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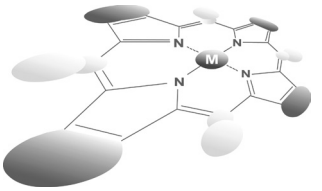

# Synthesis of long-wavelength chlorins by chemical modification for methyl pyropheophorbide-*a* and their *in vitro* cell viabilities

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**ABSTRACT:** A series of novel chlorophyll-*a* homologs with long wavelength absorption were synthesized *via* modification of methyl pyropheophorbide-*a* used as starting material. For introducing electron-withdrawing group methylenemalononitrile moiety was established on the periphery of modified chlorin by Knoevenagel reaction of malononitrile with formyl group at 3-, 15-position and 13[1]-carbonyl group on the exocyclic ring. All of chlorins containing the methylenemalononitrile structure show Qy-absorption at more than 700 nm. Moreover, we have examined a preliminary *in vitro* photodynamic anticancer effect of these new derivatives on mouse sarcoma S-180 cell line.

**KEYWORDS:** chlorin, methyl pyropheophorbide-*a*, chemical modification, visible absorption.

## INTRODUCTION

Long wavelength absorbing photosensitizers (>700 nm) have been described as potential candidates for achieving maximum tissue penetration in photodynamic therapy (PDT) [1–5]. Among such compounds with long-wavelength absorption, some naturally occurring bacteriochlorins have been reported as effective photosensitizers in preliminary *in vitro* and *in vivo* studies [6, 7]. However, most of these naturally occurring bacteriochlorins are extremely sensitive to oxidation, which result in rapid transformation into relative stable chlorin state which has an absorption maximum at or below 670 nm [4, 8]. Furthermore, if a laser is used to excite the bacteriochlorin *in vivo*, oxidation may result in the formation of a new chromophore absorbing outside the laser window, which reduces the photodynamic efficacy. In order to render PDT more generally applicable to tumor therapy, long wavelength absorbing photosensitizers, such as stable chlorins are needed, because they should also be able to localize in relatively high concentration at the tumor site relative to normal tissues. Chlorophyll-*a* is a natural chlorin pigment and his degradation products stand in marked contrast to symmetric porphyrin pigments due to substantially stabilized $S_1$ energies, a strong Qy absorption band, and unique redox reactivates. Therefore the synthesis of novel photosensitizers, possessed the basic skeleton of chlorophyll-*a*, have become the focus of research in photodynamic therapy [9]. For chlorophyll-*a* compounds, the Qy bands as a longest absorption band were strongly affected by the substitutents on the Qy axis ($N_{21}$–$N_{23}$, see Scheme 1) [10]. Considering that introducing strong electron-withdrawing group on the chromophore or expanding the conjugation system of the macrocycle can bring about obvious red shift of its long wavelength absorption, the modifications of methyl pyropheophorbide-*a* **1** (MPP*a*), which derived from methyl pheophorbide *a* (MP*a*), the initial degradative product extracted from *Spirulina pacfica alga*, were carried out for synthesis of long-wavelength photosensitizers with stable chlorin structure in our studies. Here we report the synthesis

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of chlorophyll *a* homologs, which possess absorption maximum around longer than 700 nm, by modification of methyl pyropheophorbide-*a*.

