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Synthesis of mono-, di-, tri- and tetracarboxy azaphthalocyanines as potential dark quenchers

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ABSTRACT

Mono-, di-, tri- and tetracarboxy-substituted metal-free azaphthalocyanines (AzaPc) were synthesized from 5,6-bis(diethylamino)pyrazine-2,3-dicarbonitrile and 6-(5,6-dicyano-3-(diethylamino)pyrazin-2-ylamino)hexanoic acid using a statistical condensation approach. AzaPc bearing eight diethylamino peripheral substituents was also isolated from the mixture. Analysis of the distribution of congeners in the statistical mixture using optimized HPLC method (Phenomenex Synergy RP Fusion column, acetonitrile/tetrahydrofuran/water (pH 5.5) 50:20:30) was performed. The analysis showed optimal ratios of starting materials to be 3:1 for AAAB, 1:3 for ABBB and 1:1 for AABB/ABAB types of the congeners. The distribution of the congeners corresponded well with calculated values indicating similar reactivity of both starting materials and no sterical constraint between adjacent isoindole units in the AzaPc ring. All investigated AzaPc showed no fluorescence, extremely low singlet oxygen quantum yields ($\Phi_\Delta < 0.005$) in monomeric form and strong absorption in a wide range from 300 nm to almost 700 nm. Such properties are highly promising for future investigation of these compounds as dark quenchers of fluorescence in DNA hybridization probes.

1. Introduction

Azaphthalocyanines (AzaPc) are aza-analogues of the well known and widely-investigated synthetic dyes, the phthalocyanines (Pc). The large, planar macrocyclic system of these dyes is a reason for interesting properties utilized in several applications connected either with light absorption, electron transfer or oxidation and reduction. Consequently AzaPc were recently investigated as promising non-linear optical limiting dyes [1,2] or as potential photosensitizers in photodynamic therapy [3–5]. Their fluorescence [6,7], acid-base [8,9], electron-deficient [10–13], aggregating and non-aggregating [14,15] and microporous [16] properties have also been reported. Our recent efforts have been aimed at the investigation of AzaPc dyes in a completely new application. When suitably substituted, they are able to quench the fluorescence of different fluorophores in DNA hybridization probes and they were shown to be highly efficient as modern type of non-emitting quenchers, so called "dark quenchers" [17]. The use of DNA-hybridization probes that generate fluorescence only when they bind to their targets enables real-time monitoring of the polymerase chain reaction and is widely used in genetic analysis [18]. The method is based on presence of two dyes – a fluorophore and a quencher, the latter is able to quench fluorescence of the former when they are placed close to each other in space. A broad absorption spectrum, no intrinsic fluorescence and very low singlet oxygen production in the monomeric form are highly desirable for quenchers in this application. These conditions are met for (dialkylamino)- substituted AzaPc. Besides typical B- and Q-bands in the regions of 360 nm and 650 nm, respectively, they also absorb strongly around 500 nm due to the participation of the amine lone pairs in conjugation with the AzaPc core. Moreover, excited states of (dialkylamino)-AzaPc are efficiently deactivated by ultrafast intramolecular charge transfer (ICT) from the lone pairs of the peripheral dialkylamino groups (donors) to the macrocyclic system (acceptor) [19]. As a consequence of ICT, no fluorescence occurs and the singlet oxygen quantum yield is extremely low.

The first AzaPc that were investigated as dark quenchers in DNA hybridization probes contained only one functional group, through

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which they were attached to an appropriate oligonucleotide [17]. However, other approaches in hybridization assays may require more modifiable sites to be present on the AzaPc macrocycle in order to allow binding of two or more oligonucleotide chains or other modifiers. The present work is focused on the synthesis, isolation and determination of photophysical and photochemical properties of (dialkylamino)-substituted AzaPc bearing one to four carboxyls as suitable functional groups.

2. Experimental section

2.1. Chemicals and instruments for synthesis

All organic solvents used for synthesis were of analytical grade. Anhydrous butanol was stored over magnesium and distilled prior to use. All necessary chemicals were purchased from established suppliers (Merck, Sigma–Aldrich, Acros) and used as received. TLC was performed on Silica gel 60 F254 plates (Merck, Darmstadt). Merck Kieselgel 60 (0.040–0.063 nm) was used for preparative column chromatography. Infrared spectra were measured in KBr pellets on IR-Spectrometer Nicolet Impact 400. ^{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). Chemical shifts reported are given relative to tetramethylsilane and were locked to the solvent signal. UV–vis spectra were recorded on spectrophotometer UV–2401PC, Shimadzu Europa. Mass spectra were measured on a Quattro MicroTM API (Waters, Milford, Massachusetts, USA) in positive electrospray mode (ESI-MS). Solutions for ESI MS were prepared in methanol and formic acid was added before measurements to support ionization. Adducts with Na^{+}, K^{+} and formic acid were always found in the mass spectra of the dyes as minor peaks. Compounds **1** [20] and **2** [21] were prepared in our laboratory according to published procedures.

