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Cationic azaphthalocyanines bearing aliphatic tertiary amino substituents—Synthesis, singlet oxygen production and spectroscopic studies

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Abstract

New cationic zinc azaphthalocyanines (AzaPc) were prepared using a statistical method of synthesis starting from 5,6-bis(2-diethylaminoethylsulfanyl)pyrazine-2,3-dicarbonitrile (A) and 5,6-bis(*tert*-butylsulfanyl)pyrazine-2,3-dicarbonitrile (B). All the six possible AzaPc derivatives were detected on TLC but only five of them were finally isolated using column chromatography on silica (excluding AAAB type). The adjacent (AABB) and opposite (ABAB) isomers were well separated. Singlet oxygen quantum yields (Φ_Δ) were measured using the DPBF decomposition method. Values of Φ_Δ in anhydrous dimethylformamide (DMF) drastically decrease with the number of amino groups from 0.66 for BBBB type to 0.04 for AAAA type. The same dependence was observed in anhydrous DMF for the corresponding hydrochlorides. When DMF/water 95:5 added with HCl was used, the Φ_Δ values for amino AzaPc increased to approximately 0.66. This suggests that solvation of the amino and Cl^- ions plays an important role in the separation of the AzaPc molecules although no changes were found in UV–vis spectra. At least eight cationic charges are necessary for complete monomerization of AzaPc in water. Fewer charges lead to significant decrease of the absorption in the area of the *Q*-band and to pronounced dimeric character of the absorption spectrum.

Keywords: Cationic azaphthalocyanines; Tetrapyrazinoporphyrazines; Photodynamic therapy; Singlet oxygen quantum yield; Unsymmetrical substitution; Aggregation; Fluorescence quantum yield

1. Introduction

Azaphthalocyanines (AzaPc) are aza analogues of phthalocyanines (Pc) with some carbons in their macrocyclic system replaced by nitrogens. They can be used in similar applications as the parent Pc that have found a wide use in areas such as liquid crystals, electronic devices, gas sensors, non-linear optics, industrial dyes and Langmuir–Blodgett films [1–3]. Pc have also attracted a great attention in photodynamic therapy (PDT) [4–6]. This promising cancer treatment [7,8] is based on absorption of light (in area 650–800 nm) by a photosensitizer (PS) that transfers its energy to triplet oxygen leading to the highly reactive singlet oxygen ($^1O_2$) form.

Unfortunately, due to their planar macrocyclic system, unsubstituted Pc and AzaPc tend to aggregate, which results in low solubility, difficulties with purification and characterization and ineffectiveness in PDT. Several approaches were introduced to overcome this property as discussed previously [9]. Introduction of bulky peripheral substituents [9], charged substituents [10,11] or monomerizing solvents [12,13] are widely reported in literature.

It has been found [14,15] that amphiphilic compounds are more active than either hydrophilic or lipophilic PS in PDT-mediated cell killing. The presence of both lipophilic and hydrophilic moieties appears to improve activity. In studies involving different types of water-solubilizing substituents

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(cationic, neutral and anionic), the cationic Pc appeared to be the most efficient [16], even more than the clinically used temoporfin (Foscan®) [17]. Their uptake by cells is improved too [18]. The photodynamic efficacy of cationic Pc on cells was also confirmed by other studies [19,20]. Moreover, cationic dyes (not only Pc) are generally localized in mitochondria [21], one of the most important targets in PDT the impairment of which induces rapid induction of apoptosis [7,22].

The photosensitizers described in the present work were designed in line with the above mentioned findings. We synthesized new zinc AzaPc containing two different kinds of substituents. Bulky lipophilic *tert*-butylsulfanyl substituents ensure good monomerization of AzaPc in organic solvents and consequently provide good chromatographic properties. The *tert*-butyl group was previously found to better inhibit dimerization than long alkyl chains [9]. Alkylsulfanyl are the best derivatives from the point of view of $^1O_2$ production [23]. The second substituent – diethylaminoethylsulfanyl – corresponds to the desired hydrophilic cationic part (after quarternization or converting to hydrochloride salt). Its bulkiness is also advantageous for aggregation inhibition.

In a first attempt, the cationic part was chosen to be a stable quarternary amine. However, at this point a question arose if the alkylation of tertiary amine of the final AzaPc will be quantitative. It is very difficult to separate a mixture of incompletely alkylated homologues and the presence of non-alkylated side chains could lead to misleading results. Various authors (e.g. [24–27]) reported this reaction to run with high yields under different reaction conditions and with various alkylating agents and did not report on the presence of any lower homologues. On the other hand, other authors were not able to achieve quantitative quarternization even using more drastic conditions. Thus, Martí et al. [28] found the presence of some amount of trialkylated 3,4-tetrapyridinoporphyrazine and were not able to isolate the tetracationic derivative. Our own findings (see below) about the alkylation approach were not satisfactory too. In other studies, authors obtained mainly disubstituted instead of tetrasubstituted [29] compounds even after changing amine and conditions. Obviously, the role of charge number and lipophilicity on photodynamic efficiency could be appraised only if quantitative alkylation procedures would be available.

Alternatively, we choose to convert the tertiary amines to hydrochloride salts a process that should be quantitative. Since the salts are composed of a strong base and a strong acid they will not hydrolyze in water back to the free base and the free acid at physiological pH.

2. Experimental

All organic solvents used for synthesis were of analytical grade. 1,3-Diphenylisobenzofuran (DPBF), 2,2-dimethylpropane-1-thiol, 2-(diethylamino)ethane thiol hydrochloride and 2,2′-pyridil were purchased from Aldrich. Diaminomaleonitril and anhydrous dimethylformamide for synthesis were purchased from Across Organics. Dimethylformamide for singlet oxygen and fluorescence studies was obtained from Aldrich (biotech grade, water <0.005%). Zinc phthalocyanine (ZnPc) was purchased from Eastman Organic Chemicals (New York, USA). All chemicals and solvents were used as received without further purification except for zinc acetate (Lachema, Czech Republic) that was dried in a drying gun at 78 °C and under a pressure of 13 mbar for 8 h. TLC was performed on Silica gel 60 F254 (Merck). Kieselgel 60 (0.040–0.063 mm) from Merck was used for column chromatography. Aluminium oxide-neutral (LS 5/40) (Lachema, Czech Republic) was used for column chromatography in pre-purification steps of some AzaPc. Melting points were measured on an Electrothermal IA9200 Series Digital Melting point Apparatus (Electrothermal Engineering Ltd., Southend-on-Sea, Essex, Great Britain) and are uncorrected. Infrared spectra were measured in KBr pellets on a Nicolet Impact 400 IR-Spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury, Vx BB 300 (299.95 MHz, ^{1}H and 75.43 MHz, ^{13}C) Bruker Comp. (Karlsruhe, Germany). Chemical shifts are given relative to internal $Si(CH_3)_4$. UV–vis spectra were recorded on a UV-2401PC spectrophotometer from Shimadzu (Shimadzu Europa GmbH, Duisburg, Germany) and in some cases on a Cary 1E UV–vis spectrophotometer (Varian, Australia). Fluorescence data were obtained on an AMINCO-Bowman Series 2 luminescence spectrometer (SLM-Aminco, Urbana, IL, USA). MALDI-TOF mass spectra were recorded in positive reflectron mode on a mass spectrometer Voyager-DE STR (Applied Biosystems, Framingham, MA, USA). For each sample, 0.5 μl of the mixture was spotted onto the target plate, air-dried and covered with 0.5 μl of matrix solution consisting of 10 mg of α-cyano-4-hydroxycinnamic acid in 100 μl of 50% ACN in 0.1% TFA. The instrument was calibrated externally with a five-point calibration using Peptide Calibration Mix1 (LaserBio Labs, Sophia-Antipolis, France). The ESI mass spectrometric analyses were performed with LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) using an electrospray ion source. The source voltage was maintained at 4.5 kV, the capillary voltage at 33.0 V, the tube lens offset at 55.0 V and the capillary temperature at 200 °C. Nitrogen was used as a sheath gas at flow rate of 14 arbitrary units. Helium was used as a damping gas. Solutions of analytes at a concentration of 1.0 μg/ml in methanol with 0.1% acetic acid were used for the flow injection analysis. The samples were introduced into the ion source at flow of 5.0 μl/min.

