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Design, synthesis, and in vitro photodynamic activities of benzochloroporphyrin derivatives as tumor photosensitizers

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Abstract—Novel benzochloroporphyrin derivatives (BCPDs) were designed, synthesized, and characterized. In vitro dark cytotoxicity and photodynamic efficacy of BCPDs were evaluated by MTT assay on human hepatoma BEL-7402 cells. The experimental results showed that BCPDs **15**, **16**, **17**, and **18** have strong long wavelength absorptions around 670 nm and exhibit significantly lower dark cytotoxicity than BPDMA and possess potent photocytotoxicity, IC_{50} values 1.32 μg/mL for **15**, 0.26 μg/mL for **16**, 0.47 μg/mL for **17** of 0.27 μg/mL for **18**, and 0.23 μg/mL for BPDMA. Among them, BCPDs **16** and **18** are more effective and promising PDT photosensitizers based on the studies with BEL-7402 cells and show nearly the same photodynamic efficacy as BPDMA. MG-P staining qualitative analysis also indicated that PDT with BCPDs **16** can induce apoptosis in BEL7402 cells.

Photodynamic therapy (PDT) is an attractive modality for the treatment of cancer and other diseases.[1] It involves the illumination of a photosensitizer with light of an appropriate wavelength, that is, in the range of 630–800 nm for which tissue penetration is optimal. The light-activated photosensitizer can form lethal cytotoxic active singlet oxygen ($^1O_2$) and reactive oxygen species (ROS).[2–5] By directing the light beam to the target area, PDT can be used to selectively destroy diseased tissue without harming surrounding cells.

Porphyrin-related photosensitizer such as porfimer sodium (Photofrin II), a complex mixture of hematoporphyrin derivatives, is the first clinically approved photosensitizer for the treatment of bladder cancer by PDT in 1993.[1,6] Although encouraging results have been obtained, it has some serious limitations. The molecular absorption coefficient of Photofrin II at the clinically used wavelength of 630 nm is low ($\varepsilon = 1170\ M^{-1}\ cm^{-1}$).[6] More importantly, it causes skin photosensitivity for a prolonged period of time, up to 30–90 days after treatment, due to its accumulation and retention in skin tissues.[7] Therefore, much research has been devoted to developing new photosensitive compounds with improved photophysical efficiency and fewer side effects.

In the recent decade, a number of so-called second generation photosensitizers especially related to chlorins have been reported in the literature.[8] Among them, a benzoporphyrin derivative (BPD) obtained from Diels–Alder reaction of protoporphyrin IX dimethyl ester **1** with dimethyl acetylenedicarboxylate (DMAD) as its most effective analog—ring A modified *cis*-isomer monocarboxylic acid form, so-called BPDMA **2**, has generated interest due to its low skin phototoxicity, rapid clearance from tissues, and strong absorption at long wavelengths ($\lambda_{max} = 690$ nm, $\varepsilon = 35{,}000\ M^{-1}\ cm^{-1}$) to take full advantage of greater tissue penetration.[6,9] It is undergoing clinical trials for the treatment of basal cell carcinoma[10] and has had clinical success in the treatment for age-related macular degeneration (ARMD) by PDT.[11]

BPD-MA **2** is synthesized by first treatment of protoporphyrin IX dimethyl ester **1** with DMAD followed by rearrangement with base and subsequent partial hydrolysis (Scheme 1).[12] In principle, there is a synthetic key problem in efficiently obtaining the pure BPDMA **2**. Treatment of protoporphyrin IX dimethyl ester **1** with

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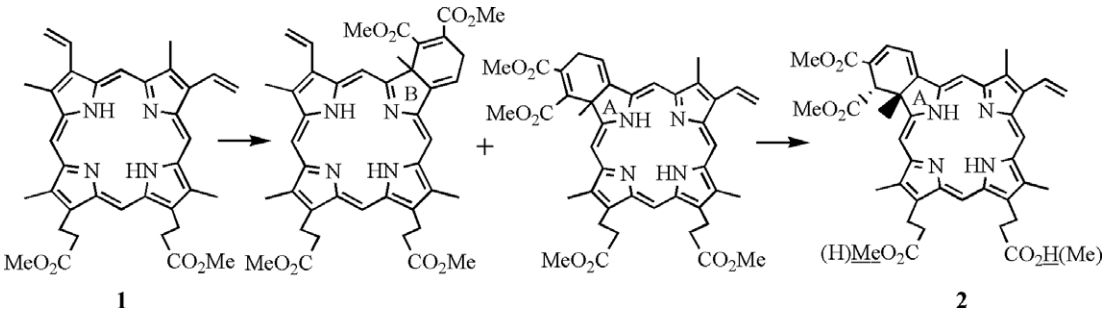