## RESULTS AND DISCUSSION

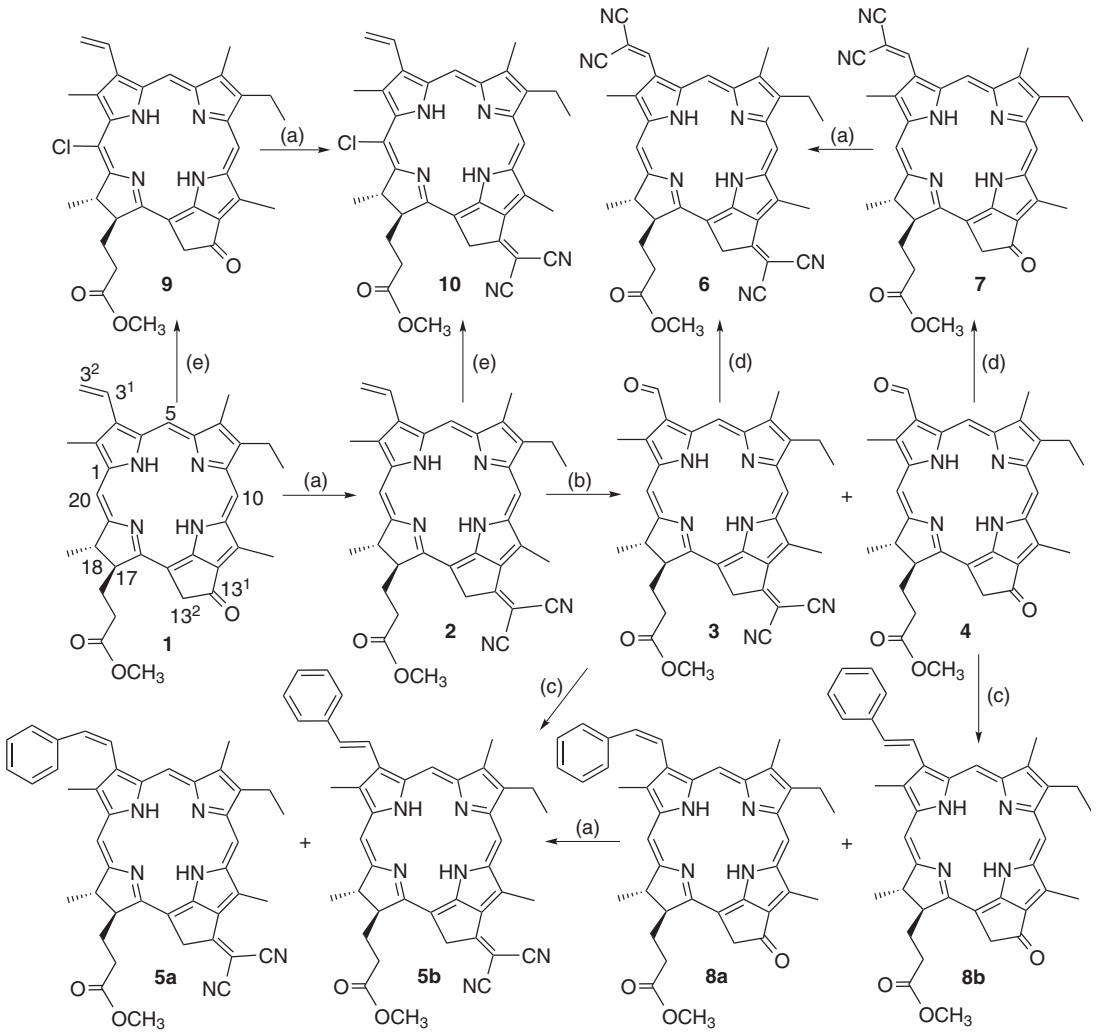
### Synthesis and characterization

Malononitrile exhibits high activity and its introduction will extend greatly the Qy peak of chlorin comparing with other active methylene compounds such as dimethyl malonate and acetylacetone [11, 12], on the other hand, in our preliminary experiment, the reaction of dimethyl malonate or acetylacetone with E-ketone gave very poor yield in same condition or no react. Therefore in this study we choose malononitrile as main building block to prepare the stable long-wavelength chlorins. The reaction of starting material **1** with malononitrile in the presence of sodium ethoxide in EtOH by refluxing to give $13^1$-dicyanomethylene chlorin **2** in 75% yield which shows Qy-absorption at 704 nm. The exocyclic carbon–carbon double bonds at 3-position of **2** was oxidized with $OsO_4$ in dichloromethane in the presence of pyridine followed by glycol cleavage, using sodium periodate to form chlorin **3** and methyl pyropheophorbide-*d* **4** (MPP*d*) whose Qy peaks were observed at 727 and 694 nm, respectively. To expand conjugated π-system of chlorin chromophore the C3-formyl of **3** reacted continuously with $Ph_3PCH_2Ph$ in dichloromethane in the presence of aqueous NaOH solution at room temperature for 30 min to give a C3-phenyl substituted chlorin mixture composed of pyropheophorbide **5a** as *cis*-isomer (19%) and **5b** as *trans*-isomer (59%), which were easily separated by chromatography and showed their Qy bands at 703 and 710 nm, respectively. The Knoevenagel reaction of **3** was performed readily in THF in the presence of TEA to give tetracyano-substituted chlorin **6** in 70% yield, whose Qy-absorption attained to 734 nm. By the same way MPP-*d* **4** was converted into dicyano-substituted chlorin **7** (Qy = 706 nm) from which chlorin **6** was also obtained by means of refluxing in THF with malononitrile. The chlorination for MPPa **1** produced 20-chlorochlorin **9** whose π-system was further extended through its 20-*meso*-position. The subsequent Knoevenagel reaction of **9** with malononitrile smoothly gave $13^1$-dicyanomethylene-substituted chlorochlorin **10** whose Qy peak appeared at 714 nm. Although this compound also could be obtained from **2** by same chlorination with NCS, the speed and yield of the reaction were relatively low in comparison with that of MPP*a* **1** due to the absorb-electron effect of the dicyanomethylene group at $13^1$-position. The Wittig reaction of MPP*d* **4** with benzyltriphenylphosphonium bromide generated a pair of *cis-trans* isomers **8a** (Qy = 667 nm) and **8b** (Qy = 673 nm) in 20% and 58% yields, respectively [11]. The condensations of these isomers with malononitrile were carried out under the same condition for preparing chlorin **2** to also give **5a** and **5b** (Scheme 1). It is worth noting that *E*-ketone and C3-formyl group exhibited different activities, thereby in the reaction of malononitrile with C3-formyl group, TEA was used as a moderate catalyst, while its condensation with *E*-ketone need a stronger base (NaOEt).

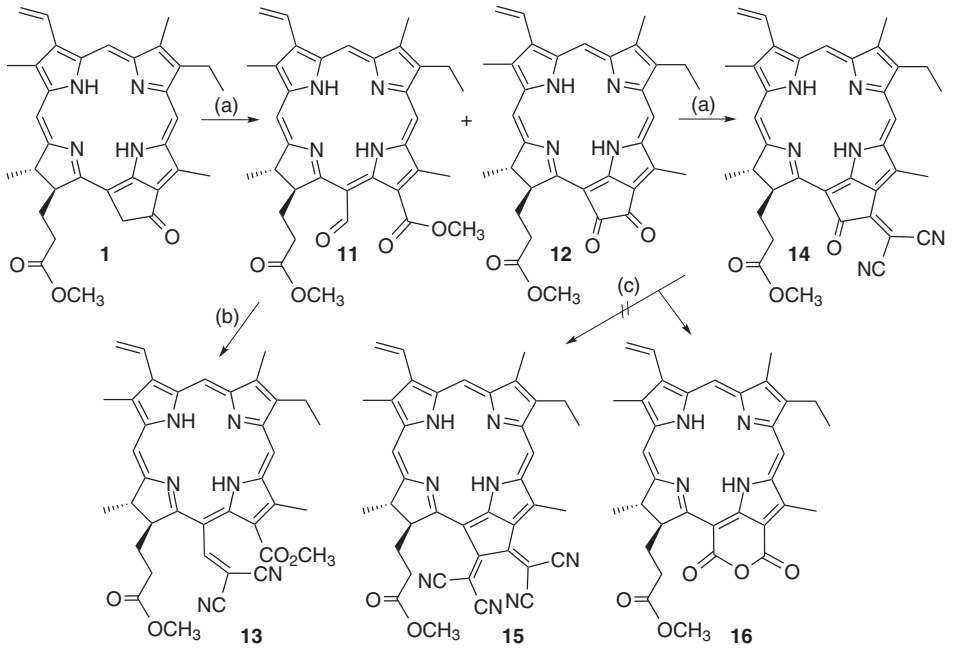
In order to introduce methylenemalononitrile moiety at *meso*-position, purpurin-5 dimethyl esters **11** and $13^2$-oxopyropheophoride-*a* **12**, obtained from the allomerization of MPPa **1** [13], were used as reaction precursors on account of the high reactivities of their C15-carbonyl groups. The former (**11**) reacted with malononitrile smoothly to form expected product **13** in 67% yield under the catalysis of TEA in THF. The corresponding reaction of the latter (**12**) did not gave designed tetracyanochlorin **15**, but diacyanochlorin **14** in 52% instead. The further reaction of **14** with malononitrile using sodium ethoxide as catalyst by refluxing in EtOH also not generated **15**, but rearranged into purpurin-18 **16** (Scheme 2).