2.1. Synthesis

Synthesis of **1** [30], **2** [9] and **3** [9] were performed according to previously published method. ^{1}H NMR signals of dyes showed strong broadening. Aromatic ^{13}C NMR signals of all AzaPc were poor, broad and very hard to detect. Both MS methods used for AzaPc (MALDI-TOF and ESI) showed a typical cluster (for a presence of Zn) at mass corresponding to counted values.

5,6-*Bis*(2-*diethylaminoethylsulfanyl*)*pyrazine*-2,3 - *dicarbonitrile* (**4**): A solution of 3.56 g (21 mmol) of 2-(diethylamino)-ethane thiol hydrochloride in water was treated with 43.5 ml of water solution of NaOH (concentration 1.00 M) and stirred at r.t. for 10 min. A tetrahydrofuran (50 ml) solution of 2.00 g

(10 mmol) of **2** was added at once and the mixture vigorously stirred for 30 min at r.t. Diethyl ether (50 ml) was added, the organic phase was separated and the water phase was washed twice with diethyl ether. Combined organic phases were evaporated (taking care not to exceed a temperature of 30 °C), dissolved in diethyl ether, filtered and washed three times with water acidified with few drops of concentrated HCl. Organic phases were discarded and water neutralized with NaOH to slightly basic reaction (pH paper). A yellow oily product was isolated by washing with diethyl ether, carefully evaporated and purified by column chromatography on silica with acetone/diethyl ether 1:1 as a mobile phase. The fractions of product were not evaporated but converted immediately to its hydrochloride salt **5**. Thus, all fractions with product were combined and bubbled with dry gaseous HCl to form white solid. Recrystalization from MeOH/diethyl ether yielded 3.95 g (84%) of white needles of **5**. All the analytical data belong to **5**. M.p. 215–217 °C (dec.). IR (KBr) 2995, 2983, 2927, 2587, 2560, 2455, 2237(CN), 1486, 1459, 1317, 1160, 1140, 983. ^{13}C NMR (CD_3OD) δ 9.5, 25.3, 50.4, 114.8, 128.5, 159.9 (one aliphatic signal overlaps with signal of solvent). ^{1}H NMR (CD_3OD) δ 4.85 (s, 2H, NH), 3.80–3.68 (m, 4H, S-CH_2), 3.54–3.43 (m, 4H, N-CH_2), 3.36 (q, 8H, $J = 7.3$ Hz, N-CH_2), 1.42 (t, 12H, $J = 7.4$ Hz, CH_3).

2,3,9,10,16,17,23,24-*Octakis*(*tert-butylsulfanyl*)-1,4,8,11,15,18,22,25-*octaazaphthalocyaninato zinc(II)* (P2-Zn0): A mixture of 306 mg (1 mmol) of **3**, 366 mg (2 mmol) of anhydrous zinc acetate and 4 ml of anhydrous dimethylformamide (DMF) was immersed into oil bath preheated to 160 °C and stirred at this temperature for 90 min. A dark green solution was poured into cold water (200 ml), left for half an hour and crude P2-Zn0 was filtered off and washed with water and methanol. Green solid was then absorbed to a small amount of silica (1.5 g) and washed with methanol on a glass frit until the solution was colorless. The product on silica was then dried up, poured on silica column and purified by chromatography with chloroform/tetrahydrofuran 20:1 as eluent to yield 87 mg (27%) of dark green solid. All spectroscopic data correspond with the ones of the same compound prepared previously by the "insertion method" we described previously [9].

2,3,9,10,16,17,23,24-*Octakis*(2-*diethylaminoethylsulfanyl*)-1,4,8,11,15,18,22,25-*octaazaphthalocyaninato zinc(II)* (P2-Zn4): The free base **4** has to be released from its hydrochloride **5** before reaction. Thus, 465 mg (1 mmol) of **5** was dissolved in water (10 ml) and 2.2 ml of 1 M NaOH was added. The free base **4** was isolated by washing with diethyl ether, drying the organic layer with anhydrous sodium sulfate and carefull co-evaporation (not exceeding 30 °C) with toluene to dryness. Anhydrous DMF (4 ml) and 366 mg (2 mmol) of anhydrous zinc acetate were added, the mixture was immersed into oil bath preheated to 160 °C and stirred at this temperature for 90 min. The resulting dark solution was poured into cold water (200 ml), left for half an hour and a very fine suspension of P2-Zn4 was filtered off. Green solid was then dissolved in water acidified with few drops of concentrated HCl, filtered and made basic with NaOH. The water suspension was extracted several times with chloroform, the organic layer was dried with anhydrous sodium sulfate, filtered and evaporated to give 100 mg of a crude product. This was pre-purified on a short neutral aluminium oxide column with THF as eluent to give 65 mg of product and then purified using chromatography on silica with THF/pyridine 3:7 as eluent. Pure product was then dissolved in minimum amount of chloroform and dropped into hexane (150 ml). Very fine suspension appeared after 24 h in refrigerator and was filtered off to yield 30 mg (7%) of dark green solid. ^{13}C NMR ($CDCl_3$) δ 11.8, 29.0, 46.8, 51.2, 145.5, 151.2, 157.8. ^{1}H NMR ($CDCl_3$) δ 4.90–4.72 (bs, 16H, S-CH_2), 4.72–3.11 (bs, 16H, CH_2), 3.11–2.32 (bs, 32H, N-CH_2), 1.58–0.76 (bs, 48H, CH_3). MALDI-TOF MS *m/z* 1633 ($M + H^+$). UV–vis (pyridine) $\lambda_{max}(\varepsilon)$ 657 (241800), 595 (33000), 386 (136600).

2.1.1. General procedure of synthesis of unsymmetrical AzaPc

The free base **4** was released from its hydrochloride **5** following the procedure mentioned above for the synthesis of P2-Zn4. Then, 785 mg (2 mmol) of free base **4**, 612 mg (2 mmol) of **3**, 1.46 g (4 mmol) of anhydrous zinc acetate and 12 ml of anhydrous DMF were mixed together, immersed into oil bath preheated to 160 °C and stirred at this temperature for 90 min. The mixture was then cooled down and poured into cold water (400 ml). A fine suspension was filtered off, thoroughly washed with water and air dried. The green solid was then extracted with chloroform and solvent evaporated to yield about 600 mg of crude product. This product was pre-purified by chromatography using a short column filled with neutral aluminium oxide and tetrahydrofuran as mobile phase. The mixture (380 mg) of the six different AzaPc and some side products was separated using column chromatography on silica. The different AzaPc were obtained as green fractions using increasing polarity of mobile phase (reported bellow as the first column) starting from chloroform/THF 20:1 for P2-Zn0. Every fraction was then purified on column at least once more using either the same or slightly changed mobile phase (the second and the third column).

