

Diketopyrrolopyrrole-Porphyrin Conjugates with High Two-Photon Absorption and Singlet Oxygen Generation for Two-Photon Photodynamic Therapy**

Julie Schmitt, Valérie Heitz,* Angélique Sour, Frédéric Bolze,* Hussein Ftouni, Jean-Francois Nicoud, Lucia Flamigni, and Barbara Ventura*

Abstract: Two-photon photodynamic therapy is a promising therapeutic method which requires the development of sensitizers with efficient two-photon absorption and singlet-oxygen generation. Reported here are two new diketopyrrolopyrrole-porphyrin conjugates as robust two-photon absorbing dyes with high two-photon absorption cross-sections within the therapeutic window. Furthermore, for the first time the singlet-oxygen generation efficiency of diketopyrrolopyrrole-containing systems is investigated. A preliminary study on cell culture showed efficient two-photon induced phototoxicity.

Photodynamic therapy (PDT) is an appealing non-invasive treatment currently used to cure skin damage and age-related macular degeneration. In PDT, upon light excitation, a photosensitizer undergoes an efficient intersystem crossing that populates the triplet excited state which reacts with molecular oxygen, thus producing singlet oxygen and/or other cytotoxic reactive oxygen species.^[1] A major drawback of PDT performed in clinics concerns the accessibility of the photosensitizer to the light source. Light excitation is usually performed in the UV-visible region and its low penetration in tissues for wavelengths below $\lambda = 700$ nm limits the treatment to superficial damage. An emerging and promising development concerns two-photon PDT.^[2] Indeed, two-photon (TP) excitation in the near infrared offers great advantages to

perform PDT in a more efficient way: 1) an increase in spatial confinement of the treatment inherent to the quadratic dependence of TP absorption with laser intensity, 2) an increase in light penetration useful to treat deeper tumors, and 3) a decrease in the photodamage as a result of the low energy of the TP excitation wavelength. Such improvements are dependent on the development of new photosensitizers characterized by a high TP absorption cross-section (σ_2) in the near infrared since the ones currently used in clinics have low TP efficiency.^[3]

Many organic or inorganic TP absorbing chromophores presenting high σ_2 values have been reported for applications in materials science^[4] and biology.^[2,5] Nevertheless, developing TP sensitizers which fulfill the specifications of PDT in terms of solubility, high singlet-oxygen production quantum yield, and low dark cytotoxicity is much more challenging. Since porphyrin and its derivatives absorb light in the visible range, generate singlet oxygen efficiently, and are already used in clinics in classical one-photon PDT,^[6] TP absorbing molecules in the near infrared were developed by extending the π system of the porphyrin core.^[7] Moreover, porphyrins associated with electron-donor or electron-acceptor moieties through conjugated systems led to promising TP-PDT results.^[8] Especially, those systems incorporating π -conjugated porphyrin dimers or self-assembled porphyrin oligomers have shown the highest σ_2 values, though the poor water solubility of these large aromatic cores limits their application unless several cationic or anionic groups are added on the periphery.^[8b,9]

Diketopyrrolopyrroles (DPPs) have been known for years as pigments presenting high thermo- and photostability and a bright red color. DPP is insoluble in most solvents, but dialkyl N-substituted DPPs showed good solubility and were used for applications in materials science, for example, molecular electronics, photovoltaics, or electroluminescence.^[10] More recently, these dyes have received attention for biological applications as TP dyes for fluorescence microscopy, after incorporation of water-solubilizing groups,^[11] or π expansion of the DPP core.^[12] Only recently, DPPs have been associated with porphyrins as organic sensitizers for dye-sensitized solar cells.^[13] In 2013, Gryko and co-workers reported a first linear system consisting of two free-base porphyrins connected to a central DPP core which presents a high TP absorption cross-section for material applications,^[14] thus evidencing that the conjugation of DPP to porphyrin systems is a promising strategy for achieving large σ_2 values. Nevertheless, to the best of our knowledge,

[*] J. Schmitt, Prof. V. Heitz, Dr. A. Sour
LSAMM, Institut de Chimie de Strasbourg, CNRS/UMR 7177
4 rue Blaise Pascal, 67000 Strasbourg (France)
E-mail: v.heitz@unistra.fr

Dr. F. Bolze, Dr. H. Ftouni, Prof. J.-F. Nicoud
CAMB, UMR 7199, Uds/CNRS, Faculté de Pharmacie
74 route du Rhin, 67401 Illkirch (France)
E-mail: frederic.bolze@unistra.fr

Dr. L. Flamigni, Dr. B. Ventura
Istituto ISOF-CNR
Via P. Gobetti 101, 40129 Bologna (Italy)
E-mail: barbara.ventura@isof.cnr.it

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Supporting information for this article (descriptions of synthetic experimental details, turbidimetric method, additional one- and two-photon photophysical characterizations, singlet-oxygen generation, imaging and TP phototoxicity experiments) is available on the WWW under <http://dx.doi.org/10.1002/anie.201407537>.