2.2. General procedure of synthesis of AzaPc (**3**–**7**)

A solution of **1** (363 mg, 1.33 mmol) and **2** (181 mg, 1.33 mmol) in dry butanol (20 cm^3) was heated to reflux and metal lithium (130 mg, 18.5 mmol) was added. The mixture was heated under reflux for next 3 h. The solvent was evaporated under reduced pressure, aq. acetic acid (50% v/v, 100 cm^3) was added and the mixture stirred at r.t. for 30 min. The resulting precipitate was filtered, washed thoroughly with water and dried. The crude mixture of AzaPc was purified by column chromatography using gradient elution starting from chloroform/acetone/methanol 10:1:1 as the first eluent to separate crude **3** and **4** as first two intense purple fractions. After compound **4** was eluted from the column the eluent was changed to more polar chloroform/acetone/methanol 10:3:3 and AzaPc **5** and **6** were collected as the next purple fractions. Compound **7** was eluted from the column using methanol as eluent. Such crude congeners were purified by column chromatography at least once more (usually twice), using the eluents mentioned below.

2.2.1. 2,3,9,10,16,17,23,24-Octakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza)phthalocyanine (**3**)

This fraction was further purified by column chromatography with ethyl-acetate/hexane 6:1. Isolated fractions containing the product were evaporated, dissolved in a minimal amount of dichloromethane and precipitated in methanol. The fine suspension was collected and dried to give 10 mg of dark purple solid (1.3%). R_f values and all analytical data corresponded to the compound prepared previously in our laboratory [9].

2.2.2. 2-(5-Carboxypentylamino)-3,9,10,16,17,23,24-heptakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza) phthalocyanine (**4**)

This fraction was purified by column chromatography with eluent hexane/ethyl-acetate/acetic acid 6:6:1. Isolated fractions containing the product were evaporated, dissolved in a minimal amount of dichloromethane and precipitated in hexane. The fine suspension was collected and dried to give pure congener **4**. Dark purple solid (25 mg, 3.1%); UV–vis (THF): λ_{max}, nm (ε) 678 (90 500), 647 (67 100), 618sh, 592sh, 508 (56 000), 364(103 000); IR (KBr): ν, cm^{-1} 2969, 2930, 2871, 1726 (CO), 1640, ^{1}H NMR (300 MHz, [D$_5$] pyridine): δ_H, ppm 13.75 (2H, br s, central NH), 7.50–7.39 (1H, m, NH), 4.15 (2H, q, J = 6.4 Hz, CH_2–NH), 4.00–3.71 (24H, m, CH$_2$–N), 3.64 (4H, q, J = 7.0 Hz, CH$_2$–N), 2.68 (2H, t, J = 7.1 Hz, CH_2–COOH), 2.12–1.91 (4H, m, CH$_2$), 1.86–1.69 (2H, m, CH$_2$), 1.26–1.04 (42H, m, CH$_3$). ^{13}C NMR (75 MHz, [D$_5$]pyridine): δ_C, ppm 176.0, 152.3, 150.6, 144.7, 141.7, 140.9, 140.8, 137.1, 44.5, 43.0, 42.8, 34.9, 29.6, 27.4, 25.5, 13.1, 12.9. ESI-MS: m/z 1150 (calcd. for [M + H]$^+$ 1150).