2,3,9,10,16,17-Hexakis(tert-butylsulfanyl)-23,24-bis(2-diethylaminoethylsulfanyl)-1,4,8,11,15,18,22,25-octaazaphthalocyaninato zinc(II) (P2-Zn1): This compound was synthesized according to the general procedure with following mobile phases: the first column THF, the second column THF, the third column THF/chloroform/pyridine 4:10:1. Yield 26 mg (2.0%). MALDI-TOF MS *m/z* 1375 ($M + H^+$). ESI MS *m/z* 1413 ($M + K^+$), 1397 ($M + Na^+$), 1375 ($M + H^+$), 688 ($M + 2H^+$). UV–vis (pyridine) $\lambda_{max}(\varepsilon)$ 657 (186500), 595 (25600), 385 (102300).

2,3,16,17-Tetrakis(tert-butylsulfanyl)-9,10,23,24-tetrakis(2-diethylaminoethylsulfanyl)-1,4,8,11,15,18,22,25-octaazaphthalocyaninato zinc(II) (P2-Zn2O): This compound was synthesized according to the general procedure with following mobile phases: the first column THF/pyridine 10:1, the second column THF/pyridine 20:1, the third column THF/pyridine 20:1. Yield 9 mg (0.7%). MALDI-TOF MS *m/z* 1461 ($M + H^+$). UV–vis (pyridine) $\lambda_{max}(\varepsilon)$ 657 (179700), 595 (24000), 385 (105700).

2,3,9,10-Tetrakis(tert-butylsulfanyl)-16,17,23,24-tetrakis(2-diethylaminoethylsulfanyl)-1,4,8,11,15,18,22,25-octaazaphth-

alocyaninato zinc(II) (P2-Zn2A): This compound was synthesized according to the general procedure with following mobile phases: the first column THF/pyridine 10:1, the second column THF/pyridine 12:1, the third column THF/pyridine 12:1. Yield 26 mg (1.9%). MALDI-TOF MS *m/z* 1461 ($M+H^+$). ESI MS *m/z* 1499 ($M+K^+$), 1483 ($M+Na^+$), 1461 ($M+H^+$), 731 ($M+2H^+$), 487 ($M+3H^+$). UV–vis (pyridine) $\lambda_{max}(\varepsilon)$ 657 (182400), 595 (24600), 385 (102600).

2.1.2. Hydrochlorides of AzaPc

The hydrochlorides were prepared by dissolving the free base of AzaPc (about 1 mg) in a mixture of tetrahydrofuran and methanol, adding excess of concentrated HCl and evaporation to dryness. Toluene was added and the mixture evaporated to dryness once more to remove remaining water and HCl. In this way we obtained hydrochlorides P2-Zn1·HCl, P2-Zn2O·HCl, P2-Zn2A·HCl and P2-Zn4·HCl. All hydrochlorides were found to be stable in solid state. Only P2-Zn4·HCl was very hydroscopic. Summary of spectroscopic data is given in Table 2.

2.2. Singlet oxygen quantum yield

Stock solutions of all the dyes (and the corresponding hydrochlorides) were prepared in DMF and in the case of P2-Zn4·HCl and P2-Zn2A·HCl also in water. The stock solutions were found to be stable in the dark for several weeks. Light irradiation was carried out using a 1000 W Xenon arc mounted in a lamp housing equipped with a rear reflector and a 3 in. diameter fused silica condenser of *f*/0.7 aperture (Oriel). The infrared and UV below 320 nm were removed using water (8 cm) and Pyrex glass filters, respectively. The light was focussed on the entrance of a monochromator (Bentham M300) with slits generally adjusted to 1 mm giving a bandpass of 2.7 nm. The monochromatic beam was focussed on the solution contained in a 10 mm × 10 mm quartz optical cell. The size of the focussed beam was adjusted to be smaller than the surface occupied by the solution at the entrance windows of the optical cell. The power of the incident light (P_0) at the irradiation wavelength (λ_{max} of the dye *Q*-band) was measured using a surface absorbing disc calorimeter (Scientech). A typical value was 0.42×10^{-3} W. The yield of singlet oxygen production by the dyes under study was derived from its reaction with DPBF [31]. The DPBF solution was saturated with oxygen before irradiation by bubbling through the solution for a few minutes. Then, stock solution of the dye and in some cases stock solution of HCl were added. Absorbance (Abs) of the dye solution at the irradiation wavelength (λ) was measured exactly before irradiation. Typical values were around 0.1. The exact value of the intensity absorbed by the solution (I_a) can be calculated from the intensity of incident light (I_0) by using the relation derived from the Beer's law, $I_a = I_0(1 - e^{-2.3Abs})$. The absorbed intensity per time unit expressed as a number of "moles" of photons, I_{ap}, can be derived according to the relation (Eq. (1)):

$$I_{ap} = [P_0/(E_P N_A)](1 - e^{-2.3Abs}) \quad (1)$$

where E_P is the energy of one photon given by the Einstein's law, $E_P = hc/\lambda$, where *c* is the velocity of light and *h* the Planck's constant. The Avogadro constant, N_A, is used to convert the number of photons to "molar" units. In all cases, irradiation times were chosen to cause consumption of about 15% of starting amount of DPBF. The solution was stirred with a small magnetic barrel during irradiation. The progress of the reaction was monitored from the decrease of the DPBF absorption at its maximum (415 nm, $\varepsilon = 23180\ M^{-1}\ cm^{-1}$ in DMF and $\varepsilon = 22490\ M^{-1}\ cm^{-1}$ in DMF/water/HCl). Abbreviation DMF/water/HCl used throughout this article means the solvent system composed of DMF/water 95:5 (v/v), added with HCl 5×10^{-4} M. Quantum yields of the photoreaction (Φ_{DPBF}) were measured by using at least six concentrations of DPBF ranging from 5×10^{-6} to 100×10^{-6} M. They were calculated by using Eq. (2):

$$\Phi_{DPBF} = (c_0 - c_t)V/(I_{ap}t) \quad (2)$$

where c_0 and c_t are the concentrations of DPBF before and after the irradiation time *t*, respectively. *V* is the volume of the reaction (2.8×10^{-3} l). The singlet oxygen quantum yields (Φ_Δ) were calculated from Φ_{DPBF} with respect to the starting concentration [DPBF] according to Eq. (3):

$$1/\Phi_{DPBF} = 1/\Phi_\Delta + (1/\Phi_\Delta)(k_d/k_r)(1/[DPBF]) \quad (3)$$

where k_d is the decay rate constant of $^1O_2$ in the solvent (without any quencher) and k_r is the quenching constant of $^1O_2$ by DPBF. When $1/\Phi_{DPBF}$ versus 1/[DPBF] is plotted, the value of $1/\Phi_\Delta$ is obtained as the intercept with axis *y* and the value $-k_r/k_d$ as the intercept with axis *x*.