All constructions for dicyanomethylene moiety were based on Knoevenagel reaction. Instead of forming expected product, only the condensation of $C13^2$-carbonyl of **14** with malononitrile was rearranged to known purpurin-18 ester **16**, whose possible formation process was outlined in Fig. 1. we postulated that in ethanol containing sodium ethoxide the resulting malononitrile carbonion failed to attack to the carbonyl at $13^2$-position, or carbon-carbon double bond at $13^1$-position either due to its steric hindrance, but the smaller ethyioxide ion as catalyst attacked the carbon atom at $13^2$-position to form Michael adduct **A**. Subsequent nucleophilic substitution at the carbon atom of $C13^1$-ethoxyl group brought about leaving malononitrile carbonion to rearrange to diketochlorin **12** which was readily converted into purpurin-18 eater **16** under strong alkaline condition [13].

The visible spectra of chlorins show that the introduction of dicyanomethylene moiety on the tetrapyrrole macrocycles can cause various degree of bathochromic shift in their Qy peaks (Fig. 2). Compared the differences of the longest absorption bands in their visible spectra between chlorins before and after Knoevenagel reaction, each change from C=O on the exocyclic E-ring to $C=C(CN)_2$, such as **1→2** (ΔQy = 36 nm) or **2→6** (ΔQy = 30 nm), increases their Qy wavelengths more than 30 nm. The transform from formyl group to dicyanomethylene structure at 3-position or 15-position, such as **3→6** (ΔQy = 7 nm), **4→7** (ΔQy = 12 nm) and **11→13** (ΔQy = 12 nm), bring about completely different red-shift distance. Besides structure modification, these results showed that the changes of Qy wavelengths of chlorins also related to the existing substituted groups in the opposite direction of introduced dicyanomethylene along $N_{21}$–$N_{23}$ axis. The $\lambda_{max}$ of *trans*-phenyl-substituted isomer **5b** was red shifted compared to those of unsubstituted vinyl compound **2** because the C3b-phenyl conjugated with the chlorin chromophore through the **3a**–**3b** double bond. However, the corresponding Qy band of *cis*-isomer



**Scheme 1.** Synthesis of dicyanomethylene bearing long-wavelength chlorins. Reagents and conditions: (a) $CH_2(CN)_2$/NaOEt/EtOH; (b) Py, $OsO_4$/THF/$NaIO_4$/$SiO_2$; (c) $Ph_3PCH_2PhCl$/NaOH; (d) $CH_2(CN)_2$/$CH_2Cl_2$/$N(Et)_3$; (e) NCS/$CH_2Cl_2$



**Scheme 2.** Synthesis of 15-*meso* dicyanomethylene bearing purpurin-5 **13**, and attempts at synthesizing tetracyano bearing chlorin **15**. Reagents and conditions: (a) LiOH/THF; (b) $CH_2(CN)_2$/$CH_2Cl_2$/$N(Et)_3$; (c) NaOEt/EtOH

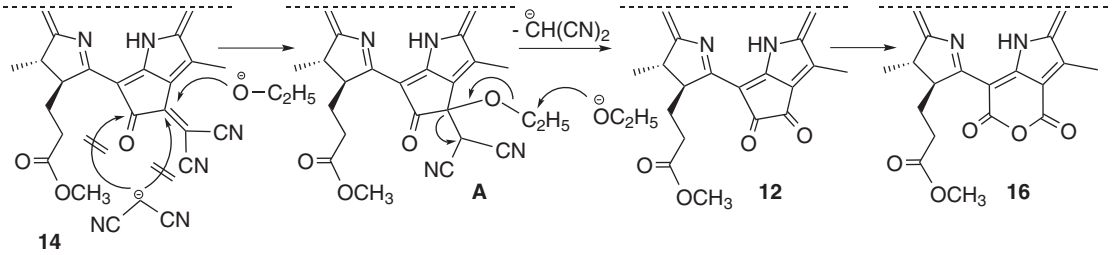



**Fig. 1.** Possible conversion process from chlorin **14** to purpurin-18 methyl ester **16**

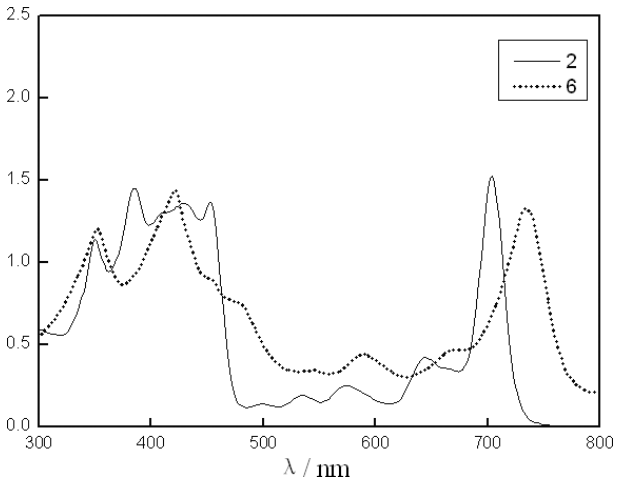



**Fig. 2.** UV-vis spectra in dichloromethane of dicyano- and tetracyano-substituted chlorin **2** and **6**

**Table 1.** Absorption properties of dicyanomethylene bearing long-wavelength chlorins

| Compound | Absorption $\lambda_{max}$, nm (relative intensity) | | | |
|---|---|---|---|---|
| | Soret | ΔSoret* | Qy | ΔQy* |
| **1** | 414 (1.00) | 0 | 668 (0.38) | 0 |
| **2** | 452 (1.00) | 38 | 704 (1.11) | 36 |
| **3** | 393 (1.00) | –21 | 726 (0.86) | 58 |
| **5a** | 452 (1.00) | 38 | 703 (1.01) | 35 |
| **5b** | 455 (1.00) | 41 | 710 (0.97) | 42 |
| **6** | 422 (1.00) | 8 | 734 (0.93) | 66 |
| **7** | 380 (1.00) | –34 | 706 (0.54) | 38 |
| **10** | 456 (1.00) | 42 | 711 (1.00) | 43 |
| **13** | 432 (1.00) | 18 | 700 (0.29) | 32 |
| **14** | 407 (1.00) | –7 | 722 (0.91) | 54 |

*ΔSoret and ΔQy represent the change of the Soret band and Qy band between the dicyano-methylene bearing long-wavelength chlorin and their starting material MPP*a* **1**.

