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ARTICLE

Synthesis and characterization of novel zinc phthalocyanines as potential photosensitizers for photodynamic therapy of cancers

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Two novel zinc phthalocyanines (Pcs): Tetramethyl Tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (**4**) and (the negatively charged) Tetrakis-2,(3)-[(3-carboxylicacid-6-sulfanylpyridine)phthalocyaninato] zinc(II) (**5**), water soluble by virtue of their ionic substituent groups were synthesized. The spectroscopic properties of both compounds were determined and their photodynamic activities were investigated in a human tumor cell model. In aqueous media the two peripherally substituted water soluble Pcs are highly aggregated. The phototoxic activity of the two novel Pcs (Pc **4** and Pc **5**; 0-20μM) was shown to be time- and dose-dependent in human pancreatic carcinoid BON cells leading to reduction of tumor cells of > 80% compared to the controls. The effectiveness of the treatment appeared to be attenuated by the aggregation of Pcs under aqueous conditions. Interestingly, even those cells that were not immediately killed by the photoactivated photosensitizer seemed to be affected by the Pc photodynamic activity, as a single PDT induced long–lasting effects on cell survival. Even 4 days after PDT, the number of the surviving cells did not reincrease or still dropped, as compared to control cells. The underlying mechanism of this observation has to be deciphered in future investigations.

Introduction

In the past decades photodynamic therapy (PDT) has emerged as a minimally invasive regimen for the treatment of cancers.[1-5] Today PDT offers an attractive new direction or addition to conventional therapies for diseases including skin lesions, age-related macular degeneration, and cancers (bladder, breast, brain, esophagus, head/neck, lung, and skin). Its therapeutic action is based on the generation of singlet oxygen ($^1O_2$) and other reactive oxygen species (ROS) that are formed upon light activation of the photosensitizer (PS) which is taken up with higher affinity in malignant than in non-malignant cells. After photoexcitation, the excited PS transfers its energy to molecular tissue oxygen to generate ROS, of which singlet oxygen is assumed to be the key cytotoxic species causing localized oxidative cell damage and death. In spite of great advances, PDT is not yet an integral part of clinical practice due to the serious drawbacks of the few regulatory approved PSs, and one among them is poor solubility in water.

Notably, tetrapyrroles such as porphyrins,[6-8] chlorins[9-12] and phthalocyanines (Pcs)[13-15] are being used as photoactive agents in PDT. It should be mentioned, that Pcs have been shown to be some of the most efficient PSs to date[16] since they have strong absorption in the far red spectral region, demonstrate high intersystem crossing (ISC) to the first excited triplet state and high efficiency of singlet oxygen generation. The photophysical properties of Pcs can be tuned by rational modification of their structure.[17] This tuning may be achieved through the addition of substituent groups on the periphery of the Pc macrocyle as well as complexing the ligand with varied metal ions giving metallated Pcs (MPcs).[17,18] The photophysical properties of the latter are highly dependent on the central metal ion employed.[17]

The suitability of Pcs for PDT is highly dependent on their water-solubility. Thus, some of the highest efficiencies of tumor targeting in photodynamic activity are achieved with water soluble Pcs.[19,20] In order to accomplish water solubility, water solubilizing groups may be introduced onto the Pc on the periphery and non-periphery of the macrocycle or even as axial ligands for Pcs that have been complexed with multivalent central metals ions such as Si^{4+}, Al^{3+} Ga^{3+} and In^{3+}.[21-23] The most common groups used to make Pcs water soluble include carboxy, sulfonate and phosphono groups.[24-26] Quaternization may also be employed for Pcs bearing pyridino substituents.[27]

This work reports on the synthesis (scheme 1) and characterization of new Tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (3) and its water soluble quaternized (positively charged) form Tetramethyl Tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (**4**) and (the water soluble negatively charged) Tetrakis-2,(3)-[(3-

carboxylicacid-6-sulfanylpyridine)phthalocyaninato] zinc(II) (**5**) phthalocyanines and their photodynamic activity on human pancreatic carcinoid BON cells.[28]

Scheme 1. Synthetic route for 4-(4-Methyl-2-pyridyloxy) phthalonitrile **(1)**, 4-(3-Carboxylicacid-6-sulfanylpyridine) phthalonitrile **(2)** and phthalocyanines **3**, **4** and **5**. (Where: (i) pentanol, zinc acetate, DBU, heating at 160 °C and (ii) dimethyl sulfate and heating at 120 °C under inert gas).

Experimental Methods

Materials

N,N-dimethyl formamide (DMF), 4-nitrophthalonitrile, 2-hydroxy-4-methylpyridine, 6-mercaptopyridine-3-carboxylic acid, ethanol, chloroform ($CHCl_3$), dichloromethane (DCM), tetrahydrofuran (THF) and acetone were obtained from Sigma Aldrich. Bio-beads S-X1 were obtained from Bio-Rad. Ultra-pure water was obtained from a Milli-Q Water System (Millipore Corp, Bedford, MA, USA).