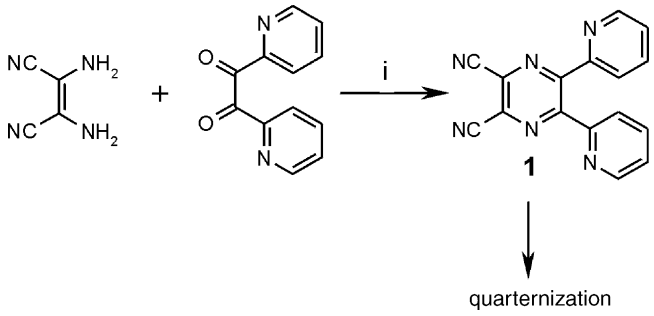
2.3. Fluorescence quantum yield

Fluorescence quantum yields of the dyes were determined relative to that of ZnPc ($\Phi_F = 0.17$ in DMF [32]) and calculated using integrated areas under the fluorescence emission spectrum of the sample and reference dye [33]. Refractive indexes were taken into account in the case of water solution of P2-Zn4·HCl ($n_{water} = 1.34$, $n_{DMF} = 1.43$). In the case of DMF/water/HCl solvent system, the refractive index was believed to be very close to that in DMF and was omitted. Fluorescence emission spectra were recorded after excitation at 386 nm, fluorescence excitation spectra were recorded observing the fluorescence signal at 710 nm. In all cases the absorbance at λ_{max} of the *Q*-band was below 0.04.

3. Results

3.1. Synthesis

We started with the preparation of precursors. The first precursor with two pyridyl moieties (**1**) was synthesized according to published method (Scheme 1) [30]. Alkylation of **1** using different strong alkylating agents (CH_3I, CH_3CH_2I) and different solvents (ethanol, DMF, chloroform) was not completely successful and main product was always monoalkylated as revealed by ^1H NMR spectroscopy. Conversion of **1** to water-soluble hydrochloride by boiling in concentrated HCl failed too. From

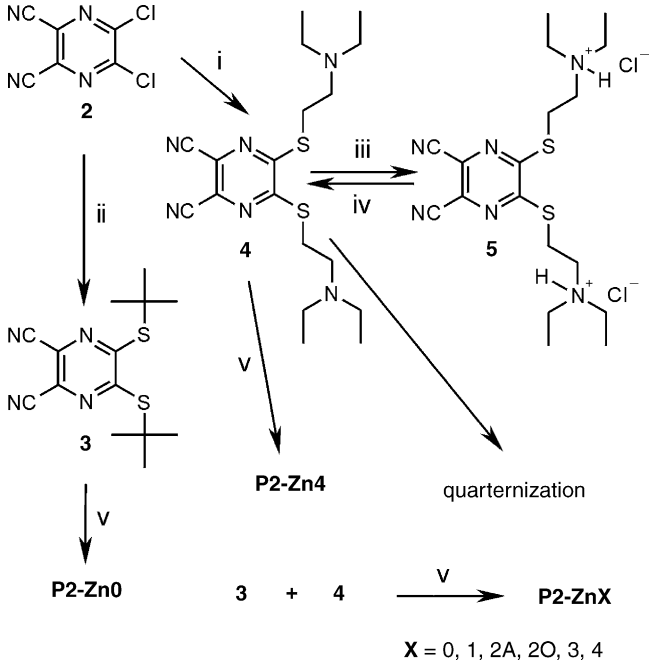


Scheme 1. Reaction conditions: (i) acetonitrile, reflux, 3 h.

these results it seems that free electron pair on pyridine nitrogen of **1** is strongly delocalized leading to very low basicity and poor reactivity. Precursor **1** is therefore unsuitable for our purposes.

Then, more reactive aliphatic tertiary amine was considered. Nucleophilic substitution (Scheme 2) of chlorine atoms in **2** with 2-diethylaminoethanethiolate lead to **4** in good yields (84%). Nevertheless, free base **4** is unstable and quickly darkens even by standing at room temperature. To overcome this problem, this compound was easily and quantitatively converted to the stable hydrochloride **5** and stored in this form and converted back to the free base just before subsequent reactions. Attempts to quarternize **4** with different alkylating agents in different solvents (see above) lead to a mixture of mono- and di-substituted derivatives (even after 7 days refluxing in DMF). Some traces of non-reacted **4** were always found on TLC.

We used a statistical method based on the reaction of two different precursors in the preparation of unsymmetrical AzaPc. This method leads to a mixture of six different compounds (P2-Zn0, P2-Zn1, P2-Zn2O, P2-Zn2A, P2-Zn3, P2-Zn4) (Fig. 1) that



Scheme 2. Reaction conditions: (i) 2-(diethylamino)ethane thiol, NaOH, THF/water; (ii) 2,2-dimethylpropane-1-thiol, NaOH, THF/water; (iii) acetone/diethyl ether, HCl (g); (iv) water, NaOH; (v) DMF, $Zn(COOCH_3)_2$.

Table 1
Mobile phases and R_f values of synthesized AzaPc on TLC

	Mobile phase	R_f
P2-Zn0	Chloroform/THF 20:1	0.90
P2-Zn1	THF	0.30
	THF/pyridine 10:1	0.60
	THF/pyridine 3:1	0.95
P2-Zn2O	THF/pyridine 10:1	0.52
	THF/pyridine 3:1	0.87
P2-Zn2A	THF/pyridine 10:1	0.27
	THF/pyridine 3:1	0.59
P2-Zn3	THF/pyridine 10:1	0.08
	THF/pyridine 3:1	0.43
P2-Zn4	THF/pyridine 3:7	0.50

were separated using standard chromatographic method with silica as a stationary phase.

We were able to find six strong green fractions corresponding to our products among some of green side products after TLC with different mobile phases (see Table 1). During searching we used standards of P2-Zn0 and P2-Zn4 prepared in a separate reaction and different strength of fractions when different ratios of starting materials **3** and **4** (3:1, 1:1, 1:3) were added to reactions. However, separation on silica column was difficult. Compounds with amino groups were strongly attached to silica at the column top. Slower fractions were usually contaminated by the faster ones that were slowly eluted from the top. This problem could not be completely solved even using several column chromatographies of isolated fractions and the compounds always contained traces of the former fractions as detected on TLC and MS. A large decrease in yield was observed after each passage on a column. The total yield of AzaPc mixture is about 30% but after the purification process it decreases to about 1–2% per fraction. Compound P2-Zn3 could not be isolated at all and was detected only by TLC. Some traces of this fraction were isolated and MS confirmed the right mass for the expected P2-Zn3 but the amount and purity were not satisfactory for further analyses and investigations. Using neutral or basic alumina as stationary phase did not improve the purification process. Therefore the final yields of AzaPc do not correspond to the expected statistical distribution. On the other hand, the statistical distribution can be observed on TLC (reactivity of both precursors is approximately the same). Moreover, longer standing of purified fractions in solution of mobile phase lead to some change in their properties and a lot of material did not move from the start when TLC was performed later. It seems that tertiary amino groups play an important role in this process because the more amino groups were present in molecule of AzaPc the stronger and faster were these changes. Since the fractions were stored in the dark no photobleaching is possible and this behavior may be concerned with the formation of the aggregates (see Section 4).