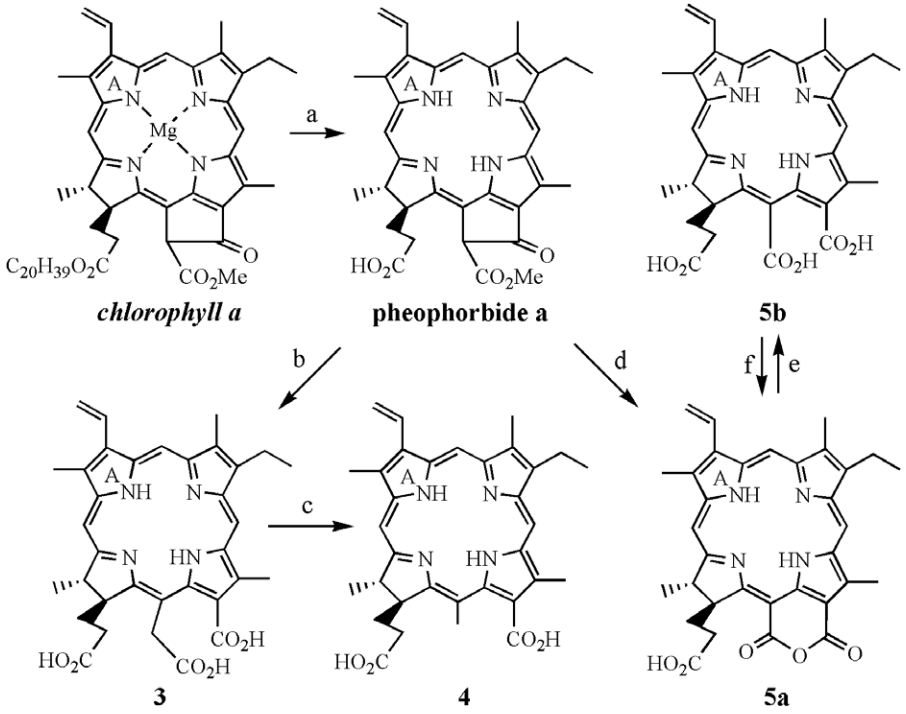
Scheme 1.

DMAD yields a mixture of ring A and ring B Diels–Alder adducts which is followed to be rearranged to a mixture of ring A and ring B *cis*-isomers by base. BPD-MA **2**, the more effective ring A modified *cis*-isomer,[13] is then separated from the isomeric mixture by column chromatography.