Phthalocyanine solutions

Stock solutions of the phthalocyanine complexes were prepared in water and stored at 4 °C. The drugs were diluted in fresh media before each experiment. In order to evaluate the effect of Pcs on the cells, the cells were incubated with either control medium or medium containing increasing concentrations of the respective Pcs.

Cell line

The human pancreatic carcinoid cell line BON was cultured in a 1:1 mixture of DMEM and HAM´s F-12 medium containing 10% of FCS (Biochrom, Berlin, Germany) and 1% L-glutamine. Cells were maintained at 37 °C in a 5 % CO_2 humidified atmosphere.[28-30] Under these conditions, the doubling time of BON cells was 34 +/- 4h. Incubation and subsequent experiments were performed while the cells were in the log phase of their growth.

Methods and Instruments

IR data were obtained by using the Perkin-Elmer spectrum 2000 FTIR spectrometer. ^{1}H NMR spectra were recorded using a Bruker AMX 600 MHz spectrometer. Elemental analyses were carried out on a Vario EL III MicroCube CHNS Analyzer. Mass spectra data were collected with a Bruker AutoFLEX III Smart beam TOF/TOF Mass spectrometer. The instrument was operated in positive ion mode using an m/z range of 400 – 3000. The voltage of the ion sources were set at 19 and 16.7 kV for ion sources 1 and 2 respectively, while the lens was set at 8.50 kV. The reflector 1 and 2 voltages were set at 21 and 9.7 kV respectively. The spectra were acquired using dithranol as the MALDI matrix, and a 354 nm Nd:YAG laser.

Steady-state absorption and fluorescence spectroscopy

The ground-state absorption spectra were recorded using a commercial spectrophotometer Shimadzu UV-2501PC. Steady-state fluorescence spectra were measured in 1 cm × 1 cm quartz cells using a combination of a cw-Xenon lamp (XBO 150) and a monochromator (Lot-Oriel, bandwidth 10 nm) for excitation and a polychromator with a cooled CCD matrix as the detector system (Lot-Oriel, Instaspec IV).

Picosecond transient absorption spectroscopy

The setup for pump-probe experiments with picosecond time resolution was described previously.[31] Briefly, to measure the transient absorption spectra, a white light continuum was generated as a test beam in a cell with a D_2O/H_2O mixture using intense 25 ps pulses from a Nd^{3+}:YAG laser (PL 2143A, Ekspla) at 1064 nm. Before passing through the sample, the continuum radiation was split to obtain a reference spectrum. The transmitted as well as the reference beams were focused into two optical fibers and were recorded simultaneously at different traces on a cooled CCD-matrix (Lot-Oriel, Instaspec IV). Tunable radiation from an OPG/OPA (Ekspla PG 401/SH, tuning range 200-2300 nm) pumped by the third harmonic of the same laser was used as an excitation beam. The mechanical delay line allowed the measurement of light-induced changes of the absorption spectrum at different delays up to 15 ns after excitation. Analysis of experimental data was performed using the compensation method.[32]

Light source and irradiation

The radiation unit used a 100 W halogen lamp (HLX 64627, Osram, Germany) as the light source. The spectral output of the lamp ranged from 400 to 800 nm. To prevent infrared irradiation, a heat-reflecting filter (Präzisions Glas & Optik, Iserlohn, Germany) with cutting-off band for $\lambda \geq 700$ nm was inserted into the optical path. The illuminated area (11×14 cm) had an average power density of incident light of 15 mW/cm^2. The light energy dose was measured with a P-9710 radiometer controlled by a silicon photocell (RW-3703-2 radiometer) from Gigahertz-Optik (Muenich, Germany). The total light energy dose was calculated by integrating the energy signal over the entire period of irradiation. Cells were illuminated for 33 or 66 min to achieve a total light dose of 30 or 60 J/cm², respectively.[5]

Measurement of growth inhibition

PS induced phototoxicity as well as dark toxic effects of the Pcs were determined by crystal violet staining of phthalocyanine (0-20 μM) treated BON cells after incubation and subsequent light irradiation (phototoxicity) or without illumination (dark toxicity). BON cells were seeded at a density of 5000 cells/well into 96-well microtiter plates and incubated with the zinc phthalocyanine photosensitizers for 6 and 12 h periods respectively. After the incubation period had lapsed the phthalocyanine medium solution was replaced with new medium solution and the plates were irradiated for 33 or 66 min by using an incoherent white light source and they were then incubated for 2 day (48 h) and 4 day (96 h) periods respectively. Crystal violet staining was carried out as previously described.[33,34] Cells were washed with phosphate buffer solution (PBS) and fixed with 1% gluteraldehyde. Following this step, cells were washed once more with PBS and then stained with 0.1% crystal violet. The unbound crystal violet dye was removed by washing. The crystal violet that had been absorbed onto the cells was solubilized with 0.2% Triton X-100. After this, light extinction was analyzed at 570 nm using an ELISA reader.