2.2.3. 2,9-Bis(5-carboxypentylamino)-3,10,16,17,23,24-hexakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza)phthalocyanine (**5**)

This compound was purified by column chromatography with hexane/ethyl-acetate/acetic acid 2:20:1. Isolated fractions containing the product were evaporated, dissolved in a minimal amount of dichloromethane and precipitated in hexane. The fine suspension was collected and dried to give pure congener **5**. This fraction was obtained as a mixture of positional isomers. Dark purple solid (35 mg, 4.4%); UV–vis (THF): λ_{max}, nm (ε) 677 (90 500), 646 (67 100), 618sh, 591sh, 499 (56 000), 363 (103 000); IR (KBr): ν, cm^{-1} 2966, 2931, 2869, 1724 (CO), 1640. ^{1}H NMR (300 MHz, [D$_5$]pyridine): δ_H, ppm 13.74 (2H, br s, central NH), 7.53–7.28 (2H, m, NH), 4.25–4.03 (4H, m, CH_2–NH), 3.99–3.76 (16H, m, CH$_2$–N), 3.62 (8H, q, J = 7.0 Hz, CH$_2$–N), 2.68 (4H, t, J = 4.6 Hz, CH_2–COOH), 2.12–1.90 (8H, m, CH$_2$), 1.90–1.69 (4H, m, CH$_2$), 1.34–1.07 (36H, m, CH$_3$). ^{13}C NMR (75 MHz, [D$_5$]pyridine): δ_C, ppm 176.1, 152.2, 150.7, 145.1, 142.0, 141.3, 136.2, 44.5, 43.0, 42.1, 35.0, 29.6, 27.4, 25.6, 13.1, 13.0. ESI-MS: m/z 1209 (calcd. for [M + H]$^+$ 1209).

2.2.4. 2,9,16-Tris(5-carboxypentylamino)-3,10,17,23,24-pentakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza) phthalocyanine (**6**)

This compound was purified by column chromatography with ethyl-acetate/acetic acid 20:1. Isolated fractions containing the product were evaporated, dissolved in a minimal amount of acetone and precipitated in diethylether. The fine suspension was collected and dried to give pure congener **6**. This fraction was obtained as a mixture of positional isomers. Dark purple solid (20 mg, 2.5%); UV–vis (MetOH): λ_{max}, nm (ε) = 681 (90 500), 651 (67 100), 618sh, 511 (56 000), 362 (103 000). IR (KBr): ν, cm^{-1} = 2965, 2930, 2868, 1734 (CO), 1637, ^{1}H NMR (300 MHz, [D$_5$]pyridine): δ_H, ppm 13.70 (2H, br s, central NH), 7.49–7.25 (3H, m, NH), 4.20–3.98 (6H, m, CH_2–NH), 3.94–3.75 (8H; m, CH$_2$–N), 3.69–3.47 (12H, m, CH$_2$–N), 2.73–2.59 (6H, m, CH_2–COOH), 2.10–1.87 (12H, m, CH$_2$), 1.86–1.64 (6H, m, CH$_2$), 1.23–1.05 (30H, m, CH$_3$). ^{13}C NMR (75 MHz, [D$_5$]pyridine): δ_C, ppm 176.2, 152.3, 150.6, 122.8, 44.5, 42.9, 42.1, 35.1, 29.6, 27.4, 25.7, 25.6, 13.1, 13.0. ESI-MS: m/z 1267 (calcd. for [M + H]$^+$ 1267).

2.2.5. 2,9,16,23-Tetrakis(5-carboxypentylamino)-3,10,17,24-tetrakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza)phthalocyanine (**7**)

This fraction was further purified by column chromatography with ethyl-acetate/acetic acid 5:1. Isolated fractions containing the product were evaporated, dissolved in a minimal amount of acetone and precipitated in diethylether. The fine suspension was

collected and dried to give pure congener **7** in yield of 15 mg of dark purple solid (1.9%). All analytical data corresponded to the compound prepared previously in our laboratory [21]. This fraction was obtained as a mixture of positional isomers.

2.3. HPLC

A Shimadzu system LC-20AD Prominence with SPD M20A diode array detector (Shimadzu, Japan) was used to perform all of the analyses. Acetic acid p.a. and triethylamine p.a were obtained from Merck (Darmstadt, Germany). Acetonitrile and tetrahydrofuran gradient-grade were obtained from Sigma-Aldrich (Prague, Czech Republic). HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it meets European Pharmacopoeia requirements. Chromatographic column Phenomenex Synergy RP Fusion with polar embedded groups, 75 × 3 mm, particle size 4 μm, (Phenomenex, USA) was used for final chromatographic analysis.

2.3.1. Sample preparation for HPLC analysis

The weighed pure standard amounts of all compounds (**3**–**7**) from 0.75 mg to 1.10 mg were dissolved together in 2 ml of a tetrahydrofuran – methanol (1:1, v/v) mixture. These solutions were diluted in ratio 1:1 and 1:10 to the same mixture. These mixtures were analyzed and calibration graphs prepared for each congener. The calibration graphs involved 3 experimental points prepared from the above mentioned solutions (stock solution, dilution 1:1, dilution 1:10). Linearity was established for each compound. Each concentration was injected in duplicate and the mean value of peak area (measured at 510 nm) was taken for the calibration curve.