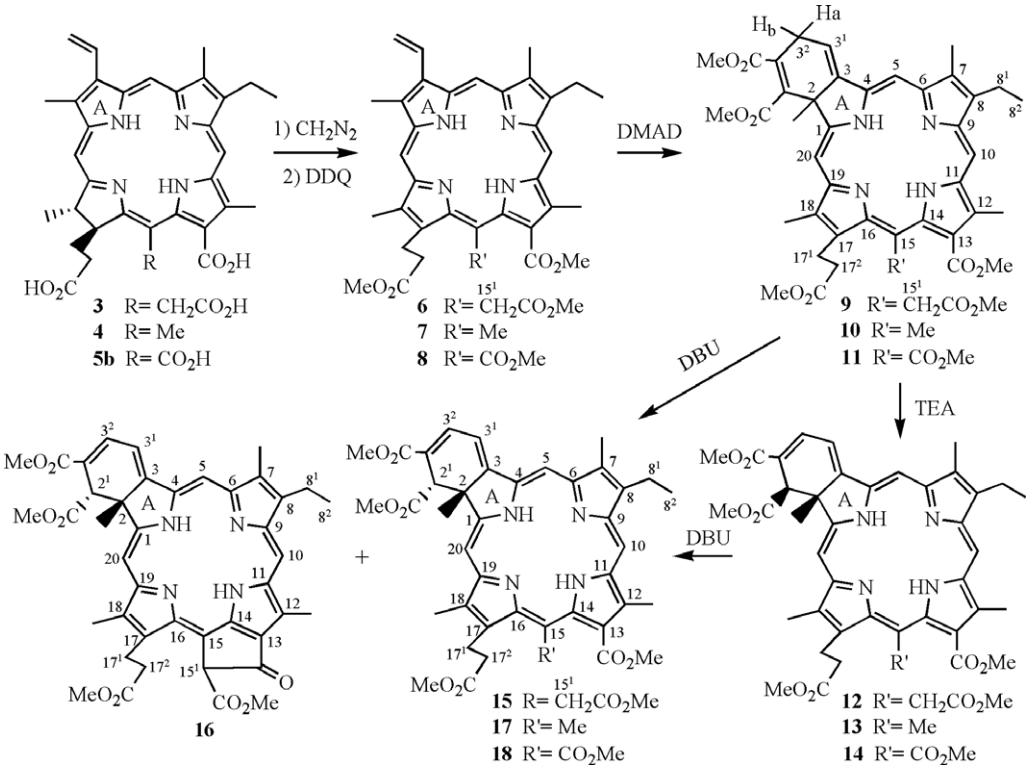
In order to solve the synthetic problem associated with preparation of biologically active BPDMA **2** and obtain novel highly potent, low toxic photosensitizers with strong absorption at long wavelength (>630 nm), we would like to use chlorin e_6 **3**, chlorin e_4 **4**, and chlorin p_6 **5b** (or purpurin-18 **5a** which is actually steady existence state of chlorin p_6 **5b**) as substrates in the Diels–Alder reaction to synthesize the novel BPDMA analogs—benzochloroporphyrin derivatives (BCPDs). Because all substrates contain only one vinyl group which is situated in the more efficacious ring A site and thus only one Diels–Alder adduct with DMAD in principle is possible. In this paper, we report the synthesis and preliminary photocytotoxicity in vitro on human hepatoma BEL-7402 cells of BCPDs.

For our study, starting materials chlorin e_6 **3**, chlorin e_4 **4**, and chlorin p_6 **5b** (or purpurin-18 **5a**) were obtained through base-degradation of pheophorbide-a which is prepared via 36% HCl degradation of *chlorophyll a* in Et_2O by the methodology developed in our laboratory using crude chlorophyll extracts in Chinese traditional herb named Silkworm excrement (Scheme 2).[14]

The synthesis pathway of a series of novel BPDMA analogs BCPDs are outlined in Scheme 3. Originally, we planned to use chlorin e_6 **3**, chlorin e_4 **4**, chlorin p_6 **5b** (or purpurin-18 **5a**) or their methyl ester to react with DMAD. However, the Diels–Alder reaction did not take place, and only chloroporphyrin e_6 trimethyl ester**6**, chloroporphyrin e_4 dimethyl ester**7**, and chloroporphyrin p_6 trimethyl ester**8**, which were prepared through methylation of chlorin e_6 **3**, chlorin e_4 **4**, and chlorin



Scheme 2. Reagents and conditions: (a) Concd HCl/Et_2O; (b) NaOH–EtOH/N_2, reflux; (c) pyridine/N_2, reflux; (d) NaOH–*i*-PrOH/O_2; (e) i—NaOH, ii—adjust to pH 6–7; (f) standing.



Scheme 3.

p_6 **5b** individually with CH_2N_2 followed by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), respectively, could undergo the Diels–Alder reaction with DMAD.