Synthetic procedures

Procedure for the synthesis of 4-(4-Methyl-2-pyridyloxy) phthalonitrile (1)

The reagents 2-hydroxy-4-methylpyridine (1.08 g, 9.9 mmol) and 4-nitrophthalonitrile (1.7 g, 9.8 mmol) were dissolved in DMF (30 ml) under argon. After stirring for 30 min at room temperature, finely ground anhydrous potassium carbonate (2.8 g, 20 mmol) was added in portions during 4 h with efficient stirring. The reaction mixture was stirred under argon atmosphere at room temperature for 3 days. Then the mixture was poured into 200 ml iced water, and the precipitate was filtered off, washed with water and then dried. The crude product was recrystallized from ethanol. Finally the pure product was dried in vacuum to give a light purple powder. Yield: 1.6 g (68.67 %). IR [(KBr) υ_{max}/cm^{-1}]: 3185 (C-H), 2229 (C≡N), 1656 (C-C), 1441, 1406 (C=C), 1360, 1279, 1206 (C-O-C), 1494(C=N). ^{1}H NMR (DMSO-d_6); δ, ppm: 8.36 (s, 1H, Ar-H), 8.29 (d, 1H, Ar-H), 8.16 (d, 1H, Ar-H), 7.63-7.68 (m, 1H, Pyridyl-H), 7.13 (d, 1H, Pyridyl-H), 7.07 (d, 1H, Pyridyl-H), 2.20 (s, 3H, Pyridyl-H). MS *m/z*: Calc: 235.14; Found: 236.08 $[M+H]^+$.

Procedure for the synthesis of 4-(3-Carboxylicacid-6-sulfanylpyridine) phthalonitrile (2)

The synthesis of **2** was similar to that of **1**, except 6-mercaptopyridine-3-carboxylic acid (2 g, 12 mmol) was employed instead of 2-hydroxy-4-methylpyridine. The amount of 4-nitrophthalonitrile used was (2 g, 12 mmol). The quantity of anhydrous potassium carbonate used was (3 g, 21.0 mmol). The phthalonitrile was precipitated out of solution by addition of the DMF slurry to acetone. Finally the pure product was dried in vacuum to give a brownish yellow solid. Yield: 1.3 g (38.46 %). IR [(KBr) υ_{max}/cm^{-1}]: 3301 (COOH), 3150 (C-H), 2240(C≡N), 1362, 1208(C-S-C), 1656 (C-C), 1439, 1409 (C=C), 1497 (C=N). ^{1}H NMR (DMSO-d_6); δ, ppm: 8.87 (s, 1H, Pyridyl Carboxyl-H), 8.82 (s, 1H, Ar -H), 7.94 (s, 1H, Pyridyl-H), 7.89 (d, 1H, Ar-H), 7.78 (d, 1H, Ar-H), 7.39 (d, 1H, Pyridyl-H), 7.17 (d, 1H, Pyridyl-H). MS *m/z*: Calc: 281.28; Found: 282.04 $[M+H]^+$.

Procedure for the synthesis of Tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (3)

A mixture of anhydrous zinc (II) acetate (0.6 g, 2.8 mmol), 4-(4-methyl-2-pyridyloxy) phthalonitrile (**1**) (0.22 g, 0.94 mmol), DBU (0.27 mL, 2 mmol) and pentanol (25 mL) was stirred at 160 ^{o}C for 5 h under nitrogen atmosphere. After cooling, the solution was mixed with n-hexane. The blue solid product was precipitated and collected by filtration and washed with n-hexane. The crude product was purified by passing through a silica gel column using chloroform to elute the product. The eluted product was then recrystalized from DMF using methanol. The product was dried in the oven at 100 ^{o}C overnight. Yield: 100 mg (42 %) UV/vis (DMSO): λ_{max} nm (log ε); 676 (5.02), 610 (4.30), 360 (4.50); IR (KBr): ν_{max}/cm^{-1}; 3098, 2606 (C-H), 1659 (C-C), 1495(C=N), 1439, 1410 (C=C), 1360, 1213 (C-O-C). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 9.06-8.96 (m, 4H, Ar-H), 8.35-8.32 (m, 4H, Pyridyl-H), 8.16-8.12 (m, 4H, Pyridyl-H), 7.97-7.90 (m, 4H, Pyridyl-H), 7.65 (d, 4H, Ar-H), 7.20-7.17 (m, 4H, Ar-H), 1.22 (s, 12H, Pyridyl Methyl-H). Calc. for $C_{56}H_{36}N_{12}O_4Zn{\cdot}2CH_3OH$: C; 65.08, H;4.14, N; 15.70. Found: C; 64.17, H; 5.09, N; 12.5. MALDI-TOF-MS *m/z*: Calc: 1006.35.41; Found: 1007.38 $[M+H]^+$.