**5a** appeared at 703 nm, which was almost the same as that of **2** (704 nm). This difference was ascribable to that *cis*-isomer has less conjugation of the C3-double bond with the chlorin chromophore because of steric repulsion between the phenyl group and C2-methyl group. The Soret/Qy absorbance ratios of chlorins including **2**, **5**–**7**, **10** and **14**, which are in the range of 0.88–1.16, were reduced after linking with electro-withdrawing groups at $13^1$-position. The corresponding ratios of chlorins **3** and **13**, which beared β, β-dicyanomethylene at 3- and 15-position respectively, were not much difference in comparison with that of their precursors **4** and **11**. It implied that rigid dicyanomethylene structure on the E-ring, except chlorin **13**, improved the absorbing intensity of Qy band more effectively than that on C3 position (Table 1).

The $^1$H NMR spectrum of chlorins introduced dicyanomethylene structure at $13^1$-position, including **2**, **3**, **10** and **14**, clearly showed the chemical shifts of all sorts of the proton signals corresponding to these of their precursor. While for the chlorins introduced dicyanomethylene structure at 3-position, after the conversion from formyl group to dicyanomethylene a 1H singlet proton signal corresponding to the proton attached to dicyanomethylene was discovered at about 8.00 ppm, at the same time the original signal for formyl group disappeared. By contrast, allmost all proton absorption signals moved to upfield except that of one hydrogen linked with central nitrogen. These regular migrations could be explained due to introduction of β, β-dicyanomethylene to reduce ring current density and (de)shielding action.

### *In vitro* photosensitizing efficacy

In the present study, viability of the cell using the methodology reported by Ahn *et al.* [14] was determined by comparison with that of MPP*a* 1 for new photosensitizers on mouse sarcoma S-180 cell line at 0.01, 0.05, 0.1, 0.5 and 1 μM after PDT (Fig. 3). For PDT treatment, it was observed that all compounds showed an improved effect for cell death or cell viability as the concentration of the photosensitizer increased. And all compounds obtained showed better effect than that of MPP*a*. Among all the dicyano- or tetracyano-substituted chlorins we obtained, compound **10** showed highest effect when compared with the others.

Table 2 shows the $IC_{50}$ values of these new photosensitizers on mouse sarcoma S-180 cell line after PDT. The reference compound MPP*a* showed a relative low effect after PDT ($IC_{50}$ > 1.0 μM), all the dicyano- or tetracyano-substituted chlorins showed lower $IC_{50}$ value than that of MPP*a*. Compound **10** among the tested photosensitizers showed relatively high PDT effect ($IC_{50}$ = 0.060 μM). Furthermore, we are aiming to explore, in greater depth, the other biological effects of these compounds for PDT.

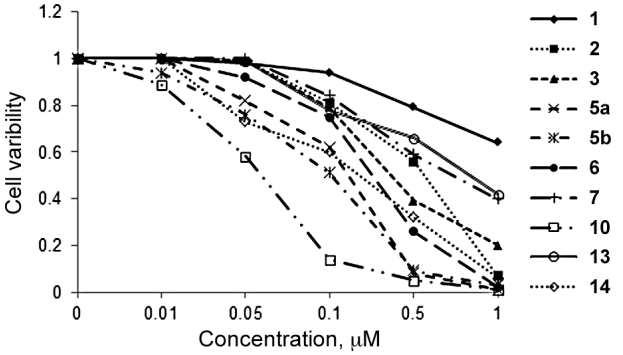



**Fig. 3.** Cell viabiliy results of photosensitizers on mouse sarcoma S-180 cell line. Compounds were tested at 0.01, 0.05, 0.1, 0.5 and 1 μM concentrations in triplicate. MPPa was used as reference compound. Mouse sarcoma S-180 cell line at 80% confluency in 96-well plates was used for *in vitro* PDT tests. Cells were illuminated with a lamp in a wavelength range of 640–700 nm, with a peak at 660 nm, for 20 min. Total light dose was 8.4 J. Statistical analyses were performed using unpaired Student's *t* test

**Table 2.** $IC_{50}$ results of photosensitizers on mouse sarcoma S-180 cell line

| Compound | **1(MPPa)** | **2** | **3** | **5a** | **5b** |
|---|---|---|---|---|---|
| $IC_{50}$, μM | >1.0 | 0.559 | 0.394 | 0.194 | 0.123 |
| Compound | **6** | **7** | **10** | **13** | **14** |
| $IC_{50}$, μM | 0.312 | 0.735 | 0.060 | 0.853 | 0.241 |

In conclusion, we have developed a new approach for the preparation of some pyropheophorbide derivatives possessing Qy-absorption at more than 700 nm by constructing two or four electron-withdrawing cyano-group on the perphery of chlorin chromophore such as at 3-positon or $13^1$-position. These long wavelength absorbing chlorins with basic skeleton of chlorophyll-a possess relative stable tetrapyrrole macrocyclic structure. Their unique optical and photochemical properties and polyfunctional groups may be valuable for the synthesis of new generation photosensitisers using in PDT. Among all the compounds we obtained, compound **10** showed relatively high PDT effect ($IC_{50}$ = 0.060 μM).

## EXPERIMENTAL

### General

All of the reactions were monitored by thin layer chromatography (TLC) using 0.20-mm silica gel plates with or without a UV indicator (60F-254). Silica gel 60 (70–230 or 230–400 mesh, Merck) was used for flash column chromatography. The melting points (uncorrected) were measured using an Electrothermal IA9000 Series digital melting point apparatus. The electronic absorption spectra were measured using a SCINCO S-3100 UV-vis spectrophotometer. The absorption maxima ($\lambda_{max}$) values are given in nanometers and relative intensity. The $^1$H NMR spectra were obtained using a Varian spectrometer (400 MHz). The chemical shifts (δ) are given in parts per million (ppm) relative to tetramethylsilane (TMS, 0 ppm), unless otherwise indicated. Elemental analyses were carried out using a Perkin Elmer 240-C microanalyzer. Materials obtained from commercial suppliers were used without further purification. Methyl pyropheophorbide *a* **1** was prepared according to the procedures described in the literature [15].

