Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2049–2052

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Photodynamic Action of Diphenyl-2,3-dihydroxychlorin: A Potential Tumor Photosensitizer

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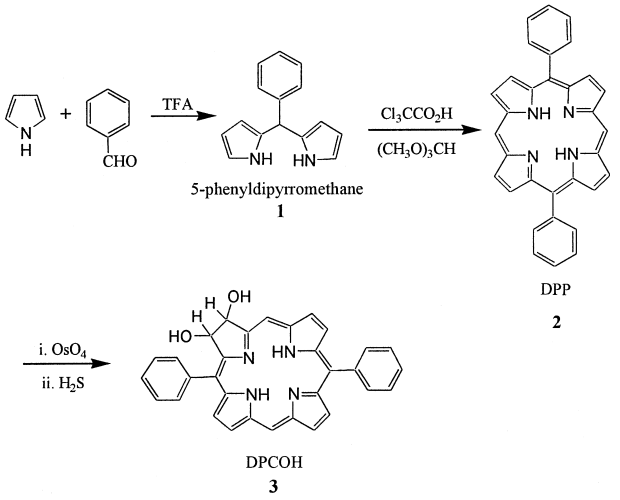
Received 26 March 2001; accepted 21 May 2001

Abstract—The synthesis, photophysical properties of diphenyl-2,3-dihydroxychlorin (DPCOH) and its photocytotoxicity to tumor cells are described. DPCOH exhibits photodynamic activity in terms of type I and type II mechanisms under irradiation. The quantum yield of $^1O_2$ in $CHCl_3$ is 0.7. For the photocytotoxicity to tumor cells, DPCOH proved to be 200 times more potent than HPD, and the dark toxicity is low (dark $IC_{90} > 32\,\mu g/mL$). © 2001 Elsevier Science Ltd. All rights reserved.

Many novel porphyrin-based photosensitizers, including chlorins, bacteriochlorins and purpurins, have been developed for the photodynamic therapy (PDT) of tumors and other illnesses.[1–3] PDT is based on the principle that porphyrins concentrated in tumor cells and upon subsequent irradiation with visible light in the presence of oxygen, specifically destroy the cells. Light-activated porphyrin-based photosensitizers can transfer energy from its triplet state by two processes, directly to molecular oxygen with generation of singlet oxygen ($^1O_2$) (type II mechanism) or by interaction with a solvent or substrate by electron or proton transfer with generation of radicals (type I mechanism).[4–6]

5,15-Diphenylporphyrin (DPP), an interesting porphyrin model compound that combines the features of two classic synthetic porphyrins, 5,10,15,20-tetraphenylporphyrin (TPP) and 2,3,7,8,12,13,17,18-octaethyl-porphyrin (OEP), was always inaccessible in quantities suitable for study because of the difficulty of synthesis. The characteristics of this molecule had not been explored until a novel method developed recently for the preparation of DPP.[7,8] Therefore it will be possible for the synthesis of DPP-based photosensitizers and investigation of their PDT application. Some initial research work about the synthesis of DPP-based chlorins has been published.[9,10] Dolphin's group declared that 5,15-diphenyl-2,3-dihydroxychlorin (DPCOH) could be prepared with the OsO_4 oxidation of DPP,[10] but they did not report the synthesis procedure, spectroscopic data and photodynamic properties.

The synthesis of DPCOH is summarized in Scheme 1. 5-Phenyldipyrromethane (**1**) and 5,15-diphenylporphyrin (**2**) were synthesized using literature methods[7,8] with the modification of workup procedures.[11] The porphyrin was oxidized[10] with OsO_4 in the present of



Scheme 1. The synthesis of DPCOH.

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PII: S0960-894X(01)00363-8

pyridine at room temperature in the dark for 3 h, and the stable intermediate osmate ester was reduced with gaseous H_2S to give DPCOH (**3**) as blue crystals in 43% yield after workup and crystallization from CH_2Cl_2/hexane.[12]

DPCOH has suitable absorption for PDT in the red region of the visible spectrum. The photosensitized generation of singlet oxygen, superoxide anion radical, hydroxyl radical, photosensitizer anion radicals and other species of radicals were detected by electron paramagnetic resonance (EPR) method at room temperature (22–24 °C), using a Bruker ESP-300E spectrometer operating at 9.8 GHz equipped with an X-band of 100 kHz field modulation. A xenon arc lamp (150 W) with an intensity of 3.6 W/cm^2 was used as the light source equipped with UV and IR cut-off filters (transmittance 470–700 nm). A water-jacketed cooling device was used to eliminate heat emission. Samples were made in cuvettes, which allowed purging the reactive volume with oxygen or argon for 30 min in the dark, according to the experimental requirements, irradiated outside the cavity in the cuvettes, and then immediately transferred into quartz capillaries designed specially for EPR analysis after exposure.

Superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) were studied by means of a spin trap of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) with EPR measurements. When CH_3CN (5% $CHCl_3$) solution containing DPCOH (0.1 mM) and DMPO (50 mM) was irradiated, an EPR spectrum appeared immediately. The EPR spectrum was characterized by three coupling constants, which are due to the nitrogen atom and two hydrogen atoms at the β and γ positions. The g factor and the determined constants (g = 2.0056, $\alpha^N = 13.0$ G, $\alpha_\beta^H = 10.0$ G and $\beta_\gamma^H = 1.4$ G) are in good agreement with the literature for DMPO–superoxide radical adduct.[13] Control experiments suggested that DPCOH, oxygen and irradiation were all necessary to produce the EPR signal. The addition of *p*-benzoquinone (4 mM), an efficient scavenger of $O_2^{\bullet-}$,[14] prior to illumination inhibited the EPR signal significantly. These observations confirmed the correct assignment of the EPR spectrum to DMPO–$O_2^{\bullet-}$ adduct. The addition of 1,4-diaza- bicyclo[2,2,2]octane (DABCO) (10 mM) inhibitors for $^1O_2$ had no effect on the EPR signal of DMPO–$O_2^{\bullet-}$, indicating that $^1O_2$ is not involved in the formation of $O_2^{\bullet-}$ by DPCOH.

Irradiation of an aerated aqueous solution of pH 7.4 (phosphate buffer, 0.5% DMSO) containing DPCOH (0.1 mM) and DMPO (50 mM) led to the formation of a four-line ESR spectrum with hyperfine splittings ($\alpha^N = \alpha^H = 14.9$ G) characteristic of the DMPO–$^{\bullet}OH$ spin adduct. The values of coupling constants were in good agreement with those found in the literature.[15] No superoxide radical was detected in aqueous solution. Control experiments ensured that no signal was obtained without light, oxygen, DPCOH or DMPO. The ESR signal intensity of the DMPO–$^{\bullet}OH$ adducts did not decrease when DABCO was added, indicating that $^1O_2$ is not involved in the formation of

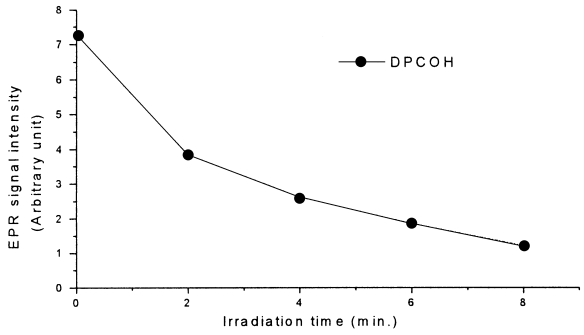


Figure 1. Spin elimination of TEMPO as a function of illumination time.

DMPO–$^{\bullet}OH$. The addition of superoxide dismutase (SOD) (30 μg/mL), an efficient scavenger of $O_2^{\bullet-}$, inhibited the formation of adduct by about 40%, indicating that the superoxide radical anion was involved in the formation of $^{\bullet}OH$ by DPCOH.

DPCOH anion radical (DPCOH$^{\bullet-}$) was also investigated by EPR method with 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl radical (TEMPO) as spin trap. Previously, Moan has developed a method to detect indirectly porphyrin anion radicals, which is based on the fact that under anaerobic conditions the porphyrin anion radical reduces TEMPO.[16] Figure 1 shows the elimination of the spin of TEMPO (0.05 mM) in anaerobic CH_3CN (5% $CHCl_3$) when exposed to light in the present of DPCOH (0.1 mM).

No significant degradation was observed in solutions exposed to air. Furthermore, no degradation was observed when solutions of TEMPO were exposed to DPCOH in the darkness or to light in the absence of DPCOH. Thus, the spin elimination is not mediated by active oxygen species because it is suppressed in aerated solution. The electron donor, such as dithiothreitol (DTT), strongly enhanced the photodegradation of TEMPO in anaerobic solutions. This confirmed that the spin elimination of TEMPO is caused by the reaction of TEMPO with DPCOH$^{\bullet-}$.