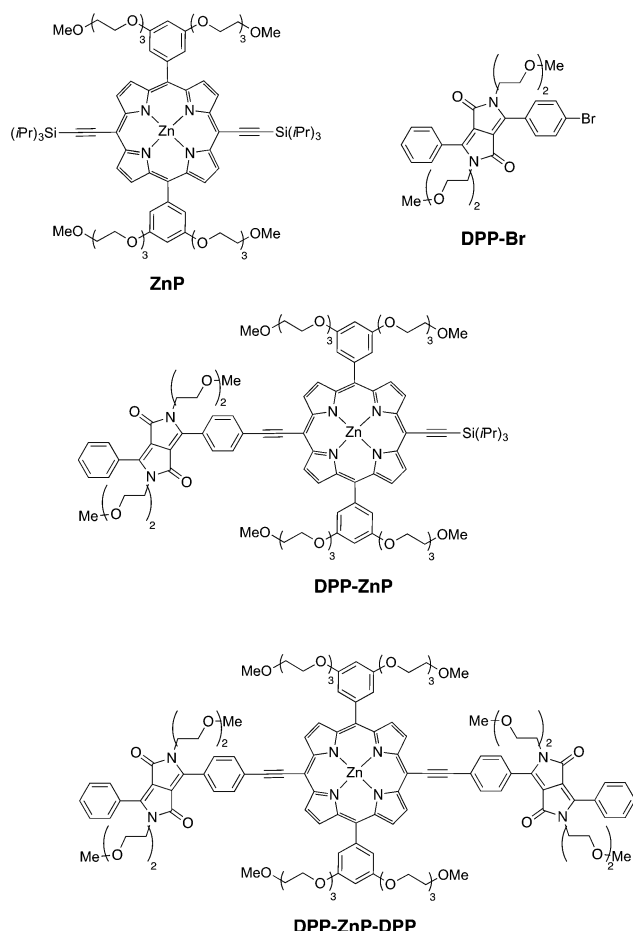


Figure 1. Structure of the DPP-porphyrin conjugates and of reference compounds **ZnP** and **DPP-Br**.

the use of DPP-porphyrin conjugates for singlet-oxygen generation has not yet been explored.

The present article reports the synthesis of new DPP/metallporphyrin conjugates, **DPP-ZnP** and **DPP-ZnP-DPP**, which fulfill several criteria for serving as efficient TP photosensitizers for PDT: high σ_2 values at two spectral regions above $\lambda = 800$ nm, efficient singlet-oxygen photosensitization, rapid cell penetration, and solubility in DMSO and water. The new DPP-metallporphyrin conjugates, **DPP-ZnP** and **DPP-ZnP-DPP**, and the reference compounds **ZnP** and **DPP-Br** are shown in Figure 1.

The conjugates, **DPP-ZnP** and **DPP-ZnP-DPP**, were prepared from the common precursor **ZnP** (Figure 2). **ZnP** has four triethylene glycol chains to increase its hydrophilicity. In addition, two protected acetylene groups in the 5- and 15-*meso* positions of **ZnP** allow the extension of the π -conjugated porphyrin system with chromophores for efficient TP absorption in the near infrared.^[7a,d,15] **ZnP**^[15] and **DPP-Br**^[16] were prepared according to literature procedure. **DPP-ZnP-DPP** was obtained in 50% yield, after classical removal of both triisopropylsilyl (TIPS) groups from **ZnP** and subsequent Sonogashira cross-coupling reaction of **1** with **DPP-Br**. **DPP-ZnP** was obtained from **ZnP** in two steps, the first one involving a statistical removal of the TIPS group (Figure 2). The reaction, performed with 0.25 equivalents of TBAF in THF, allowed the isolation of three compounds, **2**, **ZnP** and **1**, after purification. The desired intermediate **2** was obtained in 42% yield. The compound **2** reacted with **DPP-Br** in a Sonogashira cross-coupling reaction to afford **DPP-ZnP** in 77% yield after purification.