Surprisingly, the two isomers containing two *tert*-butylsulfanyl quarters and two diethylaminoethylsulfanyl quarters were well separated and two fractions of the same mass were found. There have been very few examples of chromato-

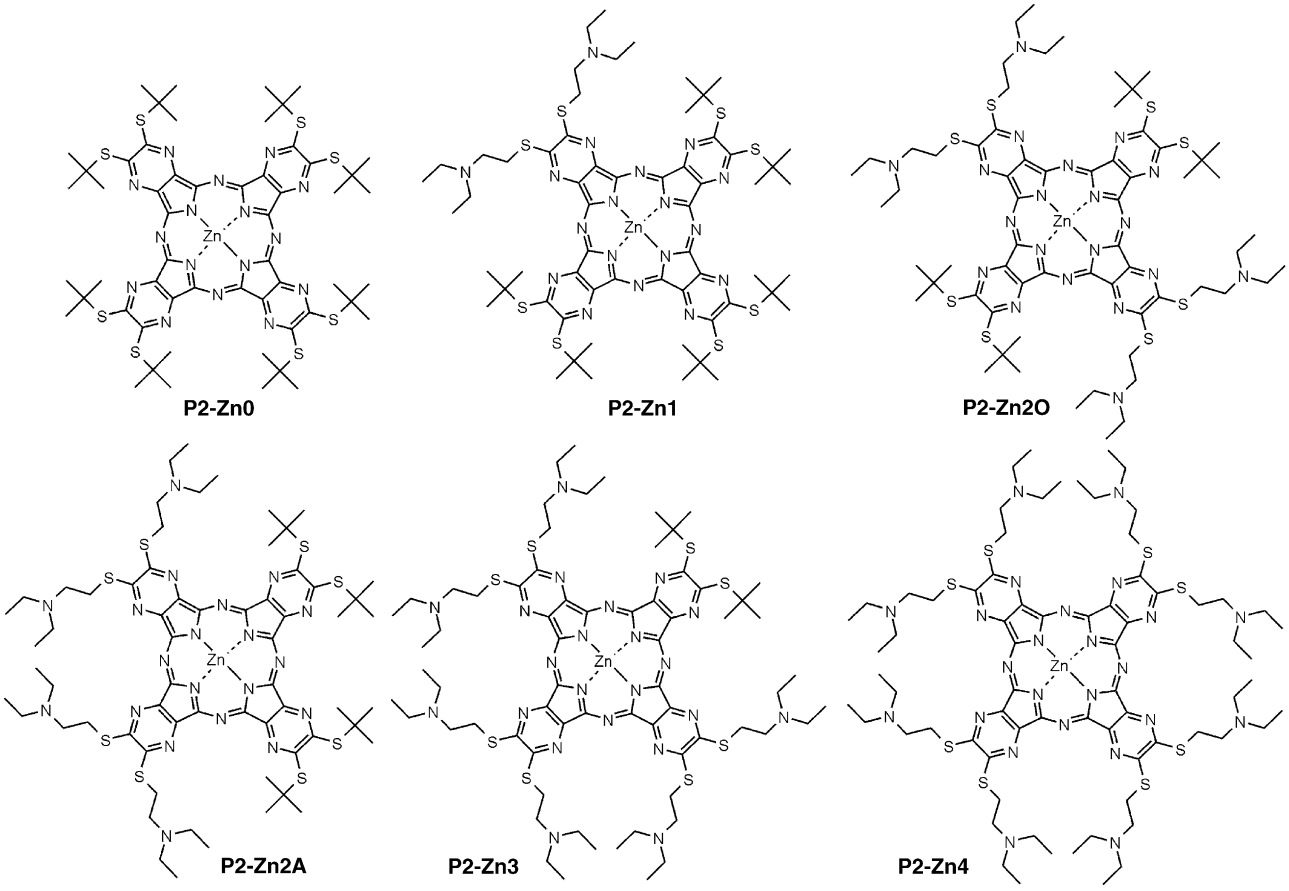


Fig. 1. Structures of AzaPc in mixture.

graphic separation of "*adjacent*" and "*opposite*" (or *cis* and *trans* or AABB and ABAB) isomers of Pc till this time [34–38]. Chromatography is not always sufficient for separation of these isomers (e.g. [39,40]) and more often selective methods for synthesis of the pure isomers are used [41]. Both isomers were well separated in our case and their R_f values were more similar to previous P2-Zn1 (for P2-Zn2O) and following P2-Zn3 (for P2-Zn2A) fractions (Table 1). Assigning these fractions to each isomer is usually derived from ^{1}H NMR analysis [34,42] or differences in UV–vis spectra arising from a different symmetry of the Pc isomers [34,35,43]. In our case none of these two methods can be used. All ^{1}H NMR signals showed strong broadening and even the ratio of peripheral substituents cannot be exactly calculated. That is also why, unsymmetrical AzaPc were identified by using MS, since NMR analyses does not bring any unequivocal identification. UV–vis spectra of all compounds in non-aggregated form display little difference because the influence of the peripheral substitutuents on the electron density distribution in the macrocyclic system is almost the same. Therefore no important decrease in symmetry of conjugated macrocyclic system occurs. Alternatively, we considered the forces that could be involved in separation of these isomers on silica. When substituents allowing H-bonding (in our case the tertiary amine) are used, hydrogen bridges with OH groups of silica are expected to account for compound separation. Because of the rigidity of the planar skeleton of AzaPc molecule, adjacently substituted compounds are probably able to provide more H-bridges per molecule. As a result, they will be less mobile on silica and present lower R_f values (Fig. 2). This could also explain why opposite P2-Zn2O is more similar in chromatographic behavior to monosubstituted P2-Zn1 than to its constitutional isomer P2-Zn2A. Analogously to previous case it is valid also for P2-Zn2A and P2-Zn3. Similar findings were found for adjacent and opposite isomers of bis(1,4-didecylbenzo)-bis(3,4-pyrido)porphyrazine [35], where the possible H-bond can arise on pyridine part of molecule.

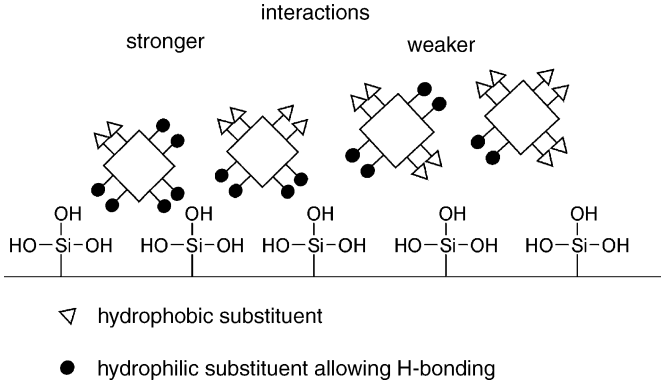


Fig. 2. Explanation of chromatographic behavior of AzaPc allowing H-bonding with silica stationary phase.

3.2. Singlet oxygen measurements

Photosensitizers excited to the triplet state can react via two main photoprocesses—types I and II. Albeit it has been pointed out that DPBF may react also with reactive species produced by photoprocess type I [44], it is generally believed that this route is not important [31,39,45]. It is generally assumed that DPBF is decomposed only by $^1O_2$ generated by type II photoprocess to produce 1,2-dibenzoylbenzene. Moreover, it is known that DPBF quenches singlet oxygen almost exclusively by chemical reaction, physical quenching being negligible [46]. Therefore, only two processes account for the disappearance of singlet oxygen in the solution (reactions (4) and (5)):

$$^1O_2 \rightarrow {}^3O_2 \quad (4)$$

$$^1O_2 + \mathrm{DPBF} \rightarrow \mathrm{DPBF_{ox}} \quad (5)$$

Reactions (4) and (5) represent the natural decay of the $^1O_2$ in the solvent with the rate k_d and the reaction of $^1O_2$ with DPBF with the quenching constant k_r, respectively. Since both reactions run simultaneously, Φ_Δ cannot be calculated directly from the Eq. (2) but must be approximated to infinite DPBF concentration using Eq. (3) (see Section 2). Reaction of singlet oxygen with DPBF (Eq. (5)) was found to run independently of DPBF concentration until approximately 20% of DPBF was consumed (excellent linearity of the dependence of DPBF decay on time). In the present study, the irradiation times were always chosen to limit the final DPBF conversion to about 15%. The plots of $1/\Phi_{DPBF}$ versus 1/[DPBF] show very good linearity in all measurements (Fig. 3), allowing accurate Φ_Δ determination (Table 2).