(Quaternized) Tetramethyl Tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (4)

Yield: 28 mg (56 %) UV/vis (DMSO): λ_{max} nm (log ε); 675 (4.91), 612 (4.17), 343 (4.51); IR (KBr): ν_{max}/cm^{-1}; 3050, 2618, 2380 (C-H), 1658 (C-C), 1498 (C=N), 1440, 1409 (C=C), 1360, 1213 (C-O-C). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.09 (bs, 4H, Ar-H), 7.96 (bs, 4H, Pyridyl-H), 7.86-7.81 (m, 4H, Ar-H), 7.52 (d, 4H, Pyridyl-H), 7.29 (d, 4H, Ar-H), 7.22 (d, 4H, Pyridyl-H), 1.62 (s, 12H, Pyridyl Methyl-H) 1.28 (s, 12H, CH_3). Calc. for $C_{60}H_{48}N_{12}O_{12}S_2Zn$: C; 57.26, H; 3.84, N; 13.35, S; 5.09. Found: C; 58.11, H; 4.85, N; 19.62, S; 6.31. MALDI-TOF-MS *m/z*: Calc: 1258.65; Found: 1261.86 $[M+3H]^{3+}$.

Procedure for the synthesis of Tetrakis-2,(3)-(3-Carboxylicacid-6-sulfanylpyridine)phthalocyaninato zinc(II) (5)

The synthesis of **5** was as outlined for **3**, except compound **2** (0.22 g, 0.77 mmol) instead of compound **1** was employed. The amounts of the other reagents were as follows: anhydrous zinc acetate (0.23 g, 1.13 mmol), dry pentanol (25 ml) and DBU (0.25 ml, 0.16 mmol). A dark green product was obtained after washing with ethanol. It was purified by multiple dissolutions into 0.1 M sodium hydroxide solution and repeatedly precipitating out using 20 % HCl. The final precipitated product was washed with methanol, acetone and chloroform. The product was finally dried in the oven overnight at 90 ^{o}C. Yield:

30 mg (30 %). UV/vis (DMSO): λ_{max} nm (log ε) 683 (5.08), 616 (4.32), 368 (4.53). IR spectrum (cm^{-1}): 3350 (COOH), 3082, 2622 (Ar-CH), 1440, 1400 (C=C), 1657 (C-C), 1498 (C=N), 1361, 1250 (C-S-C). ^{1}H NMR (DMSO-*d6*) δ: 8.35 (bs, 4H, Pyridyl-H), 8.25-8.23 (m, 4H, Ar-H), 8.16-8.14 (m, 8H, Pyridyl-H), 7.97 (s, 4H, Pyridyl-H), 7.84 (s, 4H, Ar-H), 7.33-7.26 (m, 4H, Ar-H). Calc. for $C_{56}H_{28}N_{12}S_4O_8Zn{\cdot}2CHCl_3$: C; 48.74, H; 2.13, N; 11.76, S; 8.90 %. Found: C; 47.59, H; 3.16, N; 11.65, S; 8.09 %. MS (ES-MS) m/z: Calc: 1190.58; Found: 1192.64 $[M+2H]^{2+}$.

Results and discussion

Characterization of complexes 3, 4 and 5

The new complexes **3**, **4** and **5** were characterized using UV-Vis, IR, NMR spectroscopies, MALDI-TOF mass spectrometry and elemental analysis. The analyses are all consistent with the expected as shown in the experimental section with the exception of an elevated content of nitrogen for the elemental of the quaternized complex **4**. Complex **3** exhibited solubility in organic solvents such as $CHCl_3$ and DCM, DMF and DMSO while complexes **4** and **5** were soluble in the latter two solvents and aqueous media.

The mass spectra of the Pcs were obtained by the relatively soft ionization MALDI-TOF technique with the molecular ion peaks observed at 1007.38 for **3**, 1261.86 for **4** and 1192.64 for **5**.

The ^{1}H NMR spectra of the complexes **3**, **4** and **5** show complex patterns due to the mixed isomer character of the tetra substituted MPc derivatives and perhaps also due to aggregation. Complexes **3**, **4** and **5** were found to be pure by ^{1}H NMR with all the substituent protons and the ring protons observed in their respective regions.

UV/Vis Absorption and Fluorescence Spectroscopy

The newly synthesized complexes **3, 4** and **5** showed no signs of aggregation in the respective absorption spectra in DMF (shown in Fig. 1). However strong aggregation was observed when the compounds were dissolved in water as evidenced by the intense broadening of absorption bands compared to the spectra of these compounds in DMF. Moreover, there is a hypsochromic shift of corresponding Q-band maxima when samples are dissolved in water (from 676 to 632 nm for **3,** from 675 to 641 nm for **4** and from 683 to 643 nm for **5** respectively, see Table 1). It seems that aggregation for the quaternized Pc **4** is not as intense compared to the two other Pcs, since the observed broadening of its absorption Q-band is lower than that for **3** and **5** when dissolved in water (see Fig. 1).

Fig. 1. Normalized UV/Vis absorption spectra of **3** (*a*), **4** (*b*) and **5** (*c*) dissolved in DMF and in water. Comparison of the absorption spectra of these Pcs in aqueous solution is given in (*d*).

Table 1. Absorption, emission, photophysical and lifetime data.