### Synthesis

**Preparation of $13^1$-β,β-dicyanomethylene-$13^1$-deoxopyropheophorbide-*a* methyl ester (2).** MPPa **1** (120 mg, 0.219 mmol) was dissolved in 30 mL EtOH, to which malononitrile (100 mg, 1.515 mmol) and sodium ethoxide (25 mg) was added under stirring. The solution was refluxed under $N_2$ and decrease of **1** was monitored by TLC. After disappearance of **1** the reaction mixture was poured into ice water and extracted with dichloromethane (2 × 50 mL). The combined extract was washed with water, dried over anhydrous $Na_2SO_4$. After evaporating the residue was chromatographed on silica gel (eluent: hexane/ethyl acetate, 3:1) to afford 98 mg of **2** (0.164 mmol, 75%). UV-vis ($CH_2Cl_2$): $\lambda_{max}$, nm (rel. intensity log ε) 704 (1.11), 644 (0.32), 574 (0.21), 535 (0.16), 452 (1.00), 430 (0.97), 384 (0.98), 350 (0.78). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 9.09, 8.97, 8.32 (each s, each 1H, *meso*-H), 7.82 (dd, *J* = 17.8, 12.3 Hz, 1H, $3^1$-H), 6.20 (dd, *J* = 17.8, 1.5 Hz, 1H, $3^2$-*trans*-H), 6.09 (dd, *J* = 12.3, 1.5 Hz, 1H, $3^2$-*cis*-H), 5.31 (d, *J* = 20.0 Hz, 1H, $13^2$-H), 5.26 (d, *J* = 20.0 Hz, 1H, $13^2$-H), 4.06–4.27 (m, 2H, 17 + 18-H), 3.58, 3.35, 3.27, 3.06 (each s, each 3H, $CH_3$ + $OCH_3$), 3.50 (q, *J* = 7.8 Hz, 2H, $8^1$-H), 2.39–2.58, 2.11–2.30 (m, 4H, $17^1$ + $17^2$-H), 1.70 (d, *J* = 7.2 Hz, 3H, 18-$CH_3$), 1.53 (t, *J* = 7.8 Hz, 3H, $8^2$-H), 0.78 (br s, 1H, NH), -1.18 (br s, 1H, NH). IR (KBr): ν, $cm^{-1}$ 3469 (N–H), 2962, 2869 (C–H), 1737 (C=O), 1664 (C=C), 1564, 1444, 1240, 1172, 1074, 908, 796 (chlorin skeleton). Anal. calcd. for $C_{37}H_{36}N_6O_2$: C 74.47, H 6.08, N 14.08; found C 74.59, H 5.89, N 14.28.

**Preparation of 3-formyl-$13^1$-β,β-dicyanomethylene-3-devinyl-$13^1$-deoxopyropheophorbide-*a* methyl ester (3) and methyl pyeopheophorbide-d (MPPd, 4).** Chlorin **2** (70 mg, 0.117 mmol) was dissolved in a mixed solution of THF (20 mL) and pyridine (0.3 mL) at 0 °C and stirred at same temperature for 30 min after adding 51 mg (0.200 mmol) osmium(VIII) oxide in THF (2 mL), and then stirred at room temperature for an additional 1 h. An excess of a solution of sodium hydrogensulfite (15 g) in a 50% mixture of methanol in water was added. The mixture was stirred violently for 20 min. After filtrating the brown osmium(VI) oxide precipitate, dichloromethane was added to the mixture. The organic layer was separated and dried over anhydrous sodium sulfate. The solvent was removed to give solid material

that was suspended in a mixture of THF (15 mL) and silica gel (2.5 g). After addition of a solution of sodium metaperiodate (1 g) in water (15 mL), the color of the solution changed from green to bronze within 30 min. After adding dichloromethane (20 mL), the mixture was filtered through cotton wool and then the resultant crude material was chromatographed on silica gel (eluent: hexane/ethyl acetate, 3:1) to give 54 mg chlorin **3** (0.090 mmol, 77%) and 8 mg MPPd **4** (0.014 mmol, 12%), respectively. **3.** UV-vis ($CH_2Cl_2$): $\lambda_{max}$, nm (rel. intensity log ε) 726 (0.86), 663 (0.22), 583 (0.21), 544 (0.15), 412 (0.99), 393 (1.00), 384 (0.86). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 11.36 (s, 1H, CHO), 10.06, 9.28, 8.62 (s, each 1H, *meso*-H), 5.61, 5.55 (each d, $J$ = 19.8 Hz, each 1H, $13^2$-H), 4.23–4.53, 4.13–4.20 (m, each 1H, 17, 18-H), 3.68 (q, $J$ = 7.8 Hz, 2H, $8^1$-H), 3.67, 3.63, 3.57, 3.15 (each s, each 3H, $CH_3$ + $OCH_3$), 2.49–2.69, 2.15–2.39 (m, 4H, $17^1$ + $17^2$-H), 1.74 (d, $J$ = 7.4 Hz, 3H, 18-$CH_3$), 1.58 (t, $J$ = 7.8 Hz, 3H, $8^2$-H), 0.80 (br s, 1H, NH), -1.50 (br s, 1H, NH). IR (KBr): ν, cm$^{-1}$ 3443 (N–H), 2962, 2926 (C–H), 1740, 1735 (C=O), 1654 (C=C), 1564, 1438, 1400, 1256, 1169, 1083, 940, 722 (chlorin skeleton). Anal. calcd. for $C_{36}H_{34}N_6O_3$: C 72.22, H 5.72, N 14.04; found C 72.40, H 5.56, N 14.19. Analytical data of MPP*d* **4** in line with the literature values [16].