2.3.2. Samples preparation for distribution analysis

Total amount of 0.2 mmol of precursors **1** and **2** in ratios 1:6; 1:3; 1:2; 2:3; 1:1; 3:2; 2:1; 3:1; 6:1 (total amount of pyrazinedicarbonitriles remained the same, the only change was in precursor ratio) was dissolved in 5 cm^3 of dry butanol, heated to reflux and lithium (1.4 mmol, 10 mg) was added. The reaction mixture was heated for 3 h, the solvent was evaporated under reduced pressure, aqueous acetic acid (50% v/v, 50 cm^3) was added for 30 min and the suspension was filtered. The resulting waxy purple solid was then washed with water (approximately 200–300 cm^3) until the filtrate was colorless. The solid material was dried and transferred quantitatively into a glass vial. All samples were dissolved in a methanol-tetrahydrofuran (1:1, v/v) mixture (6 cm^3) to obtain samples for HPLC analysis. A volume of 10 μl of sample solution was analyzed by the HPLC system. The synthesis and analysis was performed twice for each ratio of precursors. The molar concentration of each congener in the mixture was calculated from the peak area in HPLC (510 nm) using calibration curve. The percentage amount of each congener was calculated considering a sum of the molar concentrations of all congeners as 100%.

2.4. Singlet oxygen and fluorescence measurements

Singlet oxygen quantum yields were determined in DMF according to previously published procedure using decomposition of a chemical trap of singlet oxygen 1,3-diphenylisobenzofuran (DPBF) [22]. Absorption of the dyes in the Q-band region during measurements was approximately 0.1. Zinc phthalocyanine (ZnPc, from Sigma–Aldrich) was used as the reference ($\Phi_\Delta = 0.56$ in DMF [23,24]).

Fluorescence quantum yields were determined in DMF by comparative method [25] using ZnPc as reference ($\Phi_F = 0.30$ in chloronaphthalene [26]). Absorption of the dyes in the Q-band maximum was approximately 0.05 in order to avoid an inner filter effect. The samples were excited at two wavelengths – 366 nm and 610 nm. All measurements (both singlet oxygen and fluorescence) were performed three times and the presented data represent the mean of these three experiments. Because of solubility reasons compound **7** was dissolved in MeOH and appropriately diluted with DMF. Both singlet oxygen and fluorescence quantum yields of compound **7** were then determined in DMF containing maximum 1% of MeOH (v/v).

3. Results and discussion

3.1. Synthesis

The choice of peripheral substituents of the final AzaPc was reached following rational considerations. The AzaPc macrocycle must be substituted with dialkylamino substituents in order to undergo efficiently the ICT responsible for the absence of fluorescence and low singlet oxygen production [19]. Bulky peripheral substituents (e.g., diethylamino) are expected to reduce the typical aggregation of the AzaPc planar macrocyclic system and to allow the simple purification and determination of its photophysical and photochemical properties in the monomeric form. Carboxy groups should be attached via a spacer because a longer distance to the macrocyclic core should allow future modifications by avoiding steric constraints in close proximity to the macrocycle. Given the abovementioned requirements, diethylamino and 5-carboxypentylamino substituents were chosen and compounds **1** and **2** (Fig. 1) prepared as starting materials.

Syntheses of unsymmetrical Pc and AzaPc are not as simple as in the case of symmetrically substituted ones and they often require specific approaches [27,28]. A polymer-support method [29,30] and the subphthalocyanine method [31] were used to prepare the AAAB type selectively, and selective strategies for AABB [32–34] and ABAB [32,35,36] type derivatives were also developed. However, the most widespread and the simplest method for the synthesis of all derivatives in a one–pot reaction is a statistical condensation of two different precursors A and B [37–39]. This approach gives rise to six different AzaPc molecules (AAAA, AAAB, ABAB, AABB, ABBB and BBBB) that may be called “congeners” due to the similarity of the compounds and the same origin [40].