It was not possible to detect any fluorescence signal for the Pcs **3**, **4** and **5** in water since their fluorescence intensities lay below the signal-to-noise ratio of the steady-state fluorescence setup employed for this study. This observation is due to aggregation of the Pc molecules in aqueous solution (due to the likely formation of non-fluorescent aggregates, where fast and very efficient radiationless deactivation of the first excited singlet state follows photoexcitation of a Pc chromophore).[35-37]

The situation is completely different for the same Pcs when dissolved in DMF (fluorescence spectra are shown in Fig. 2). The fluorescence maximum is located at 687 nm for **3**, at 686 nm for **4** and at 695 nm for **5**, respectively (Table 1).

Fig. 2. Normalized fluorescence spectra of the Pcs **3**, **4** and **5** dissolved in DMF.

Picosecond Transient Absorption Spectroscopy (ps-TAS)

In order to bring to light the dynamics of the excited state population – depopulation, transient absorption experiments with picosecond time resolution (ps-TAS) were carried out. As readily seen from Fig. 3, the transient absorption (TA) spectra of the Pcs dissolved in DMF have a strong negative signal with a maximum around 680 nm which corresponds to the bleaching of the ground state absorption. The recovery of the ground state population after photoexcitation occurs mono-exponentially with a characteristic time (**τ**) of 2.71±0.15 ns (**3**), 2.93±0.18 ns (**4**) and 2.49±0.17 ns (**5**) respectively (results shown in Table 1).

Fig. 3. Transient absorption spectra at different delay times after excitation (*a*, *c*, *e*) and ground state bleaching (mono-exponential fit is shown as red line) (*b*, *d*, *f*) of the Pcs **3** (*a*, *b*), **4** (*c*, *d*) and **5** (*e*, *f*) dissolved in DMF.

Furthermore, it was observed that even 15 ns after excitation, a ground state bleaching signal still remained. This means that excitation energy is partly stored on a long-lived excited state, namely the first excited triplet state. It is well-known that the lifetime of a triplet state is much longer compared to the first excited singlet state (normally it lies in microsecond or even millisecond range) due to the fact that the T_1-S_0 transition is spin-forbidden.[17,38] In DMF the ISC $S_1 \rightarrow T_1$ quantum yield was calculated to be 0.50, 0.51 and 0.62, for the phthalocyanines **3**, **4** and **5** respectively (see Table 1). It should be emphasized, that the ISC quantum yield correlates with that of the singlet oxygen generation (as both normally have very close values). The quantum yield for singlet oxygen production is a very important parameter for the use of Pcs as PSs in PDT of cancers.[17,38,39] Since all Pcs under investigation show

appreciable ISC quantum yield, they could be considered promising candidates for use as photosensitizers in PDT.
Unfortunately, the situation changed dramatically, when the samples were dissolved in water. It was not even possible to measure the TA spectra of Pc **5**, due to high scattering of the excitation light on the aggregates. It was possible however, to carry out pump-probe experiments for the Pcs **3** and **4**, and the results are presented in Fig. 4.

Fig. 4. Transient absorption spectra (*a*, *c*) and ground state bleaching (mono-exponential fit is shown as red line) (*b*, *d*) of Pcs **3** (*a*, *b*) and **4** (*c*, *d*) dissolved in water.

The TA spectra for the Pc **3** in aqueous solution were very noisy and also, the intensity of its ground bleaching was quite low. It seems that the depopulation of the first excited singlet state occurs extremely fast and practically follows an excitation pulse (25 ps, FWHM). The ISC quantum yield value was estimated to be practically 0.
The change in optical density (ΔOD) signal of Pc **4** dissolved in water is much more pronounced compared to that of compound **3**, and this is expected as **4** is the water soluble quaternized form. Nevertheless, the ground state bleaching decays very fast with a characteristic time of 25±10 ps, and the quantum efficiency of population of the first excited triplet state via ISC was estimated to be only 2%. This 2% efficiency of triplet state population is rather low and thus less likely to result in significant PDT efficiency.
In summary, it was found that the Pcs **3**, **4** and **5** have a very high tendency to aggregate when dissolved in water. This aggregation tendency in water results in a quenching of their fluorescence as well as ISC quantum yields compared to the corresponding results of the phthalocyanines when dissolved in DMF.

In vitro determination of Pc-induced phototoxicity

The photodestructive potency of water soluble complexes **4** and **5** (high aggregation tendency in water) was investigated in BON cells, which were used as a model cell line of human pancreatic carcinoid tumors.[30] Water soluble complexes were targeted for synthesis and investigation in this work because favorable and high efficiencies of tumor targeting in photodynamic activity are achieved with the use of such Pcs. For both compounds dark- and phototoxicity measurements were carried out at concentrations ranging from 0 to 20 µM (Fig. 5). BON cells were incubated with complexes **4** and **5** for 6 h and 12 h prior to illumination, which was performed with a light dose of 30 or 60 J/cm², respectively. Irradiation dose-dependently decreased the cell numbers of BON cells pretreated with either complex **4** or **5** compared to untreated controls (Fig. 5). Non-photoactivated Pcs (0 – 20 µM) did not affect cell proliferation of BON cells (figure not shown).

