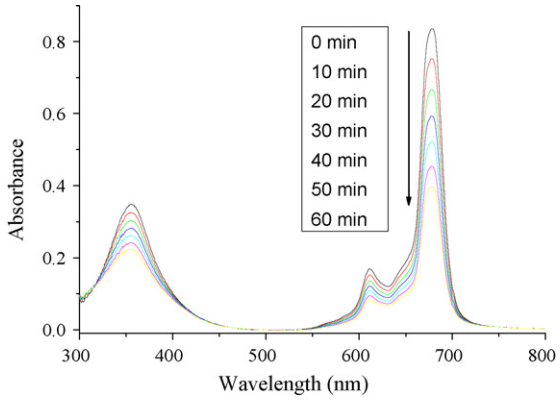

Fig. 4. Absorption spectra changes of compound **3b** in DMF at 10 min intervals under irradiation with red light.

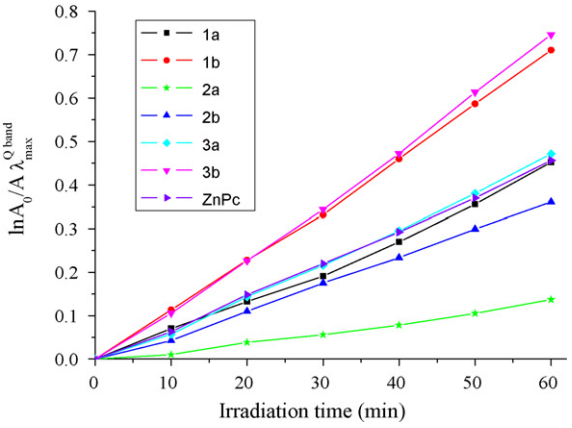

Fig. 5. First-order plots for the photodegradation of phthalocyanines in DMF.

3.4. In vitro photodynamic activities

The photodynamic activities of the prepared ZnPcs in Cremophor EL emulsions were investigated against human gastric carcinoma MGC803. In an initial screening where the cells were incubated with the same concentration of phthalocyanine (8 μM), all these phthalocyanines are found to be essentially nontoxic in the absence of light. Upon illumination with a red light, the compounds exhibit different photocytotoxicities, as shown in Table 3. When 8 μM of phthalocyanine was used, the cell viability was found

Table 2
Photochemical data for phthalocyanines **1a–3a** and **1b–3b** in DMF.

Compound	1a	1b	2a	2b	3a	3b	ZnPc
Φ_Δ [a]	0.76	0.64	0.68	0.63	0.70	0.58	0.56
$k \times 10^{-3}$ (min^{-1})	7.38	11.82	2.29	6.14	7.90	12.49	7.59

[a] Using ZnPc in DMF as the reference (Φ_Δ = 0.56) [34].

Table 3
Comparison of the photocytotoxicities of tetra-substituted phthalocyanines (8 μM) towards MGC803. The cells were illuminated with a red light (λ > 610 nm, 50 mW cm^{-2}, 60 J cm^{-2}). Data are expressed as means ± SD (n = 3).

Phthalocyanine	Viability (%)
1a	86.10 ± 5.98
1b	99.20 ± 3.73
2a	5.07 ± 0.70
2b	8.00 ± 1.97
3a	5.60 ± 0.48
3b	30.98 ± 5.86

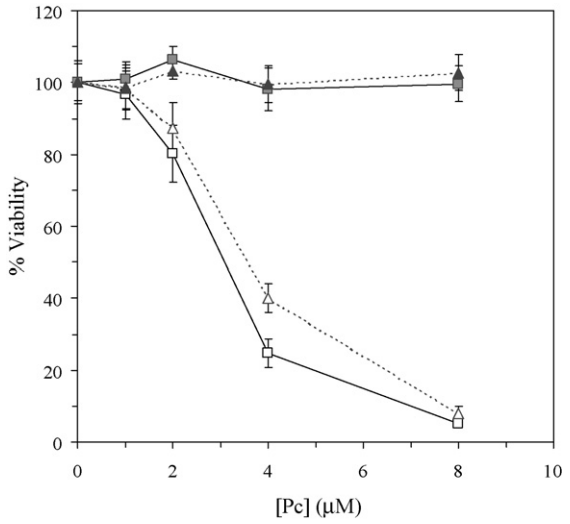

Fig. 6. Effects of **2a** (squares, solid line) and **2b** (triangles, dotted line) on MGC803 in the absence (closed symbols) and presence (open symbols) of light (λ > 610 nm, 50 mW cm^{-2}, 60 J cm^{-2}). Data are expressed as mean ± SD (n = 3).

to be 86% for **1a**, 99% for **1b**, 5% for **2a**, 8% for **2b**, 6% for **3a**, 31% for **3b**.