**Preparation of *cis*-$3^2$-phenyl-$13^1$-β,β-dicyanomethylene-$13^1$-deoxopyropheophorbide-*a* methyl ester (5a) and *trans*-$3^2$-phenyl-$13^1$-β,β-dicyanomethylene-$13^1$-deoxopyropheophorbide-*a* methyl ester (5b).** Formyl chlorin **3** (65 mg, 0.109 mmol) and benzyltriphenylphosphonium chloride (93 mg, 0.240 mmol) was dissolved in 10 mL dichloromethane and a solution of NaOH (70 mg) in $H_2O$ (3 mL) was added with stirring. The solution was stirred at room temperature under $N_2$ and the decrease of **3** was monitored by TLC. After disappearance of **3**, the reaction mixture was poured into ice water and dichloromethane. The aqueous phase was extracted with several portions of dichloromethane and the combined organic phases were washed with aqueous aq. 2% HCl, aq. 4% $NaHCO_3$ and water and evaporated *in vacuo* to dryness. The residue was chromatographed on silica gel (eluent: hexane/ethyl acetate, 4:1) to give 14 mg chlorin **5a** (0.021 mmol, 19%) and 43 mg chlorin **5b** (0.064 mmol, 59%). **5a.** UV-vis ($CH_2Cl_2$): $\lambda_{max}$, nm (rel. intensity log ε) 703 (1.01), 644 (0.34), 574 (0.23), 533 (0.19), 495 (0.16), 452 (1.00), 434 (0.94), 386 (0.88), 350 (0.72). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 9.10, 9.02, 8.27 (each s, each 1H, *meso*-H), 7.70 (m, 2H, Ph-H), 7.54 (d, $J$ = 11.4 Hz, 1H, $3^1$-H), 7.52 (m, 1H, Ph-H), 7.35 (d, $J$ = 11.4 Hz, 1H, $3^2$-H), 6.94–7.25 (m, 3H, Ph-H), 5.52, 5.38 (d, $J$ = 20 Hz, each 1H, $13^2$-H), 4.18–4.35, 4.06–4.15 (m, 2H, 17 + 18-H), 3.53 (q, $J$ = 7.8 Hz, 2H, $8^1$-H), 3.64, 3.53, 3.00, 2.90 (each s, each 3H, $CH_3$ + $OCH_3$), 2.46–2.64, 2.20–2.35 (m, each 1H, $17^1$ + $17^2$-H), 1.75 (d, $J$ = 7.2 Hz, 3H, 18-$CH_3$), 1.58 (t, $J$ = 7.8 Hz, 3H, $8^2$-H), 0.78 (br s, 1H, NH), -0.86 (br s, 1H, NH). IR (KBr): ν, cm$^{-1}$ 3448, 3180 (N–H), 2929, 2866 (C–H), 1741 (C=O), 1638 (C=C), 1528, 1400, 1173, 1071, 1040, 968, 728 (chlorin skeleton). Anal. calcd. for $C_{43}H_{40}N_6O_2$: C 76.76, H 5.99, N 12.49; found C 76.60, H 5.81, N 12.24. **5b.** UV-vis ($CH_2Cl_2$): $\lambda_{max}$, nm (rel. intensity log ε) 710 (0.97), 649 (0.27), 577 (0.12), 496 (0.08), 455 (1.00), 434 (0.89), 390 (0.84), 353 (0.52). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 9.12, 9.10, 8.36 (each s, each 1H, *meso*-H), 8.13 (d, $J$ = 16.3 Hz, 1H, $3^1$-H), 7.78 (d, $J$ = 7.2 Hz, 2H, Ph-H), 7.55 (d, $J$ = 16.3 Hz, 1H, $3^2$-H), 7.44–7.57 (m, 3H, Ph-H), 5.54 (d, $J$ = 21 Hz, 1H, $13^2$-H), 5.40 (d, $J$ = 21 Hz, 1H, $13^2$-H), 4.24–4.40, 4.08–4.17 (m, each 1H, 17 + 18-H), 3.63, 3.34, 3.32, 3.09 (each s, each 3H, $CH_3$ + $OCH_3$), 3.60 (q, $J$ = 7.8 Hz, 2H, $8^1$-H), 2.40–2.63, 2.17–2.36 (m, each 2H, $17^1$ + $17^2$-H), 1.75 (d, $J$ = 7.2 Hz, 1H, 18-$CH_3$), 1.61 (t, $J$ = 7.4 Hz, 3H, $8^2$-H), 0.06 (br s, 1H, NH), -0.98 (br s, 1H, NH). IR (KBr): ν, cm$^{-1}$ 3449 (N–H), 2924 (C-H), 1736 (C=O), 1686 (C=C), 1560, 1459, 1341, 1086, 1040, 976, 669 (chlorin skeleton). Anal. calcd. for $C_{43}H_{40}N_6O_2$: C 76.76, H 5.99, N 12.49; found C 76.89, H 6.13, N 12.67.

**Preparation of $3^2$,$3^2$-dicyano-$13^1$-β,β-dicyanomethylene-$13^1$-deoxopyropheophorbide-*a* methyl ester (6).** Formyl chlorin **3** (40 mg, 0.067 mmol) was dissolved in 15 mL dichloromethane containing TEA (1.5 mL), to which malononitrile (70 mg, 1.060 mmol) was added under stirring. The solution was stirred under $N_2$ for 2 h. The reaction mixture was poured into ice water and extracted with dichloromethane (2 × 15 mL). The combined extract was washed with water, dried over anhydrous $Na_2SO_4$. The combined organic phases were evaporated to druness and the residue was chromatographed on silica gel (eluent: hexane/ethyl acetate, 3:1) to afford 20 mg **6** (0.0031 mmol, 46%). UV-vis ($CH_2Cl_2$): $\lambda_{max}$, nm (rel. intensity log ε) 734 (0.93), 590 (0.31), 545 (0.24), 422 (1.00), 353 (0.83). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 9.46, 9.12, 8.69 (each s, each 1H, *meso*-H), 9.25 (s, 1H, $3^1$-H), 5.71 (d, $J$ = 21.0 Hz, 1H, $13^2$-H), 5.55 (d, $J$ = 21.0 Hz, 1H, $13^2$-H), 4.38–4.56 (m, 1H, 18-H), 4.21–4.43 (m, 1H, 17-H), 3.76, 3.62, 3.48, 3.26 (each s, each 3H, $CH_3$ + $OCH_3$), 3.69 (q, $J$ = 7.5 Hz, 2H, $8^1$-H), 2.51–2.73, 2.16–2.40 (m, each 2H, $17^1$ + $17^2$-H), 1.81 (d, $J$ = 7.2 Hz, 3H, 18-$CH_3$), 1.68 (t, $J$ = 7.5Hz, 3H, $8^1$-$CH_3$), 0.79 (br s, 1H, NH), -1.35 (br s, 1H, NH). IR (KBr): ν, cm$^{-1}$ 3450 (N–H), 2978 (C–H), 1735 (C=O), 1637 (C=C), 1561, 1475, 1225, 1084, 982, 722 (chlorin skeleton). Anal. calcd. for $C_{39}H_{34}N_8O_2$: C 72.43, H 5.30, N 17.33; found C 72.59, H 5.17, N 17.50.