The IC_{50} value of Pc **5** phototoxicity (12 h Pc incubation) calculated two days after PDT was found to be 5.7±1.6 µM. The Pc **5**-induced cell number reduction on day 4 was not adequate to allow for the calculation of its IC_{50} value. BON cells showed maximal effects with a decrease in cell number of approximately 80% at complex **5** concentration of 10 µM and a light dose of 60 J/cm² (Fig. 5d). The fact that the number of cells in complex **5** PDT treated samples did not markedly re-increase even after 4 days indicated that there was no appreciable re-proliferation, which is of particular interest for a compound that is well-suited for PDT. It shows that even those cells that were not immediately killed by the photoactivated photosensitizer were nevertheless affected by the PDT. If the underlying mode of action involved is apoptosis, necrosis, a stop or at least delay in cancer cell mitosis, or a combination of each, this has to be deciphered in future investigations; as the applied crystal violet-method does not measure these events, but solely determines changes in the cell number as compared to control cells.
Pc **4** showed lower phototoxicity compared to Pc **5** with the result that the cell number reduction induced by Pc **4** was not sufficient to allow for the calculation of its IC_{50} value. BON cells showed reasonable effects with a decrease in cell number of approximately 50% at complex **4** concentration of 20 µM and a light dose of 60 J/cm² (Fig. 5b). It was also noted that the number of cells in complex **4** PDT treated samples did not show a considerable re-increase even after 4 days. The IC_{50} values obtained in this work were higher than those obtained in work by other researchers using ZnPc and AlPc derivatives.[21,22] However, PDT conditions and especially illumination (laser, wavelength, and light dose) are different. Therefore, a direct comparison of IC_{50} values measured here with those found in the literature is not appropriate. In order to estimate the effectiveness of our Pcs, additional experiments were conducted with the clinically approved photosensitizer Photofrin (0-10 µM) using the same PDT-conditions (6 h incubation and cell number detection 48 h after PDT with 30 or 60 J/cm² irradiation doses). Photofrin-PDT dose-dependently reduced the BON cell number by up to 85% (data not shown) which is in the range of the cell number reduction induced by PDT from the Pcs used in this work (>80 % with Pc **5**).

Fig. 5. Effects of photoactivated Pcs on human cancer cells. BON cells incubated for 6 h or 12 h with complex **4** (*a*, *b*) and complex **5** (*c*, *d*) were irradiated with 30 or 60 J/cm² light dose. Changes in the cell number were determined by the crystal violet staining-method 48 and 96 h after PDT treatment. Cell number changes are shown as a percentage of irradiated but Pc-untreated control cells, which were set at 100%. Data are given as mean values +/- SEM of n = 4 independent experiments.

The differences in photodynamic activity of the two complexes **4** and **5** may be a result of differences in charge borne by each complex. Complex **4** is tetra positively charged by virtue of it being a quaternized nitrogen compound, while complex **5** is negatively charged because of the ionizable carboxyl groups. These differences in charge borne by complexes **4** and **5** might possibly influence and favor the uptake of **5** over **4** thereby

resulting in enhanced photodynamic activity of complex **5**. It seems that the cellular environment reduces probability of aggregation possibly leading to moderate generation of cytotoxic singlet oxygen upon photoexcitation of the phthalocyanine chromophores. However, the cytotoxic capacities of complexes in the cells might possibly be associated with the subcellular localization of the Pcs once taken into the cell.[40,41]

Conclusions

Two water soluble novel Zn(II)Pc complexes **4** and **5** were synthesized and *in-vitro* studies carried out to determine their photodynamic activity. The two complexes did not exhibit dark toxicity but led to phototoxic cell killing after light activation. The Pcs **4** and **5** have shown moderate photodynamic activity in *in-vitro* experiments. Compound **5** demonstrated even more pronounced phototoxic and growth inhibitory effects as compared to compound **4**. This may be due to a more favored cellular uptake of the tetra negatively charged complex **5**. Further investigations will have to clarify the exact uptake mechanism and kinetic behavior of complex **5** in human tumor cells. As the photodestructive potency of compound **5** is promising for novel approaches in PDT it merits further elucidation.

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Notes and references

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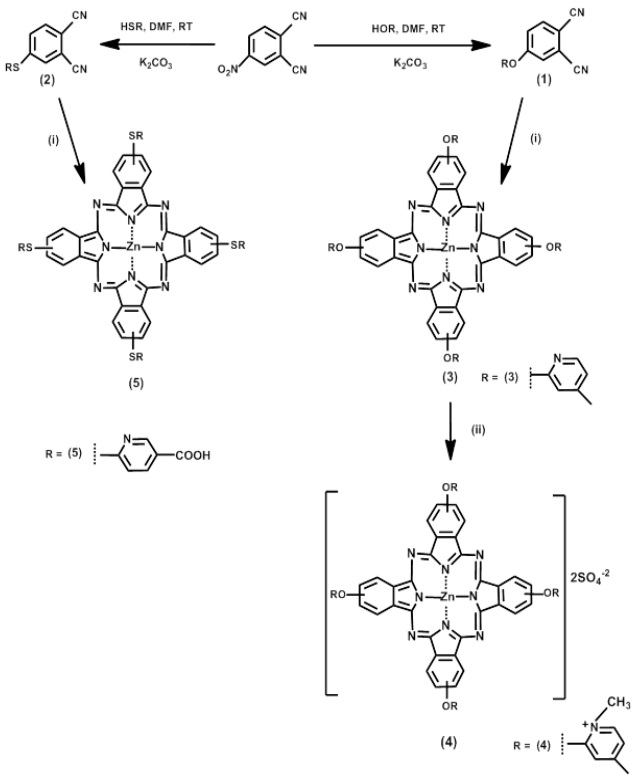