The one-photon absorption and the fluorescence spectra of compounds **ZnP**, **DPP-ZnP**, and **DPP-ZnP-DPP** were recorded in CH_2Cl_2 and DMSO. Absorption data in the two solvents are collected in Table S1 in the Supporting Information. Addition of one or two DPP units to the porphyrinic

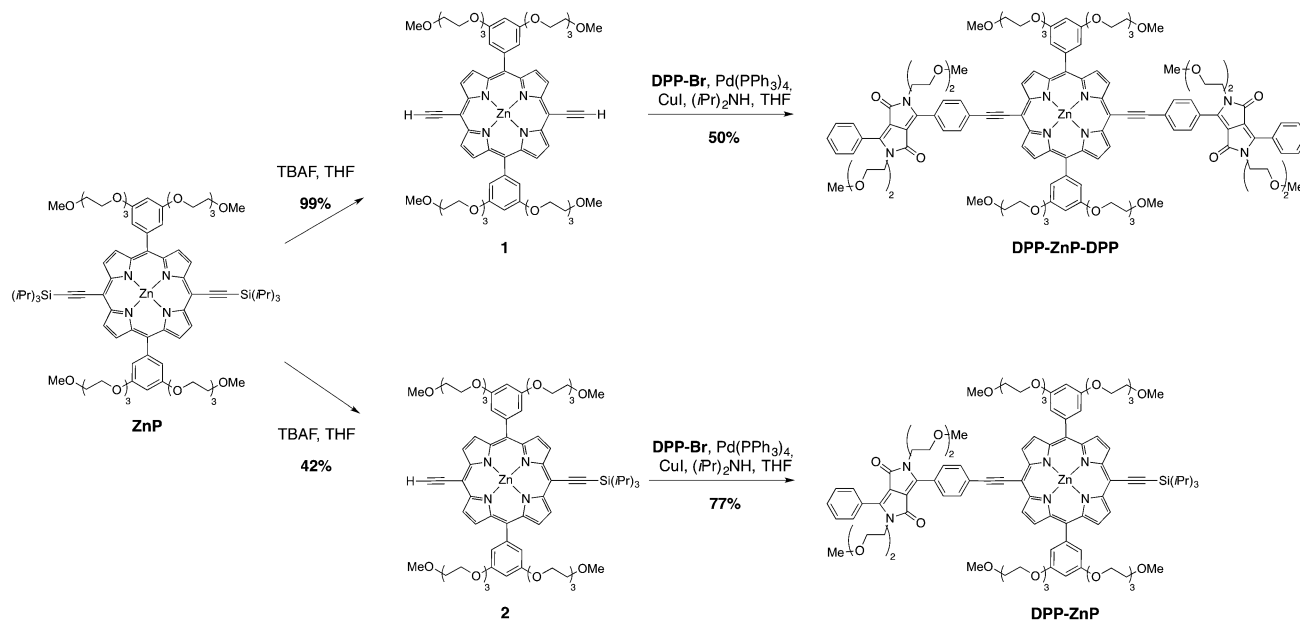


Figure 2. Synthesis of **DPP-ZnP** and **DPP-ZnP-DPP**.

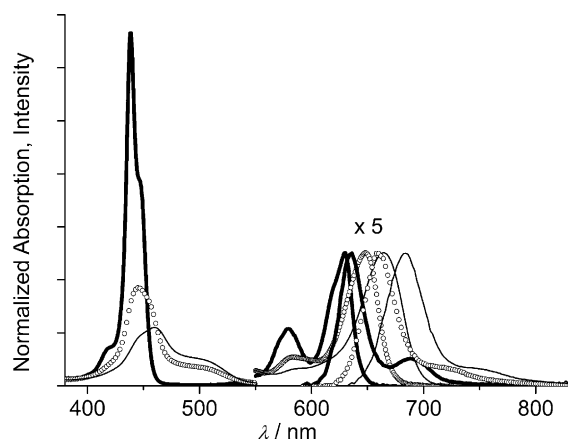


Figure 3. Normalized absorption and emission spectra of **ZnP** (thick line), **DPP-ZnP** (open circle), and **DPP-ZnP-DPP** (thin line) in CH_2Cl_2 . Absorption spectral portions in the $\lambda = 550\text{--}830\text{ nm}$ region are multiplied by a factor five.