Treatment of **6**, **7**, and **8** individually with DMAD in refluxing toluene under an atmosphere of nitrogen gave the desired Diels–Alder adducts **9**, **10**, and **11** in 40.9%, 36.1%, and 43.4% yield, respectively. Rearrangement of **9**, **10** and **11** individually with triethylamine (TEA) gave the conjugated BCPDs *trans*-isomers **12**, **13**, and **14** in quantitative yield, respectively. Reaction of **13** and **14** individually with 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) produced the conjugated BCPDs *cis*-isomers **17** and **18** in 87.5% and 100% yield, respectively. The BCPDs *cis*-isomers **17** and **18** can also be obtained by direct treatment of the Diels–Alder adducts **10** and **11** individually with DBU in 81.3% and 94.7% yield, respectively.

However, rearrangement of **9** and **12** individually with DBU both gave the same mixture of two compounds. Separated individually by silica gel column chromatography ($CH_3COCH_3/CH_3OH/CH_2Cl_2$, 7:7:100), the desired DBU rearrangement conjugated BCPD *cis*-isomer **15**, the slower moving band, was obtained as a major product in 70% and 80% yield, respectively; the undesired DBU rearrangement conjugated BCPD *cis*-isomer **16**, the faster moving band, was obtained as a minor product in 20% yield.

Compared to the resonances for the Diels–Alder adducts **9** and **12**, the ^{1}H NMR spectrum of **16** showed the presence of only one proton at 15^1-position at δ 6.74 (s, 1H) ppm and the shortage of one methoxy proton, which suggested the formation of an additional exocyclic ring system. Compared to the resonances for various protons in the DBU rearranged major product **15**, the ^{1}H NMR spectrum of **16** showed no significant shifts in the resonances of the protons at 3^1-, 3^2-, 2^1-, 2-, 17^1-, and 17^2-positions, which appeared at δ 7.81 (d, 1H, J = 5.7 Hz), 7.46 (d, 1H, J = 5.7 Hz), 5.04 (s, 1H), 1.81 (s, 3H), 4.08 (m, 2H), and 2.90 (m, 2H) ppm, respectively. These results supported the formation of a rearranged *cis*-isomer with an additional five-membered exocyclic ring between 13^1- and 15^1-positions. The mechanism for the formation of the exocyclic ring is a base-induced Dieckmann condensation. Despite extensive studies of Diels–Alder reactions in porphyrin systems by us and others, this is the first example which illustrates the formation of such a novel ring system.

All new compounds, the Diels–Alder adducts **9**, **10**, **11** and the BCPDs *trans*-isomers **12**, **13**, **14** and BCPDs *cis*-isomers **15**, **16**, **17**, **18**, essentially belong to side-chain modified 'Chlorin' derivatives from naturally occurring sources and their structures were characterized by ^{1}H NMR, ESI-MS, and elemental analysis studies.[15] The UV–visible spectral data of compounds **9**–**18** in CH_2Cl_2 are listed in Table 1.

As shown in Table 1, the UV–visible spectra of the Diels–Alder adducts **9**, **10**, and **11** showed the intense Soret bands (416 nm, 416 nm and 413 nm, respectively), which were all slightly red-shifted than that of the intermediate chloroporphyrins **6**, **7**, and **8** (409 nm, 410 nm, and 406 nm, respectively), and the long wavelength

Table 1. UV–vis data of Diels–Alder adducts **9**–**11**, BCPDs *trans*-isomers **12**–**14** and BCPDs *cis*-isomers **15**–**18**

Compound	λ_{max} (nm) ($\varepsilon \times 10^{-4}$, M^{-1} cm^{-1})				
	Soret band	Visible bands			
9	416 (6.70)	521 (0.39)	552 (0.42)	597 (0.24)	653 (0.72)
10	416 (24.05)	521 (1.27)	554 (1.30)	597 (0.76)	653 (2.20)
11	413 (14.68)	519 (0.90)	550 (1.09)	600 (0.46)	655 (1.99)
12	440 (7.07)	—	—	581 (1.45)	668 (0.90)
13	440 (3.72)	—	—	583 (0.79)	670 (0.45)
14	434 (8.05)	—	—	578 (1.89)	670 (1.20)
15	443 (7.74)	—	—	596 (2.24)	675 (1.06)
16	444 (7.46)	—	—	620 (2.21)	666 (0.92)
17	451 (16.21)	—	—	591 (3.76)	677 (2.71)
18	439 (11.30)	—	—	586 (3.09)	677 (2.26)