Scheme 1. Synthetic route for 4-(4-Methyl-2-pyridyloxy) phthalonitrile **(1)**, 4-(3-Carboxylicacid-6-sulfanylpyridine) phthalonitrile **(2)** and phthalocyanines **3**, **4** and **5**. (Where: (i) pentanol, zinc acetate, DBU, heating at 160 °C and (ii) dimethyl sulfate and heating at 120 °C under inert gas).

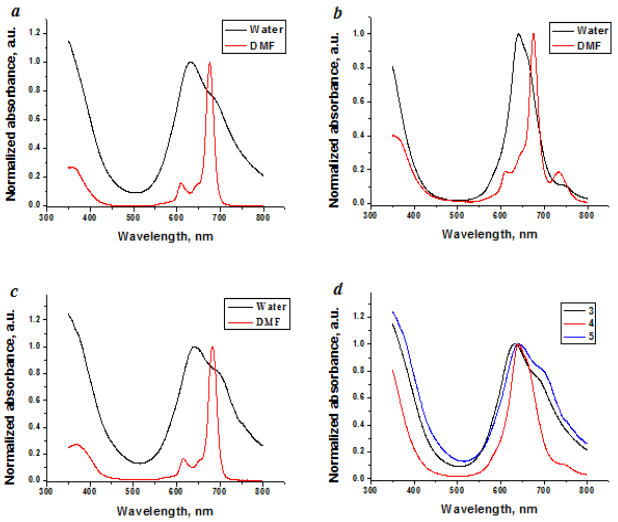


Figure 1. Normalized UV/Vis absorption spectra of **3** (*a*), **4** (*b*) and **5** (*c*) dissolved in DMF and in water. Comparison of the absorption spectra of these Pcs in aqueous solution is given in (*d*).

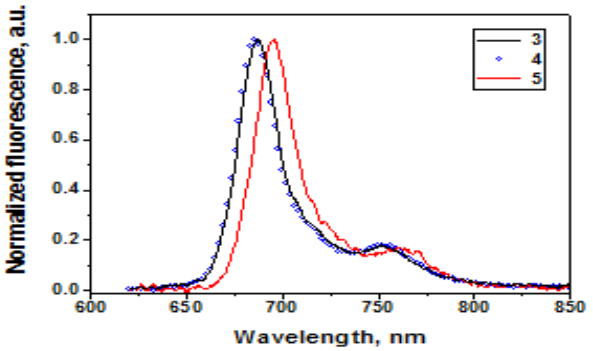


Figure 2. Normalized fluorescence spectra of the Pcs **3**, **4** and **5** dissolved in DMF.

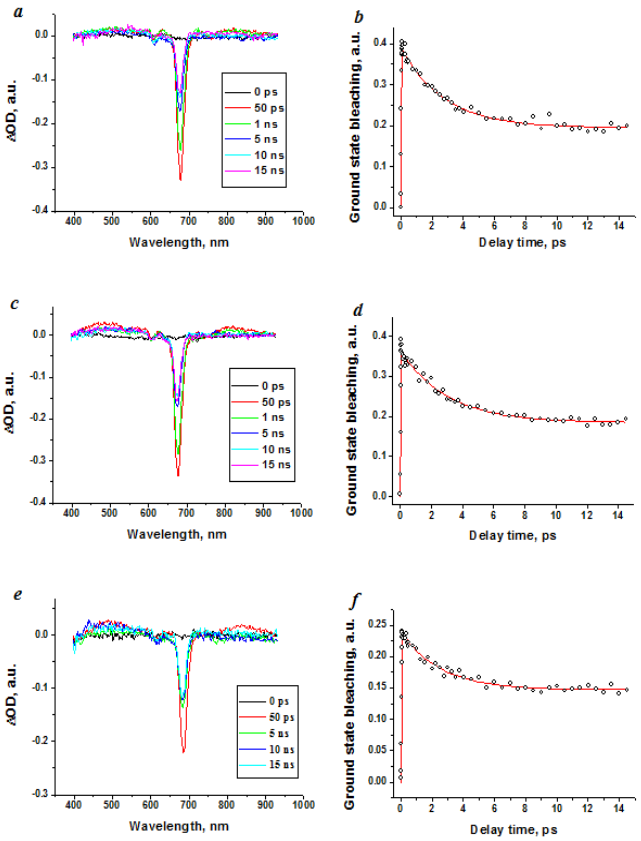


Figure 3. Transient absorption spectra at different delay times after excitation (*a*, *c*, *e*) and ground state bleaching (mono-exponential fit is shown as red line) (*b*, *d*, *f*) of the phthalocyanines **3** (*a*, *b*), **4** (*c*, *d*) and **5** (*e*, *f*) dissolved in DMF.