The photoactivities were further evaluated for the compounds **2a**–**b** and **3a**–**b**. Figs. 6 and 7 show the effects of these compounds on the MGC803 cell lines both in the absence and presence of light. It can be seen that all the four compounds are not cytotoxic in dark, but exhibit a substantial photocytotoxicity. The IC_{50} values, defined

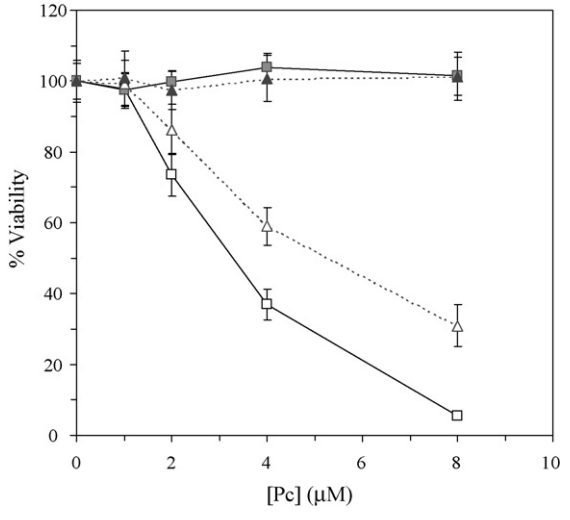

Fig. 7. Effects of **3a** (squares, solid line) and **3b** (triangles, dotted line) on MGC803 in the absence (closed symbols) and presence (open symbols) of light (λ > 610 nm, 50 mW cm^{-2}, 60 J cm^{-2}). Data are expressed as mean ± SD (n = 3).

Table 4
Comparison of the IC_{50} values of phthalocyanines against MGC 803.

Compound	IC_{50} (μM)
2a	3.05
3a	3.29
2b	3.78
3b	5.30

as the dye concentration demanded to kill 50% of the cells, can be obtained from the dose-dependent survival curves in the presence of light. These data are summarized in Table 4. It can be concluded that the photodynamic activity of these compounds follows the trend: **2a** > **3a** > **2b** > **3b** ≫ **1a** > **1b**. For all three substituted groups, the tetra-α-substituted ZnPcs exhibit a higher photocytotoxicity than the corresponding tetra-β-substituted counterparts. The ZnPcs containing carboxyl groups (**2a**–**b** and **3a**–**b**) are much more efficient than those containing the ester groups (**1a**–**b**). Among all these compounds, the ZnPc non-peripherally substituted with 4-carboxylphenoxy (**2a**) shows the highest photodynamic activity with the IC_{50} value of 3.05 μM.

To understand further the different photoactivities of these compounds, we examined their absorption spectra in cellular culture medium (RPMI 1640 medium supplemented with 10% fetal bovine serum). As shown in Fig. 8, the compounds tetra-substituted with ester-containing groups (**1a** and **1b**) show two broaden signal peaking at ca. 640 nm and 680 nm, indicating [16,39] that they are highly aggregated in the medium. By comparison, the α-substituted **1a** is less aggregated than the β-substituted counterpart **1b**, owing to the former showing the higher absorption at ca. 680 nm. The very weak photocytotoxicity for **1a** and **1b** should be mainly due to their high aggregating trend in growth medium, since aggregation of phthalocyanine provides an efficient nonradiative energy relaxation pathway, thereby shortening the excited state lifetimes and greatly reducing the photosenstizing efficiency [16,40–42].

The absorption spectra of **2a** and **3a** in the culture medium are similar (see Fig. 9), showing an intense and sharp Q band at 694 nm and 698 nm, respectively. The typical feature of non-aggregated phthalocyanines [16,41–43] suggests that the tetra-α-substituted ZnPcs with carboxyl-containing groups (**2a** and **3a**) predominantly exist as a non-aggregated form in the biological medium. The non-aggregated nature is an important desirable feature for PDT. The long-wavelength and strong absorption of these two compounds is also desirable as it allows a deeper light penetration [40].

Compared with the α-substituted **3a**, the β-substituted **3b** shows a substantial lower Q-band absorption, along with a weak peak (at 647 nm) between the Q and vibronic band (Fig. 9(b)),

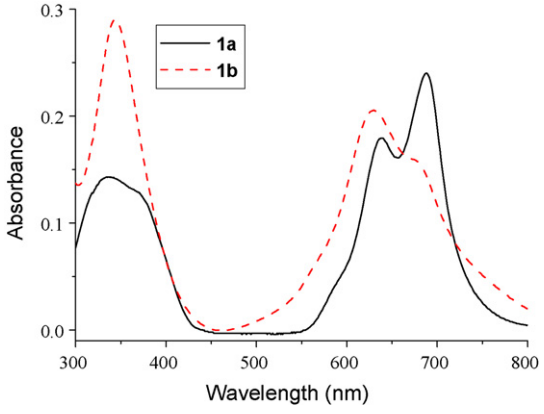

Fig. 8. Electronic absorption spectra of **1a** and **1b** (4 μM), formulated with Cremophor EL, in the cellular culture medium.