In our case, statistical condensation of **1** and **2** in a molar ratio 1:1 in butanol with lithium butoxide gave the mixture of AzaPc **3**–**7** (Fig. 1) that were separated chromatographically on a preparative silica column. The different lipophilicities of precursors **1** and **2** facilitated the separation, because the resulting macrocycles had different silica-binding properties and, as a result, different R_f values. The R_f values in chloroform/acetone/methanol 10:1:1 were 0.98, 0.8, 0.4–0.6 (three overlapping spots), 0.13, 0.01 for **3**, **4**, **5**, **6** and **7**, respectively. Since the precursor **2** is unsymmetrically substituted, it gave rise to positional isomers in some of the fractions – one single compound for **3** and **4**, five isomers for **5** (three for adjacent, two for opposite type), and four isomers for **6** and **7**. Unfortunately, the differences between the isomers were too small even to be detected on TLC or HPLC. The only exception was compound **5** where three isomers were detected on TLC and also two on HPLC (see below) but their separation was not successful.

The yields of pure fractions were in the range 1.3–4.4%, and the overall yield of transformation of pyrazinedicarbonitriles to AzaPc macrocycles was around 13%. Assignment of the fractions to each compound was based on their mass spectra and on the number of aliphatic hydrogens in ^1H NMR spectra. Also IR spectra showed more intense bands of C=O stretch vibrations at approximately 1726 cm^{-1} for compounds bearing more carboxy groups. The first

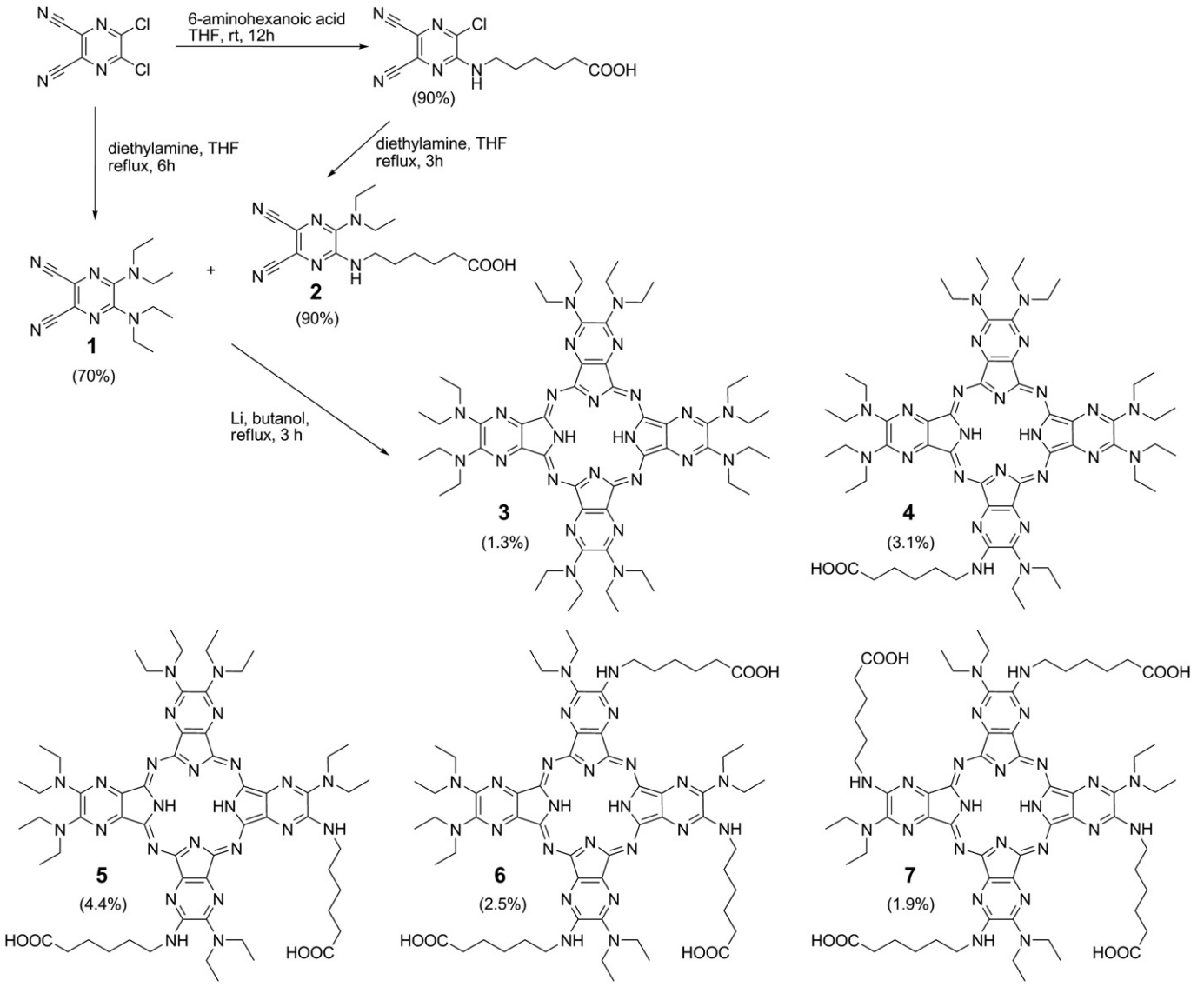

Fig. 1. Scheme of the synthetic pathway to AzaPc **3**–**7**. Only one isomer is shown for each AzaPc.