**Preparation of $3^2$,$3^2$-dicyanopyropheophorbide-*a* methyl ester (7).** MPP*d* **4** (42 mg, 0.076 mmol) was dissolved in 20 mL dichloromethane, to which malononitrile (70 mg, 1.060 mmol) and TEA (1.5 mL) was added under stirring. The solution was stirred under $N_2$ for 2 h. The reaction mixture was poured into ice water and extracted with dichloro-methane (2 × 15 mL). The combined extracts was washed with water, dried over anhydrous $Na_2SO_4$. After evaporating the solution the residue was chromatographed on silica gel (eluent: hexane/ethyl acetate, 3:1) to afford 34 mg **7** (0.057 mmol,

76%). UV-vis ($CH_2Cl_2$): $\lambda_{max}$, nm (rel. intensity log ε) 706 (0.54), 638 (0.08), 590 (0.11), 522 (0.11), 434 (0.54), 431 (0.49), 380 (1.00). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 9.58, 9.19, 8.79 (s, each 1H, *meso*-H), 9.23 (s, 1H, $3^1$-H), 5.26 (d, $J$ = 20.0 Hz, 1H, $13^2$-H), 5.19 (d, $J$ = 20.0 Hz, 1H, $13^2$-H), 4.34 (q, $J$ = 7.3 Hz, 1H, 18-H), 4.12 (d, $J$ = 7.2 Hz, 1H, 17-H), 3.67, 3.62, 3.51, 3.28 (each s, each 3H, $CH_3$ + $OCH_3$), 3.70 (q, $J$ = 7.6Hz, 2H, $8^1$-H), 2.52–2.70, 2.20–2.43 (m, each 2H, $17^1$ + $17^2$-H), 1.84 (d, $J$ = 7.2 Hz, 3H, 18-$CH_3$), 1.71 (t, $J$ = 7.6 Hz, 3H, $8^1$-$CH_3$), -0.16 (br s, 1H, NH), -2.08 (br s, 1H, NH). IR (KBr): ν, $cm^{-1}$ 3449, 3167 (N–H), 2963 (C–H), 1740 (C=O), 1672 (C=C), 1542, 1510, 1400, 1223, 1075, 1021, 980, 799 (chlorin skeleton). Anal. calcd. for $C_{36}H_{34}N_6O_3$: C 72.22, H 5.72, N 14.04; found C 72.06, H 5.60, N 13.91.

**Preparation of 20-chloropyropheophorbide-*a* methyl ester (9).** 150 mg of NCS was partialy added to a solution of MPPa **1** (200 mg, 0.330 mmol) in methylene chloride (50 mL), and the reaction mixture was stirred for 5 h under nitrogen in the dark. The resulting solution was then poured into 200 mL of iced water and extracted with methylene chloride (3 × 100 mL). The combined extract was washed with 10% aqueous $NaHCO_3$, water and dried over $Na_2SO_4$ and evaporated to dryness. The residue was chromatographed on silica gel (eluent: hexane/ethyl acetate, 2:1) to afford 237 mg chlorin **9** as dark-red solid in 80% yield. mp 210–214 °C. UV-vis ($CHCl_3$): $\lambda_{max}$, nm (rel. intensity log ε) 676 (0.41), 620 (0.07), 550 (0.12), 518 (0.09), 416 (1.00). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 9.52, 9.55 (each s, each 1H, *meso*-H), 7.92 (dd, $J$ = 17.8, 11.5 Hz, 1H, $3^1$-H), 6.27 (dd, $J$ = 11.5, 1.5 Hz, 1H, $3^2$-H), 6.14 (dd, $J$ = 17.8, 1.5 Hz, 1H, $3^2$-H), 5.24 (d, $J$ = 3.2 Hz, 2H, $13^2$-H), 4.80 (q, $J$ = 7.0 Hz, 1H, 18-H), 4.24 (dd, $J$ = 9.0, 2.8 Hz, 1H, 17-H), 3.68 (q, $J$ = 7.6 Hz, 2H, $8^1$-$CH_2$), 3.67, 3.60, 3.59, 3.24 (each s, each 3H, $OCH_3$ + $CH_3$), 2.05–2.70 (m, 4H, $17^1$ + $17^2$-H), 1.70 (t, $J$ = 7.6 Hz, 3H, $8^2$-$CH_3$), 1.64 (d, $J$ = 7.0 Hz, 3H, 18-$CH_3$), 0.48 (br s, 1H, NH), –1.94 (br s, 1H, NH). Other analytical data are consistent with the literature data [17].

**Preparation of 20-chloro-$13^1$-β,β-dicyanomethylene-$13^1$-deoxopyropheophorbide-*a* methyl ester (10).** This compound as a red solid was obtained from compound **9** by reacting with malononitrile in the yield of 59% according to the method for preparing compound **2**. UV-vis ($CHCl_3$): $\lambda_{max}$, nm (rel. intensity log ε) 711 (1.00), 651 (0.17), 585 (0.17), 545 (0.08), 456 (1.00), 385 (0.79). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 9.17, 9.35 (each s, each 1H, *meso*-H), 7.85 (dd, $J$ = 17.8, 11.5 Hz, 1H, $3^1$-H), 6.28 (dd, $J$ = 11.5, 1.4 Hz, 1H, *cis*-$3^2$-H), 6.07 (dd, $J$ = 17.8, 1.4 Hz, 1H, *trans*-$3^2$-H), 5.44 (d, $J$ = 21.0 Hz, 1H, $13^2$-H), 5.28 (d, $J$ = 21.0 Hz, 1H, $13^2$-H), 4.66 (q, $J$ = 7.1 Hz, 1H, 18-H), 4.04 (dd, $J$ = 9.2, 2.4 Hz, 1H, 17-H), 3.62 (q, $J$ = 7.6 Hz, 2H, $8^1$-H), 3.63, 3.54, 3.50, 3.19 (each s, each 3H, $CH_3$ + $OCH_3$), 2.53–2.61, 2.15–2.25, 1.85–1.93 (each m, 4H, $17^1$ + $17^2$-H), 1.64 (t, $J$ = 7.6 Hz, 3H, $8^2$-$CH_3$), 1.55 (d, $J$ = 7.1 Hz, 3H, 18-$CH_3$), -1.32 (each br s, each 1H, NH), -1.51 (each br s, each 1H, NH). IR (KBr): ν, $cm^{-1}$ 3448, 3145 (N–H), 2956 (C–H), 1736 (C=O), 1665 (C=C), 1619, 1437, 1401, 1257, 1170, 1010, 976, 710, 708, 597 (chlorin skeleton). Anal. calcd. for $C_{37}H_{35}ClN_6O_2$: C 70.41, H 5.59, N 13.32; found C 70.26, H 5.72, N 13.25.