π system causes a significant broadening of the Soret band, a red-shift of both Soret and Q-bands, and an increase in the Q-bands intensity along the series (see Figure 3 and Figure S13 and Table S1). Similar behavior has been reported for extended porphyrins or porphyrins conjugated with aromatic units.^[4b, 7a,d, 17]

All systems exhibit fluorescence arising from the lowest singlet excited state of the porphyrin, with emission bands that mirror the porphyrin Q-bands (Figure 3 and Figure S13). For the DPP-containing compounds, upon excitation both on the porphyrin Q-bands and in the $\lambda = 400\text{--}450\text{ nm}$ region, where both porphyrin and DPP chromophores absorb, only the fluorescence of the porphyrin moiety is detected (for a complete characterization of model **DPP-Br** in the two solvents, see Figure S14 and Table S3). The emission quantum yields obtained upon excitation in the two wavelength regions are identical and a good superimposition of excitation and absorption spectra on the entire absorption range is observed (see Figure S15), thus confirming a complete energy transfer from the DPP to the porphyrin unit. Time-resolved luminescence measurements in the picosecond regime upon excitation at $\lambda = 355\text{ nm}$ evidenced that the quenching of the DPP emission in **DPP-ZnP** and **DPP-ZnP-DPP** is faster than the experimental resolution (10 ps), thus indicating a rate higher than 10^{11} s^{-1} . The emission quantum yields, ranging from 0.11 to 0.16 in CH_2Cl_2 (Table 1) and from 0.14 to 0.19 in DMSO

Table 1: Emission properties and singlet oxygen production quantum yields of the compounds in aerated dichloromethane at room temperature.

	λ_{max} [nm] ^[a]	ϕ_{h} ^[b]	τ [ns] ^[c]	ϕ_{Δ} ^[d]
ZnP	636, 688	0.11	1.68	0.74
DPP-ZnP	658, 724	0.15	1.06	0.58
DPP-ZnP-DPP	684, 752 sh	0.16	0.78	0.50

[a] From corrected emission spectra. [b] Fluorescence quantum yields; see the Supporting Information for details. [c] Fluorescence lifetimes, excitation at $\lambda = 465$ and 560 nm . [d] Singlet-oxygen production quantum yields.

(Table S2), are significantly high for Zn-porphyrin fluorescence, but in line with values reported for *meso*-acetylene conjugated Zn-porphyrins.^[9d, 18] As compared to **ZnP**, the emission quantum yield of **DPP-ZnP** and **DPP-ZnP-DPP** increases up to 30–40 % in both solvents.

The singlet-oxygen production quantum yields measured in CH_2Cl_2 are reported in Table 1. Figure S16 shows the singlet-oxygen phosphorescence spectra recorded from optically matched solutions of the three porphyrinic compounds and the chosen standard. Even though the yields gradually decrease in the series, the measured values for **DPP-ZnP** and **DPP-ZnP-DPP**, of the order of 0.50–0.60, are significantly high and these arrays can be considered as promising in view of the desired phototoxicity.

TP absorption properties of **ZnP**, **DPP-Br**, **DPP-ZnP**, and **DPP-ZnP-DPP** were experimentally determined in CH_2Cl_2 by their TP-induced fluorescence emission. Their TP excitation spectra in CH_2Cl_2 are illustrated in Figure 4 (for DMSO solutions, see Figure S17).

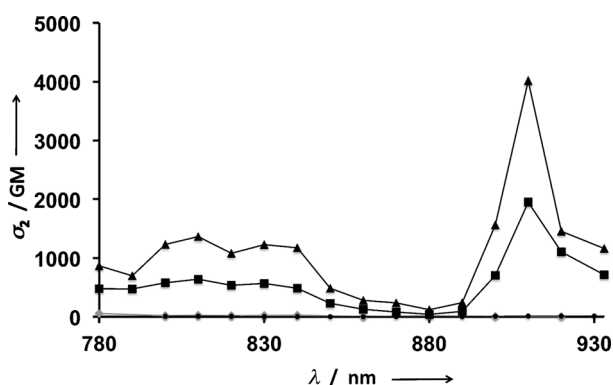


Figure 4. TP excitation spectra of **ZnP** (grey diamond), **DPP-Br** (black circle), **DPP-ZnP** (black square), and **DPP-ZnP-DPP** (black triangle) in CH_2Cl_2 .