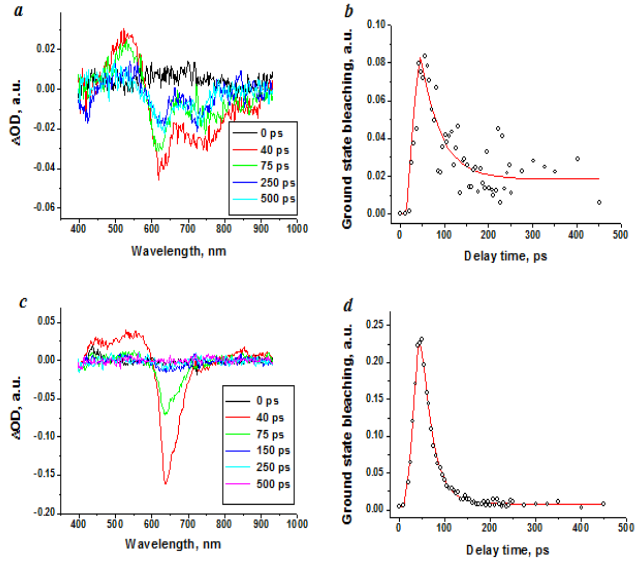


Figure 4. Transient absorption spectra (*a*, *c*) and ground state bleaching (mono-exponential fit is shown as red line) (*b*, *d*) of Pcs **3** (*a*, *b*) and **4** (*c*, *d*) dissolved in water.

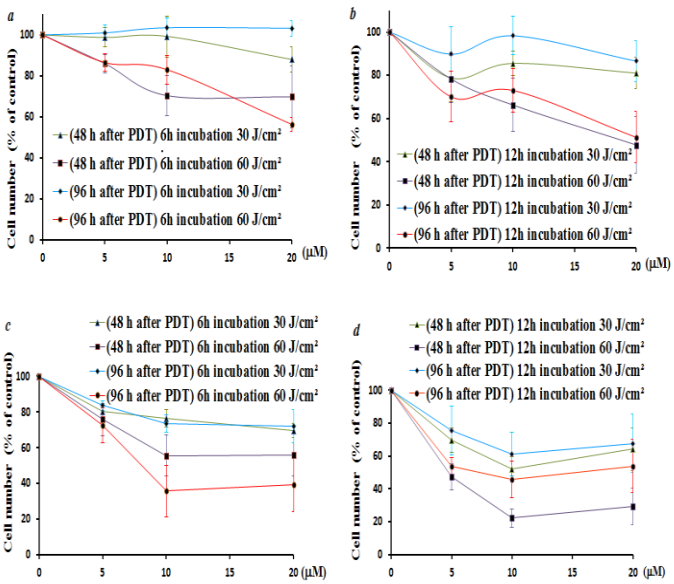
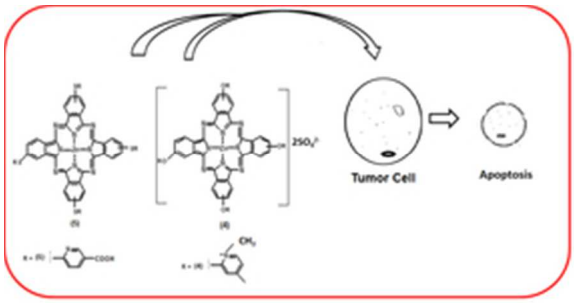


Figure 5. Effects of photoactivated Pcs on human cancer cells. BON cells incubated for 6 h or 12 h with complex **4** (*a*, *b*) and complex **5** (*c*, *d*) were irradiated with 30 or 60 J/cm² light dose. Changes in the cell number were determined by the crystal violet staining-method 48 and 96 h after PDT treatment. Cell number changes are shown as a percentage of irradiated but Pc-untreated control cells, which were set at 100%. Data are given as mean values +/- SEM of n = 4 independent experiments.

Table 1. Absorption, emission, photophysical and fluorescence lifetime data.

Complex	Solvent	Log ε	Q band[a] λ_{max} (nm)	Emission λmax(nm)	Φ_{ISC}	τ (ns)
3	**DMF**	**5.02**	**676**	**687**	**0.50**	**2.71 ± 0.15**
4	**DMF**	**4.91**	**675**	**686**	**0.51**	**2.93 ± 0.18**
5	**DMF**	**5.08**	**683**	**695**	**0.62**	**2.49 ± 0.17**

[a]When dissolved in water, the Q-band maxima of the complexes showed a hypsochromic shift in the following order: from 676 to 632 nm for **3,** from 675 to 641 nm for **4** and from 683 to 643 nm for **5** respectively.



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