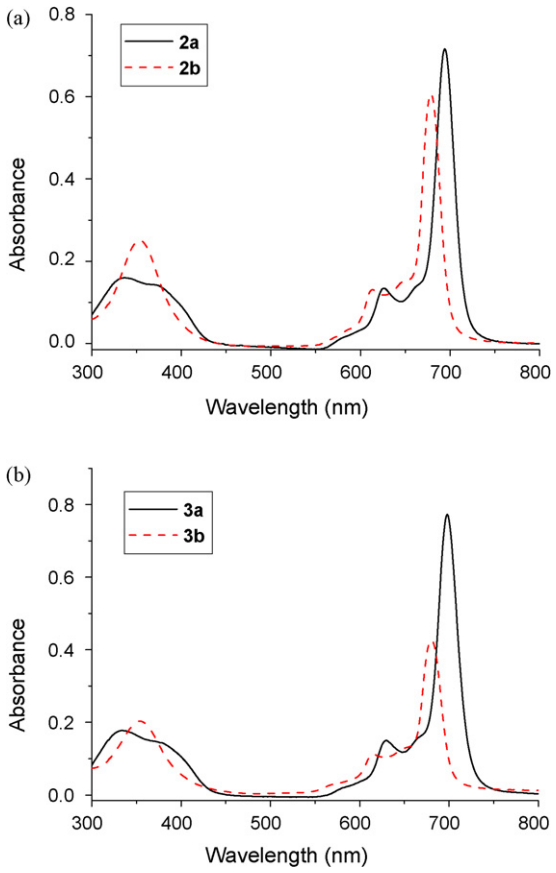

Fig. 9. Electronic absorption spectra of phthalocyanines (4 μM), formulated with Cremophor EL, in the cellular culture medium.

suggesting that the extent of monomeration for **3b** is lower than those for **3a** in growth medium. It can be seen in Fig. 9 that compound **2b** exhibits a slightly higher aggregation tendency than the corresponding **2a**, although **2b** also shows a rather high Q-band absorption in the medium. In a word, substitution at the α-position of ZnPc skeleton may more efficiently prevent phthalocyanine from aggregating in physiological media than that at β-position, which is likely due to the larger hindrance effect of α-substituents. Therefore, the observations that the tetra-α-substituted ZnPcs are more photoactive than the tetra-β-substituted counterparts, should be attributed to their less aggregation properties. Furthermore, slightly higher singlet oxygen quantum and photo-stabilities of tetra-α-substituted compounds may also be desirable factors.

To reveal whether the different photoactivity of compounds **2a–b** and **3a–b** is also related to the cellular uptake, we employed an extraction method to quantify the cellular uptake of these compounds for MGC803. The results are shown in Table 5, which suggests that the cellular uptake follows the trend: **2a** ≈ **2b** > **3a** ≈ **3b**. It is clear that the introduction of 4-carboxylphenoxy groups leads ZnPc having a higher cellular uptake than the 4-(2-carboxyl-ethyl)phenoxys, which may account for the observed trend of photo-activity of these compounds (**2a** > **3a**; **2b** > **3b**). Of course, the trend that compounds **2a–b** are more photostable than the corresponding compounds **3a–b** (see Table 2), should also be a favorable factor.

It can be seen that there is not obvious difference on cellular uptake between non-peripherally and peripherally tetra-substituted phthalocyanines. Therefore, the fact that the tetra-α-substituted ZnPcs have a higher photocytotoxicity than the β-substituted counterparts, should be not related to the cellular uptake.

Table 5
Percentage of cellular uptake of compounds **2a–b** and **3a–b** by MGC803.

Phthalocyanine	Uptake (%)
2a	0.59 ± 0.07
2b	0.52 ± 0.04
3a	0.23 ± 0.02
3b	0.30 ± 0.08

4. Conclusion

A novel series of zinc (II) phthalocyanines tetra-substituted with carboxyl groups at α- or β-positions were synthesized and characterized. The compounds containing α-substituted groups have a red-shift in Q-band absorption as well as emission spectrum with a low fluorescence quantum yield than the corresponding ZnPcs containing β-substituents. When compared with the tetra-β-substituted ZnPcs, the corresponding tetra-α-substituted analogues exhibit a less aggregating trend in the cellular growth medium, and a slightly higher singlet oxygen quantum yield and higher photo-stability, but do not show obvious difference in cellular uptake. As a result, the tetra-α-substituted zinc phthalocyanines exhibit a higher photocytotoxicity toward MGC803 cells than the tetra-β-substituted counterparts. Among all these compounds, the ZnPc non-peripherally substituted with 4-carboxylphenoxy (**2a**) shows the highest photodynamic activity with the IC_{50} value of 3.05 μM.

Acknowledgements

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