The σ_2 of **ZnP** without an appended DPP is low, approximately 20 GM from $\lambda = 780$ to 920 nm , as expected for simple porphyrin systems. Addition of one DPP system on the porphyrinic core induces the appearance of a new band at $\lambda = 910\text{ nm}$ for **DPP-ZnP** with an increase of two orders of magnitude of the σ_2 (from 8 to 2000 GM for **ZnP** and **DPP-ZnP**, respectively) and a smaller increase in the σ_2 in the $\lambda = 800\text{--}840\text{ nm}$ region (18 and 1400 GM for **ZnP** and **DPP-ZnP**, respectively, at $\lambda = 810\text{ nm}$). The addition of a second DPP doubles the σ_2 to 4000 GM at $\lambda = 910\text{ nm}$ for **DPP-ZnP-DPP** as compared to **DPP-ZnP**. The related system described by Gryko et al.,^[14] with two porphyrin units linked by one DPP central core, shows a slightly lower σ_2 of 3000 GM at $\lambda = 950\text{ nm}$, thus indicating that the DPP can be successfully used as a central or side electroactive group.

In view of biological applications, the water solubility of **DPP-ZnP** and **DPP-ZnP-DPP**, estimated using the turbidimetric method (see the Supporting Information), were $90\text{ }\mu\text{M}$ and $60\text{ }\mu\text{M}$, respectively. Thus, HEK cells were incubated with **DPP-ZnP** and **DPP-ZnP-DPP** ($1\text{ }\mu\text{M}$ in culture medium with 1 % DMSO) and observed with a confocal microscope. A

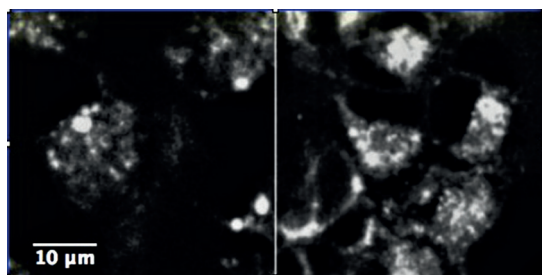


Figure 5. Confocal images of HEK cells incubated with 1 μM **DPP-ZnP** (left) and **DPP-ZnP-DPP** (right) in culture medium with 1% DMSO (λ_{ex} = 405 nm, λ_{em} from 600 to 700 nm, 3.5 mW laser power).

rapid cellular penetration was noticed^[9d] (see Figure S18) and a stable fluorescent signal was obtained after two hours (Figure 5). The distribution was heterogeneous in the cell cytoplasm, without nuclear penetration (see Figure S19 for colocalisation studies). After one night, neither cell death nor abnormal cellular shapes were detected, thus indicating low dark toxicity (see Figure S20 for quantitative cell toxicity tests).

TP excited microscopy and TP fluorescence lifetime imaging were also performed on HEK cells incubated with **DPP-ZnP** and **DPP-ZnP-DPP** and showed the same fluorescence behavior as that seen in confocal microscopy (see Figure S21).

A preliminary TP-PDT type of experiment was performed on a HeLa cell culture. After 2 hours of incubation with **DPP-ZnP**, the cells were irradiated at $\lambda = 910$ nm for 300 scans. After 4 hours, the cell viability was estimated with the combination of Hoechst and Cytos orange nuclear stains. Important cell death, above 90%, was detected (Figure 6). An extensive study is in progress.

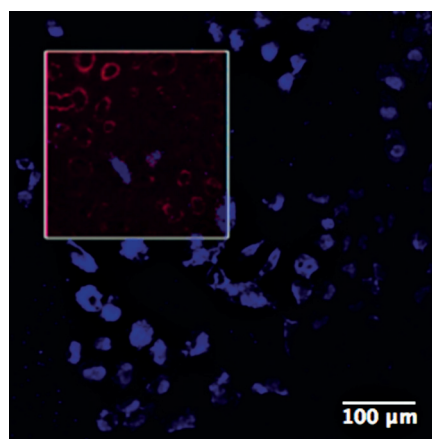


Figure 6. TP-PDT on HeLa cells incubated 2 h with 1 μM of **DPP-ZnP** in culture medium with 1% DMSO. TP-PDT was performed only within the square region (white box 250 $\times$ 250 μm , λ_{ex} = 910 nm, P = 5.9 mW back pupil of the objective, 300 scans). In red, TP fluorescence of the sensitizer in cells before irradiation. In blue, global fluorescence of Hoechst nuclear stain, indicating living cells 4 h after irradiation of the square region (no cells with compromised cytoplasmic membranes were detected with Cytos orange).

In summary, synthesis and characterization of two DPP-porphyrin conjugates presenting large two-photon absorption cross-sections in the therapeutic window as well as efficient singlet-oxygen generation are reported. Both display rapid cellular penetration and preliminary TP excitation results from cell cultures incubated with **DPP-ZnP** show high phototoxicity. Further developments of DPP-containing systems are thus of great interest for TP-PDT and theranostic applications.

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