**Preparation of 15-β,β-dicyanomethylenepurpurin-5 methyl ester (13).** This compound as a red solid was obtained from compound **11** by reacting with malononitrile in the yield of 71% according to the method for preparing compound **6**. UV-vis ($CHCl_3$): $\lambda_{max}$, nm (rel. intensity log ε) 700 (0.29), 620 (0.07), 564 (0.20), 502 (0.08), 432 (1.00), 358 (0.67) nm. $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 9.59 (s, 1H, $15^1$-H), 8.45, 9.34, 9.62 (each s, each 1H, *meso*-H), 7.88 (dd, $J$ = 17.7, 11.5 Hz, 1H, $3^1$-H), 6.28 (d, $J$ = 17.7 Hz, 1H, *trans*-$3^2$-H), 6.16 (d, $J$ = 11.5 Hz, 1H, *cis*-$3^2$-H), 4.50 (d, $J$ = 10.7 Hz, 1H, 17-H), 4.36 (q, $J$ = 7.3 Hz, 1H, 18-H), 3.66 (q, $J$ = 7.6 Hz, 2H, $8^1$-H), 4.25, 3.37, 3.51, 3.32, 3.18 (each s, each 3H, $CH_3$ + $OCH_3$), 1.95–2.08, 2.38–2.41, 2.58–2.69 (each m, 4H, $17^1$ + $17^2$-H), 1.78 (d, $J$ = 6.6 Hz, 3H, 18-$CH_3$), 1.67 (t, $J$ = 7.6 Hz, 3H, $8^2$-$CH_3$), 0.20 (br s, 1H, NH), -0.20 (br s, 1H, NH). IR (KBr): ν, $cm^{-1}$ 3423, 3163 (N–H), 2958 (C–H), 1740, 1735 (C=O), 1672 (C=C), 1606, 1400, 1256, 1084, 924 (chlorin skeleton). Anal. calcd. for $C_{38}H_{38}N_6O_4$: C 71.01, H 5.96, N 13.08; found C 71.24, H 5.79, N 13.20.

**Preparation of $13^1$-β,β-dicyanomethylene-$13^2$-oxo-$13^1$-deoxopyropheophorbide-*a* methyl ester (14).** This compound as a red solid was obtained from compound **12** by reacting with malononitrile in the yield of 68% according to the method for preparing compound **6**. UV-vis ($CHCl_3$): $\lambda_{max}$, nm (rel. intensity log ε) 722 (0.91), 618 (0.08), 512 (0.28), 472 (0.36), 425 (0.35), 407 (1.00). $^1$H NMR (400 MHz; $CDCl_3$): δ, ppm 8.94, 9.50, 9.80 (each s, each 1H, *meso*-H), 8.08 (dd, $J$ = 17.8, 11.5 Hz, $3^1$-H), 6.36 (dd, $J$ = 17.8, 1.0 Hz, 1H, *trans*-$3^2$-H), 6.30 (dd, $J$ = 11.5, 1.0 Hz, 1H, *cis*-$3^2$-H), 5.11 (d, $J$ = 8.0 Hz, 1H, 17-H), 4.63 (q, $J$ = 7.5 Hz, 1H, 18-H), 3.75 (q, $J$ = 7.6 Hz, 2H, $8^1$-H), 3.62, 3.51, 3.50, 3.35 (each s, each 3H, $CH_3$ + $OCH_3$), 1.95–2.07, 2.18–2.43, 2.67–2.80 (each m, 4H, $17^1$ + $17^2$-H), 1.88 (d, $J$ = 7.3 Hz, 3H, 18-$CH_3$), 1.70 (t, $J$ = 7.6 Hz, 3H, 8-$CH_3$), 0.51 (br s, 1H, NH), -1.98 (br s, 1H, NH). IR (KBr): ν, $cm^{-1}$ 3449 (N–H), 2989 (C–H), 1741, 1735 (C=O), 1638 (C=C), 1542, 1528, 1308, 1080, 726 (chlorin skeleton). Anal. calcd. for $C_{37}H_{34}N_6O_3$: C 72.77, H 5.61, N 13.76; found C 72.60, H 5.40, N 13.91.

### MTT assay for *in vitro* photosensitizing efficacy

The photosensitizing activities of long-wavelength dicyanomethylene bearing compounds (**2**, **3**, **5a**, **5b**, **6**, **7**, **10**, **13**, **14**) and MPPa **1** were determined in the mouse sarcoma S-180 cell line. The cells were grown in α-MEM with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin. Cells were maintained in 5% $CO_2$, 95% air, and 100% humidity. Cells were plated in 96-well plates at a density of 5 × $10^3$ cells per well

in complete medium. After an overnight incubation at 37 °C, the photosensitizers were added at varying concentrations and incubated at 37 °C for 3 h in the dark. Prior to light treatment the cells were replaced with drug-free complete medium. Cells were then illuminated with a lamp in a wavelength range of 640–700 nm, with a peak at 660 nm, for 20 min. Total light dose was 8.4 J. After PDT, the cells were incubated for 48 h at 37 °C in the dark. Following the 48 h incubation, 10 μL of 4.0 mg/mL solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) (Sigma) dissolved in PBS was added to each well. After a 4 h incubation at 37 °C, unreacted MTT and medium were removed and 100 μL DMSO was added to solubilize the formazan crystals. The 96-well plate was read on a microtiter plate reader (ELISA-reader, BioTek, Synergy HT, USA) at an absorbance of 570 nm. The results were plotted as percent survival of the corresponding dark (drug no light) control for each compound tested after subtracting medium only control absorbance. Each data point represents the mean from 3 separate experiments with 6 replicates at each dose, and the standard errors were less than 10%.

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