The $^1O_2$ generation photosensitized by DPCOH in $CHCl_3$ was also investigated by EPR measurements using 2,2,6,6-tetramethyl-4-piperidone (TEMP) as a spin trap. Moan and Wold had reported that TEMP could easily react with $^1O_2$ to yield a nitroxide, TEMPO, which can be detected by EPR spectroscopy.[15] Irradiation of oxygen-saturated $CHCl_3$ solution containing DPCOH (0.1 mM), and TEMP (10 mM) afforded typical three-line EPR spectrum with $\alpha^N = 16.0$ G and g = 2.0056, which were in agreement with the literature.[15] Under similar conditions but in the absence of DPCOH, oxygen or light TEMPO formation did not occur. Further support for this was provided by a DABCO inhibiting experiment; in the presence of $^1O_2$ scavenger DABCO, the EPR signal of TEMPO was suppressed.

The photooxidation of 9,10-diphenyl-anthracene (DPA) to its endoperoxide derivative by singlet oxygen is

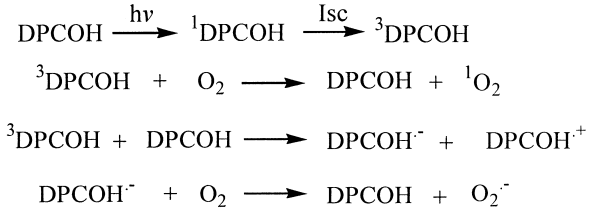
usually used to detect singlet oxygen formation and its quantum yield during photosensitization.[17] The photooxidation of DPA sensitized by DPCOH was carried out on a 'merry-go-round' apparatus, using a medium-pressure rare earths mercury lamp (400 W) as the light source. Filters were used in combination to isolate the 410 nm emission of the lamp (maximum transmittance, 45%). The reactions were followed spectro-photometrically by observing the decrease in the 374 nm absorption peak of DPA (where DPCOH has lower absorptivity) as a function of irradiation time.

In order to determine the quantum yield of $^1O_2$ generation, haematoporphyrin was used as the reference ($\Phi_\Delta = 0.79$). During the measurements the concentration of DPCOH remained constant at 10 μM and the absorption of haematoporphyrin at 410 nm used was adjusted to be the same as those of DPCOH solution (0.68 in a 10×10 mm cell). DPCOH can efficiently photosensitize the generation of $^1O_2$ with about 0.89 times the effectiveness of haematoporphyrin. The ϕ^1O_2 of DPCOH in $CHCl_3$ is thus estimated to be 0.70.

In general, with the excitation of light, the photosensitizer is first excited into a short-lived singlet state, and then converted into the longer-lived triplet state via an intersystem crossing process, and photosensitization then occurs only in the triplet state.[18] The energy transformation from triplet DPCOH to oxygen produces the $^1O_2$, and the DPCOH triplet state may transfer its electron to the DPCOH in the ground state to give DPCOH cation and anion radicals. The electron transfer from anion radicals to oxygen generates $O_2^{\bullet -}$, and superoxide anion radical may lead to the generation of $^\bullet$OH in aqueous solution (Scheme 2).

MDAMB543 human galactophore carcinoma cells were used for the photocytotoxicity experiment in vitro. HPD (haematoporphyrin derivatives used in PDT, purchased from Beijing Institute of Pharmaceutical Industry, containing about 30% Hp, 8% protoporphyrin, 20% hydroxyethyl vinyl deuteroporphyrin and 40% dihaematoporphyrin ether ester and higher oligomers) and DPCOH were dissolved in DMSO, and diluted to the desired concentrations with medium. Exponentially growing 543 human galactophore carcinoma cells (1×10^6/mL) were incubated with varying concentrations of the photosensitizers in RPMI-1640 medium containing 10% fetal calf serum in 96-well microtiter plate for 4 h at 37 °C and 5% CO_2. The plate was then illuminated (light source: copper vapour laser, 510 nm, 20 mW/cm^2) for a period of time, according to the experimental requirements. Incubation was continued for a further 24 h in the dark and the number of surviving cells was analyzed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay.[19]

The maximum concentration of DMSO in the incubation medium was 0.8% (v/v), and any effects of DMSO on cloning efficiency were controlled by a series of wells containing the appropriate concentration of DMSO, but no sensitizer. Preliminary studies had indicated that the presence of fetal calf serum did not significantly influence toxicity. Control experiment suggested that the survival of cells was not influenced by irradiation with laser (510 nm, 20 mW/cm^2) for 320 s without photosensitizer.