absorption maxima in visible bands at 653 nm, 653 nm, and 655 nm, respectively, which were absent in the intermediate chloroporphyrins **6**, **7**, and **8**. Compared to the Diels–Alder adducts **9**, **10**, and **11**, the absorption bands of the TEA rearranged BCPDs *trans*-isomers **12**, **13**, **14**, and the DBU rearranged BCPDs *cis*-isomers **15**, **16**, **17**, and **18** were obviously red-shifted and broader due to the extended conjugation. They showed the intense broad Soret bands around 440 nm and the long wavelength absorption maxima in visible bands around 670 nm.

The preliminary in vitro dark cytotoxicity and photodynamic efficacy of BCPDs *cis*-isomers **15**–**18** were evaluated by MTT assay on human hepatoma BEL-7402 cells (Fig. 1).[15]

Cells were pre-incubated with various concentrations of BCPDs **15**–**18** in the dark for 24 h at 37 °C followed by irradiation with a laser (670 nm) at a fluence of 10 J/cm^2 (56 mW/cm^2 for 3 min). Then, hepatoma BEL-7402 cells were incubated for 24 h at 37 °C. The cell survival fraction was measured for photo-induced growth inhibition by photosensitizers using MTT assay. Experiments were carried out in triplicates. Figure 1 shows the concentration-dependent cell survival curves for BCPDs **15**–**18** and BPDMA.

As shown in Figure 1, the cell survival rates of BCPDs **15**–**18** were all higher than 92% in the absence of light for concentrations up to 2.5 μg/mL, which revealed the minimal cytotoxicity in darkness (so-called 'dark cytotoxicity'). However, the cell survival rates of positive control BPDMA ranged from 97% to 5% in darkness under the wide concentrations ranging from 0.03125 μg/mL to 2.5 μg/mL and its IC_{50} value (calculated by SPSS 10.0 for windows) was 1.35 μg/mL (95% confidence limit was 1.19 ~ 1.54 μg/mL, which showed significantly higher dark cytotoxicity on BEL-7402 cells than that of BCPDs **15**–**18**).

In addition, BCPDs **16**, **17**, and **18** exhibited significantly higher photocytotoxicity on BEL-7402 cells than BCPD **15**. Calculated by SPSS 10.0 for windows, IC_{50} values were 0.26 μg/mL (95% confidence limit was 0.16 ~ 0.47 μg/mL) for BCPD **16**, 0.47 μg/mL (95% confidence limit was 0.22 ~ 1.05 μg/mL) for BCPD **17**, 0.27 μg/mL (95% confidence limit was 0.24 ~ 0.32 μg/mL) for BCPD **18**, and 1.32 μg/mL (95% confidence limit was 1.15 ~ 1.52 μg/mL) for BCPD **15**, while IC_{50}

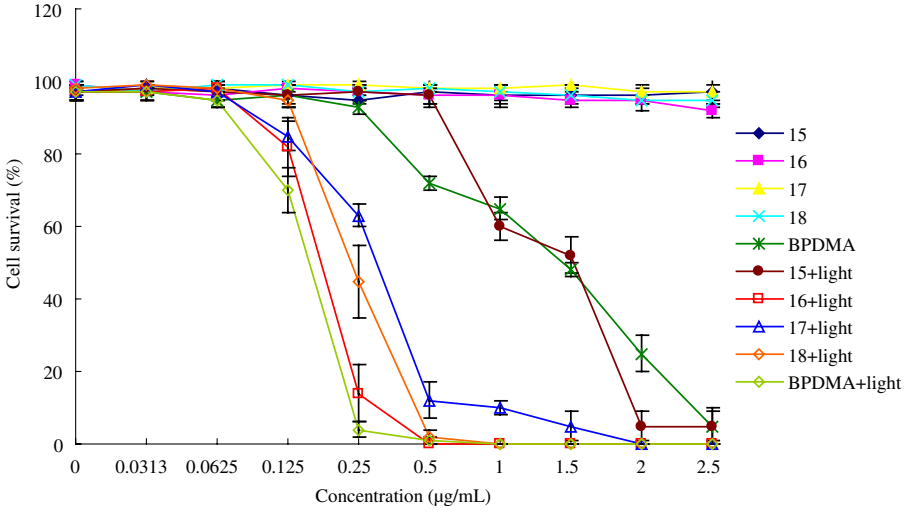


Figure 1. Cytotoxic effect of BCPD **15**–**18** on BEL-7402 cells in the absence of light and by irradiation for 3 min with a laser at a power density of 56 mW/cm^2 and wavelength 670 nm.