The intercept with the axis x gives the value $-k_r/k_d$ (in DMF $k_r/k_d = 2.67 \pm 0.3 \times 10^4\ M^{-1}$, in DMF/water/HCl $k_r/k_d = 3.28 \pm 0.2 \times 10^4\ M^{-1}$). Since the k_r/k_d value does not depend on the DPBF concentration or on the Φ_Δ value of the various dyes, all plots should intercept at one point. As shown in Fig. 3 and Table 2 (last column), this fact is observed in all cases, which confirms the accuracy of the measurements. Using already published values of k_d ($5.3 \times 10^4\ s^{-1}$ [47] and $4.0 \times 10^4\ s^{-1}$ [48]) we obtained the value $k_r = 1.1–1.4 \times 10^9\ M^{-1}\ s^{-1}$. This is in excellent agreement with the previously published value $k_r = 1.1 \times 10^9\ M^{-1}\ s^{-1}$ [49]. The value of Φ_Δ obtained for the reference compound ZnPc is also in excellent agreement with the published value ($\Phi_\Delta = 0.56$ in DMF [39], $\Phi_\Delta = 0.567$ in the present work).

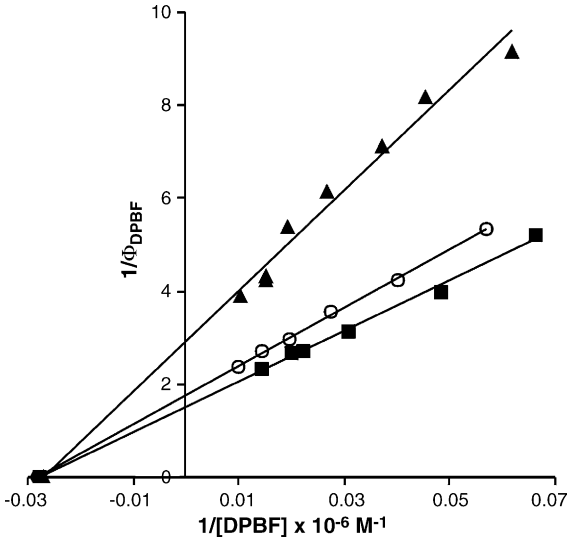


Fig. 3. Dependence of $1/\Phi_{DPBF}$ on 1/[DPBF] in DMF for P2-Zn0 (■), ZnPc (◯) and P2-Zn1 (▲).

Table 2
Photophysical and photochemical data of prepared compounds

Compound	Absorbance		Fluorescence		Φ_Δ	k_r/k_d (M^{-1})
	λ_{max} (nm)	Log ε ($M^{-1}\ cm^{-1}$)	λ_{max} (nm)	Φ_F		
DMF						
P2-Zn0	654	5.45	660	0.224	0.664	2.77×10^4
P2-Zn1	655	5.27	661	0.126	0.339	2.74×10^4
P2-Zn1·HCl	655	5.26	661	0.031	0.206	2.73×10^4
P2-Zn2A	655	5.26	661	0.035	0.139	2.62×10^4
P2-Zn2A·HCl	655	5.26	661	0.018	0.089	2.94×10^4
P2-Zn2O	655	5.27	661	0.039	0.142	2.49×10^4
P2-Zn2O·HCl	655	5.25	661	0.019	0.081	2.54×10^4
P2-Zn4	655	5.38	661	0.004	0.039	2.65×10^4
P2-Zn4·HCl	655	5.33	661	0.004	0.035	2.44×10^4
ZnPc	669	–	674	–	0.567	2.82×10^4
ZnPc (standard)	670 [39]	–	675 [32]	0.17 [32]	0.56 [39]	–
DMF/water/HCl						
P2-Zn0	654	5.45	660	0.226	0.678	3.21×10^4
P2-Zn1·HCl	657	5.26	665	0.132	0.639	3.08×10^4
P2-Zn2A·HCl	657	5.27	665	0.130	0.669	3.27×10^4
P2-Zn2O·HCl	657	5.26	665	0.128	0.645	3.31×10^4
P2-Zn4·HCl	657	5.38	665	0.065	0.467	3.52×10^4
Water + HCl						
P2-Zn4·HCl	650	5.32	656	0.098	–	–

Focusing on the data in Table 2, a decrease of Φ_Δ in DMF is observed with increasing number of amino groups in the molecules. Such results were not expected because there should not be any great influence of peripheral chains on Φ_Δ. The same dependence is found in the group of hydrochlorides in DMF with even lower Φ_Δ values for the hydrochloride when compared with the corresponding free base. Almost identical observations were made with the AzaPc dyes in the form of free base in pyridine (data not shown). It must be pointed out that anhydrous DMF (water <0.005%) was used for these measurements. When the same measurements were performed in a system DMF/water/HCl (HCl is added to ensure complete ionization of all amino groups (for explanation see Section 3.4)), the Φ_Δ values of all amino AzaPc increased significantly reaching the values of amino-free P2-Zn0. The reaction of DPBF with $^1O_2$ is not affected by addition of HCl, since the value of Φ_Δ for P2-Zn0 is the same in both solvent systems (within experimental error). No changes in the spectra or concentration of DPBF were found after addition of HCl into solution, even after several hours of standing in the dark or after irradiation in the absence of AzaPc under the same conditions used for Φ_Δ determination.

3.3. Fluorescence quantum yield

Fluorescence quantum yields (Φ_F) were determined in the same solvent systems (Table 2). The behaviour of the AzaPc corresponded to the results obtained for Φ_Δ. Significant decrease with increasing number of amino groups in the molecule in DMF and lower values for the corresponding hydrochlorides were observed. Increase of Φ_F for amino AzaPc in DMF/water/HCl was also observed but the value did not reach that of amino-free P2-Zn0 as it happened in the case of Φ_Δ in the same solution.

Typical fluorescence emission and excitation spectra together with UV–vis absorption spectra are shown in Fig. 4. The spectra of all examined AzaPc in DMF and DMF/water/HCl present the same shape. Small shifts only due to a solvatochromic effect were found. No shifts were found for free-bases and corresponding hydrochlorides in anhydrous DMF. Small red shift in both fluorescence and absorption spectra of hydrochlorides was found when DMF/water/HCl system was used. This supports the view expressed in the discussion about a solvation of the amino groups and ion pair formation. Moreover, no solvatochromic shift was observed for P2-Zn0 where no solvatable moiety is present. In all cases, the absorption and fluorescence excitation spectra present exactly the same shape at the concentrations used for either Φ_F or Φ_Δ measurements (Fig. 4). Whereas the extinction coefficients at λ_{max} of the *Q*-band did not change with dilution, it was suggested that no classical dimers are present in both type of experiments.