Scheme 2.

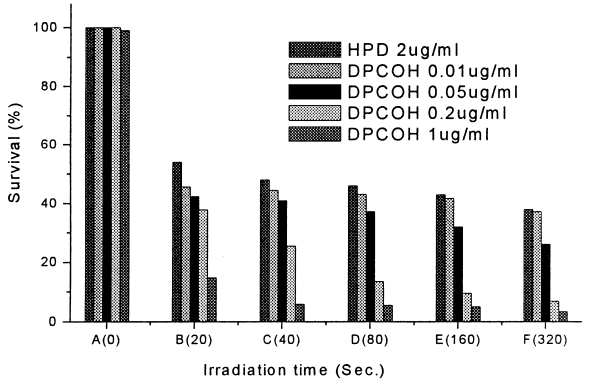


Figure 2. Percentage of cell survival versus irradiation time.

MTT colorimetric assay measures the activity of mitochondrial hydrogenases. This quick and reliable assay has been shown to correlate well with other cell-viability tests, including the clonogenic assay and [^{3}H]thymidine incorporation in the case of PDT inactivation of cells with haematoporphyrin derivative[20,21] and benzoporphyrin derivatives.[19] We employed MTT assay to evaluate the effect of the concentrations on the phototoxic potential of DPCOH. Figure 2 displays survival cell counts as a function of irradiation time. HPD was used as a control. Compared with HPD, DPCOH exhibits 200 times more potent photocytotoxicity with the irradiation and following a further 24 h incubation in the dark. Irradiation of the cell suspensions containing 0.01 μg/mL DPCOH with laser (510 nm, 20 mW/cm^2) for 20 s led to 55% inhibition, while 2 μg/mL HPD only introduced 46% tumor cells dead under the same condition.

The dark toxicity characteristic of DPCOH was determined following a similar procedure for cells exposure to graded doses of the photosensitizer for 24 h. Precautions were taken to avoid exposure of the cells to light throughout the period that they were exposed to the photosensitizer. The result suggest that the dark IC_{90} (90% inhibition) of DPCOH is larger than 32 μg/mL.

In summary, OsO_4 oxidation of DPP giving the corresponding more amphiphilic dihydroxy-chlorin is simple and efficient. Our study has shown that $^1O_2$, $O_2^{\bullet -}$, $^\bullet$OH and DPCOH$^{\bullet -}$ can be generated during the irradiation of DPCOH. It could be inferred that the photodynamic action of DPCOH involves both type I and type II

mechanisms. The $^1O_2$ quantum yield of DPCOH was estimated to be 0.7 in $CHCl_3$. The elementary biological experiments show that DPCOH exhibits very high phototoxic activity and low dark toxicity to 543 human galactophore carcinoma cells. It has great potential for use as a PDT agent.

Acknowledgements

The National Natural Science Foundation of China (39830090) and the National Key Laboratory for Structural Chemistry of Unstable and Stable Species supported this work.

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12. **3**: mp 260–262 °C; UV–vis λ_{max} (log ε) in benzene: 364 nm (4.54), 402 nm (5.19), 504 nm (4.13), 531 nm (3.89), 587 nm (3.67), 638 nm (4.55); fluorescence in benzene: $F_{max} = 641$ nm; fluorescence quantum yields: $\phi_F = 0.10$ (nitrogen-saturated), 0.08 (air-saturated) at room temperature; IR (KBr) cm^{-1}: 3423, 2925, 1610, 1419, 1034, 962; ^1H NMR (200 MHz, $CDCl_3$): δ −2.0 (br s, 2H), 5.90 (d, 1H), 6.45 (d, 1H), 7.75 (m, 6H), 8.12 (d, 1H), 8.24 (d, 2H), 8,48 (d, 1H), 8.55 (d, 1H), 8.65 (d, 1H), 8.89 (d, 1H), 8.95 (d, 2H), 9.10 (d, 1H), 9.28 (s, 1H), 9.96 (s, 1H); FAB-MS: *m*/*z* 496 (M^+). Anal. calcd for $C_{32}H_{24}N_4O_2$: C, 77.40; H, 4.87; N, l2.06. Found: C, 77.18; H, 4.73; N, l2.11.
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