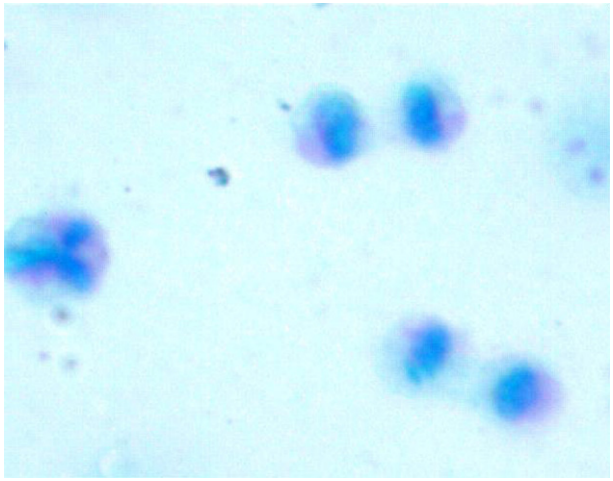


Figure 2. The images of BEL-7402 cell apoptosis induced by BCPD 16-mediated PDT at a fluence of 10 J/cm^2 (56 mW/cm^2 for 3 min) with a laser (670 nm) at concentration of 2.0 μg/mL.

value was 0.23 μg/mL (95% confidence limit was 0.13 ~ 0.46 μg/mL) for BPDMA. Among them, BCPDs **16** and **18** were determined to be more effective PDT photosensitizers based on the studies with BEL7402 cells and had nearly equal photodynamic efficacy to BPDMA.

To further address the BEL-7402 cell death caused by BCPD **16**-mediated PDT, the apoptosis morphology, which was captured by Olympus IX 41, was observed through Methyl Green–Pyronin (MG-P) staining (Fig. 2). Cells were pre-incubated with concentration of BCPD **16** at 2.0 μg/mL in the dark for 24 h at 37 °C followed by irradiation with a laser (670 nm) at a fluence of 10 J/cm^2 (56 mW/cm^2 for 3 min).Then, hepatoma BEL-7402 cells were incubated for 24 h at 37 °C. The cells were fixed by ethanol, stained by MG-P, and subsequently photographed by Olympus IX 41.

In principle, Methyl Green–Pyronin staining is effective in the identification of plasma cell and RNA in tissue sections and cytological preparations. DNA is stained green to blue by Methyl Green and RNA is colored red by Pyronin. Apoptotic cells display positive response to both Methyl Green and Pyronin. Necrotic cells show positive response to Methyl Green, but negative response to Pyronin. Normal cells exhibit negative response to both Methyl Green and Pyronin. As shown in Figure 2, hepatoma BEL-7402 cells upon treatment with BCPD **16**-mediated PDT showed typical apoptosis morphology when stained by MG-P.

In summary, a series of novel benzochloroporphyrin derivatives (BCPDs) have been designed, synthesized, and characterized. The potentials of BCPDs as PDT-sensitizers were evaluated by in vitro photocytotoxicity measurement using MTT assay on human hepatoma BEL-7402 cells. All title BCPD tested exhibit significantly lower dark cytotoxicity than BPDMA. Notably, BCPDs **16**, **17**, and **18** show significantly higher photocytotoxicity on BEL-7402 than BCPD **15**. Moreover, BCPDs, **16** and **18** are more effective PDT photosensitizers based on the studies with BEL-7402 cells. MG-P staining qualitative analysis also showed that PDT with BCPD **16** can induced apoptosis in BEL-7402 cells. Further developments such as partial hydrolysis and improvements using BCPDs are in progress and more extensive and deeper biological studies are ongoing.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.086.

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15. See Supplementary Information.