3.4. Aggregation behavior of AzaPc hydrochlorides in water

Prepared hydrochlorides of AzaPc showed different water-solubility and different aggregation behavior in water that were associated with the number of protonated amino groups. P2-Zn1·HCl was not soluble in water at all, so the studies were performed with exclusion of this compound. Insolubility of AzaPc with only two protonated amino-groups is not unexpected, since similar observations were found also for cationic benzonaphthoporphyrazines [39]. Solubility in water is improved with the presence of two next charged amino groups and compounds P2-Zn2O·HCl and P2-Zn2A·HCl showed very good solubility. Both isomers present similar properties. Their absorption spectra, however, showed characteristics typical of the presence of dimeric species in water and did not change with dilution even if complete protonation was assured by adding excess of HCl (Fig. 5, the lowest spectrum). It is therefore concluded that presence of only four positive charges in the molecule of AzaPc

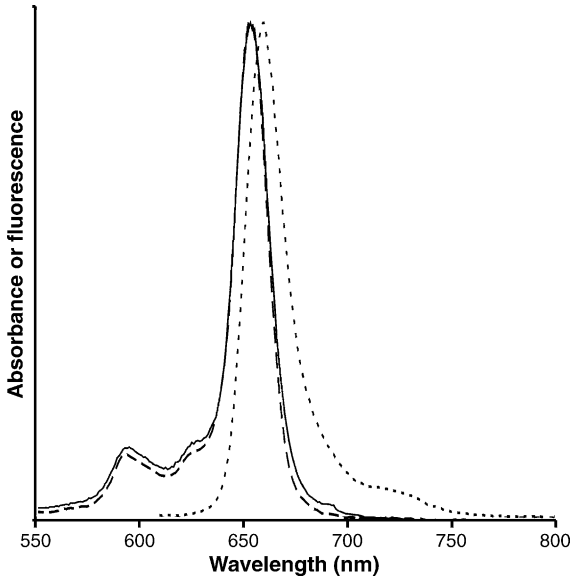


Fig. 4. Fluorescence emission (dotted line), fluorescence excitation (full line) and UV–vis absorption spectra (dashed line) of P2-Zn1 in DMF.

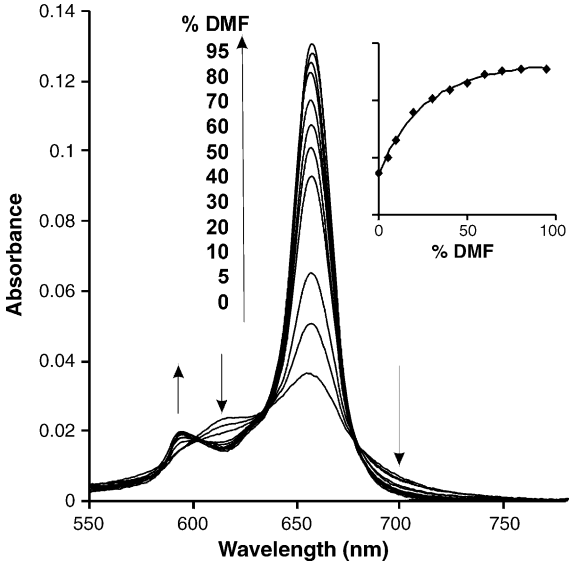


Fig. 5. Changes in UV–vis spectra of a P2-Zn2A·HCl water solution (with addition of HCl) of the constant concentration (approximately 7×10^{-7} M) with an increasing amount of DMF in the solution. Inset: an increase of absorbance at *Q*-band maximum with the increasing amount of DMF in the solution.

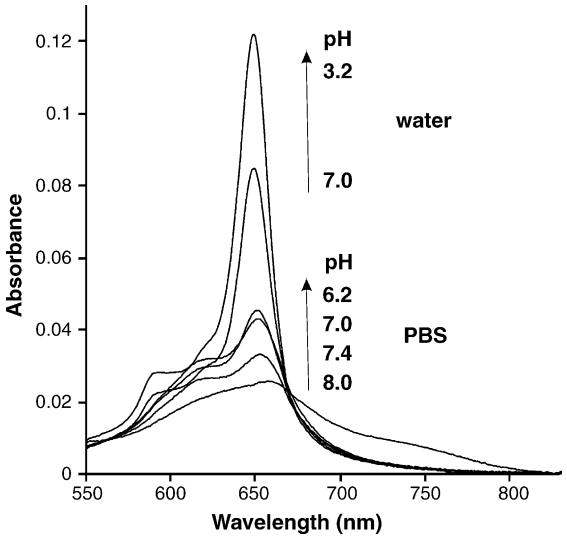


Fig. 6. Changes in UV–vis spectra of a P2-Zn4·HCl solution in water and PBS of different pH. Concentration in all cases was the same (approximately 5×10^{-7} M).

is not enough to prevent dimerization in water. The quantity of monomer in solution can be increased by addition of DMF (Fig. 5) reaching a maximum at about 90–95% of DMF in the solution (Fig. 5, inserted graph). Three isosbetic points were found (681, 638 and 600 nm) suggesting a simple transition from dimeric to monomeric form.

P2-Zn4·HCl showed also very good solubility. In water at pH 7.0, its absorption spectrum even displays monomeric character (Fig. 6). However, it seems that at least eight charged amino groups are necessary for complete monomerization of this AzaPc in water. After addition of HCl (final concentration 5×10^{-4} M) to give a pH of about 3.2, a significant increase of *Q*-band was observed indicating the presence of some amount of dimer in the solution at pH 7.0 (Fig. 6). Further addition of HCl did not improve the value of the extinction coefficient. From these and following observations it seems that the salt composed of HCl and tertiary amino group will slightly hydrolyze in water at neutral pH, releasing free amino groups. As the neutral free amino groups do not participate in the electrostatic repulsion forces responsible for monomerization, partial dimerization is observed in water. This interpretation is further supported by the following experiments. When oxygen or nitrogen was bubbled through solution of P2-Zn4·HCl in the dark a rapid decrease of the *Q*-band extinction coefficient was observed in both cases. The effect was found to depend on the time of gas bubbling. The characteristics of the spectra were restored to the starting shape after adding excess of HCl. Thus, these results cannot be attributed to decomposition. More likely, free HCl in solution is displaced during bubbling and the equilibrium between ionized and non-ionized forms shifts in favor of the latter form. From the Hendersson–Hasselbach's equation and using a pK_a value of 9.8 (pK_a = 9.82 for the similar known compound 1,2-bis(diethylaminoethylsulfanyl)phthalonitrile [26]) it can be calculated that at pH 7.4 one alkylamino chain would be at 99.6% under its ionized form. The percentage of molecules with all eight amino groups ionized would be $0.996^8 \times 100 = 96.8\%$. We must point out, that the value pK_a = 9.8 is only an approximation. If the real pK_a is only 0.5 lower, we get about 90% of P2-Zn4·HCl molecules completely ionized and the rest can lead to slight dimerization. The dependence of the dimerization on the pH of the solution can also be observed in PBS (phosphate buffer saline) as shown in Fig. 6. In this case, a more pronounced dimeric character of the spectrum was observed when the pH was increased and even precipitation from the solution can appear. In this case, the aggregation process is more complex (including the precipitation) so that no isosbestic points were found. Ionic strength of the solution may play a role in aggregation too since the spectra at the same concentration and pH showed marked differences in water and PBS (Fig. 6).

4. Discussion

It is well known and documented that AzaPc and Pc tend to form aggregates. In diluted solutions they exist in equilibrium usually between monomer and dimer forms. It depends on several factors (solvent, substitution of macrocycle, temperature, concentration, charge, etc.) whether the equilibrium is shifted more, or even exclusively, towards one or another form. However, in most cases these changes can be observed by UV–vis spectroscopy since these forms have different absorption spectra [50,51]. Also in the case of our compounds the presence of dimers can be observed (for P2-Zn0 see Ref. [9], and for amino AzaPc e.g. Figs. 5 and 6). A good resolution of the second maximum of the *Q*-band around 595 nm and the lack of increase of the extinction coefficient at the main maximum of the *Q*-band upon dilution usually indicate the presence of only monomeric species. Moreover, except for few cases, dimers do not fluoresce and do not produce $^1O_2$. Therefore, the fact that absorption and fluorescence excitation spectra are identical can be considered as a strong indication for the dye being present only in the monomeric form [52]. Both above mentioned conditions were fulfilled in the case of all Φ_F and Φ_Δ measurements and no classical dimers were observed. The decrease of Φ_F and Φ_Δ with increasing amount of amino substituents in anhydrous DMF and in pyridine (both known as good monomerizing solvents) could be attributed to some deactivating effect of these groups. However, the results obtained in the DMF/water/HCl system (HCl was added to insure complete protonation of all amino groups) support the following explanation. Some interactions between the molecules of amino AzaPc (free base) in DMF or pyridine could take place leading to aggregates like dimers, but with no or little UV–vis spectra changes. In fact, the importance of spectral modification upon dimerization strongly depends on the respective orientation of the transition dipoles on each moiety of the dimer and on the strength of their coupling [53]. Therefore, although the spectra in DMF and DMF/water/HCl showed monomeric character and the same extinction coefficients (only a small solvatochromic shift was observed (Fig. 7)) some interactions cannot be excluded, which would account for decrease in Φ_F and Φ_Δ values.

These interactions would not exist for P2-Zn0 that presents $\Phi_\Delta = 0.67$ and $\Phi_F = 0.23$ corresponding to the values of the monomer molecule. The aggregation is here efficiently inhib-

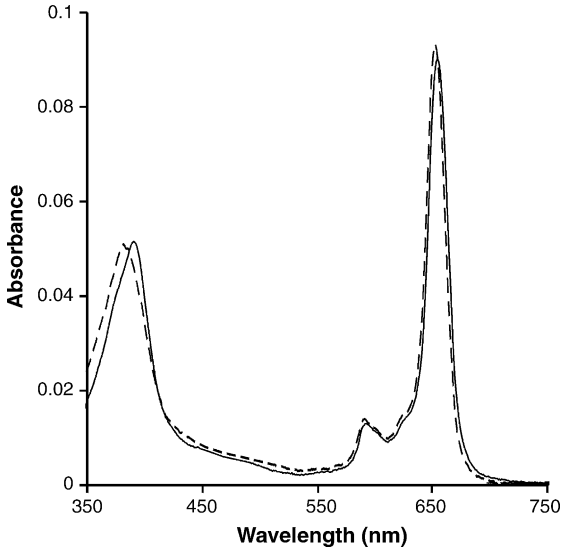


Fig. 7. UV–vis absorption spectra of P2-Zn2A·HCl of the same concentration (approximately 5×10^{-7} M) in anhydrous DMF (dashed line) and in DMF/water/HCl (solid line).

ited by the bulkiness of the eight *tert*-butylsulfanyl substituents. The diethylaminoethylsulfanyl substituent is more flexible and the bulky tertiary amino group has more chance to be deflected from the macrocycle plane allowing the AzaPc molecules to get closer. The monomerization in organic solvents is therefore ensured mainly by the *tert*-butylsulfanyl substituents and the contribution of the diethylaminoethylsulfanyl substituent to monomerization is less. The effect of increasing the number of the diethylaminoethylsulfanyl substituents agrees with view if we consider non-aqueous solvents. The hydrochlorides of amino AzaPc will behave in the same way as the free amino groups. Indeed, the amino groups will be associated with HCl to produce an ion pairs with no global charge. Therefore no electrostatic repulsion forces will occur. In addition, since these complexes are organic salts they are expected to be less soluble in DMF and the aggregation forces will be stronger. As a consequence, the hydrochlorides of AzaPc will have lower Φ_F and Φ_Δ than the corresponding free bases. This effect is further illustrated in the case of P2-Zn4·HCl that slowly (within weeks) aggregates and produces typical dimeric UV–vis spectra in DMF stock solution while the free base P2-Zn4 in DMF stock solution remains monomeric. Also a weak heavy atom effect of the chlorine atom cannot be completely ruled out since the chlorine is associated closely with the AzaPc molecule.

The presence of some amount of water (in our case 5%) will lead to solvation of the Cl^- and NH^+R_3 ions that will dissociate. The repulsion forces associated with the release of the cationic charge on the protonated amino group would permit disaggregation. Moreover, the ionized tertiary amino groups will increase their volume through solvation by water molecules, which will favor the separation of the dye molecules in the solution. All molecules will stay in monomeric form now and the Φ_F and Φ_Δ values will correspond to purely monomeric P2-Zn0. In the case of P2-Zn4·HCl the values Φ_F and Φ_Δ did not reach the maximum values measured with P2-Zn0. At this moment we have no explanation for lower values Φ_F of completely separated protonated amino AzaPc (P2-Zn1·HCl, P2-Zn2O·HCl, P2-Zn2A·HCl) in DMF/water/HCl when compared to P2-Zn0. However, it seems that the value $\Phi_F = 0.13$ could be the maximum for these compounds since all of them reached the same value.

As pointed out by one of the reviewers, another explanation must be considered too. Amines are well known as quenchers of singlet and triplet excited state [54]. If so happened in this case, P2-Zn0 should exhibit the largest values while the quantum yields should be decreased with increasing number of amino groups. Photoinduced electron transfer (PET) causing the quenching of excited states is disabled in the case when the amine nitrogen is protonated and no lone pair is available for PET [55]. This would explain restoration of the quantum yield values in DMF/water/HCl. However, this explanation would also require that the hydrochloride form of AzaPc would dissociate in anhydrous DMF that appears unlikely although this possibility cannot be entirely excluded.

In pure water solution, the presence of charged solvated amino groups appears to be the main reason for monomerization of AzaPc molecules. As suggested by our results, the presence of four cationic charges lead to good solubility in water medium but does not impede dimerization. When compared to other results obtained with cationic Pc, the distance of the cationic charge from the macrocyclic system appears to be important. In our case, the flexible aliphatic chain made of three atoms allows deflection from the plane. Similar results were found for tetracationic Pc with the charge attached through a two carbon chain [56]. In this case also, a large proportion of dimer was found in water solution. Besides, when the charge is a part of the macrocycle (alkylated tetrapyridoporphyrazines), its effect is much stronger and four charges are enough for complete monomerization while three charges are not sufficient [28]. So, in the case of flexible charged side chains, more of them are necessary (at least eight or even more with our compounds). Contrary to the results with cationic compounds where the monomerization in water seems to follow a linear dependence with an increasing number of charges, sulphonated Pc showed a different behavior. In this case monomerization depends more on the symmetry of these anionic compounds than on the number of negative charges [57].

5. Conclusion

We have synthesized several new AzaPc bearing cationic charges in solution. Their Φ_Δ depends on the complete monomerization of the molecules in the solvent. The protonation and solvation of the amino group by water strongly influence the aggregation state and increase the Φ_Δ and Φ_F values. The protonation of the tertiary amino groups in the water solution using HCl is not quantitative and depends on the pH of the solution. The monomerization of the AzaPc molecules in water medium is very sensitive to the amount of protonated amino groups in the molecule, reaching almost complete separation with eight charges. However, as suggested by the slightly lower Φ_Δ and Φ_F values, even this amount of charged substituents is not sufficient to insure full separation of the AzaPc molecules.

These molecules appear to have interesting potential with regards to biological applications. In this case, the monomerization will be improved by interaction of lipophilic part of the AzaPc molecules with lipidic bilayer of biological membranes. The amount of cationic charges present in the molecules might target the photosensitizer to subcellular structures such as mitochondria or nucleus. Preliminary results on cells showed about three times higher phototoxicity of P2-Zn4·HCl as compared to P2-Zn0, which encourages us to further investigate this class of compounds.

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