and the last eluted fractions (corresponding to **3** and **7**, respectively) were also compared with the compounds prepared by simple tetramerization of only one precursor **1** or **2**. The R_f values were in perfect agreement in all mobile phases tested.

3.2. Optimization of the yield of different congeners using HPLC

The 1:1 ratio of the starting compounds **1** and **2** allowed isolation of all congeners in reasonable amounts for analyses and determination of photophysical and photochemical properties. A percentage distribution, however, changes with the changing ratio of the starting materials. For this reason we investigated how the changes in the precursors' ratios influenced the percentage distribution of the congeners in order to optimize yield of each AzaPc. For this reason, an HPLC method for the analysis of the mixture of AzaPc **3**–**7** was developed and used for the studies.

HPLC method optimization was focused both on separation and quantitative determination. Six HPLC columns with different polarities were tested (monolithic column Chromolith Performance RP-18, 100 × 4.6 mm, column Supelco Discovery C-8, 125 × 3 mm, particle size 5 μm, column Zorbax Eclipse SB–CN 150 × 4.6 mm, particle size 5 μm, column Zorbax Eclipse SB-Phenyl 75 × 4.6 mm, particle size 3.5 μm, column Zorbax TMS 250 × 4.6 mm, particle size 5 μm and column Phenomenex Synergy RP Fusion with polar embedded groups, 75 × 3 mm, particle size 4 μm). The strategy of HPLC conditions optimization was to achieve a good peak resolution among the compounds of interest and relatively short time of analysis without gradient elution. Mobile phases used for method development contained mixtures of acetonitrile, methanol or tetrahydrofuran with water or buffer. The retention behavior of the analytes on the stationary phase was quite different and corresponded well with the structure of compounds. Compound **7** with four carboxy groups in the molecule showed very short retention times while compound **3** without the polar substitution showed strong retention on reversed-phase columns. Separations on C-8 and C-18 columns resulted in poor retention of compound **6** and **7** and the retention of compound **3** prolonged the time of analysis to more than 25 min. Moreover, low peak symmetry was observed during the elution of compounds **3** and **4**. Changes in the mobile phases' composition did not lead to satisfactory results. Columns containing reversed phases with higher polarity – Zorbax Eclipse silica "stable bond" modified with phenyl (SB-Phe), nitrile (SB–CN) or trimethylsilyl silica (TMS) were tested in

the next steps. The mobile phases were mixtures of acetonitrile with water in different ratios. Compounds **6** and **7** showed better retention and separation than on C-18 columns and, moreover, retention of compound **3** was drastically decreased and it was eluted in 6–7 min using the SB–CN column. On the other hand, it was not possible to use this chromatographic system due to strong peak tailing of all compounds and the low peak symmetry of all compounds disabled a precise quantification of the compounds in the mixture. The last tested column was a reversed-phase (C-18) Phenomenex Synergy RP Fusion with polar embedded groups inside the stationary phase. This unique mixed-mode stationary phase containing reverse phase chains with polar groups showed balanced retention of both hydrophobic and hydrophilic compounds. Different mobile phases were optimized with column Phenomenex Synergy RP Fusion and the influence of pH on separation was tested. The optimal conditions for successful separation were found to include a mobile phase containing acetonitrile/tetrahydrofuran/water in ratio 50:20:30 with the pH of the water phase adjusted to value 5.5 with help of 0.1 M acetic acid and triethylamine. The flow rate of the mobile phase was 1.0 ml min^{-1}, and chromatographic analysis was performed at ambient temperature. A volume of 10 μl of sample solution was analyzed by HPLC. Under the optimal conditions the retention time of the last compound (**3**) was 13.6 min and all compounds were well separated (Fig. 2). The retention times of compounds **3**, **4**, **5**, **6** and **7** were 13.6, 5.9, 2.9, 1.5 and 0.9 min, respectively. The resolution of all separated peaks higher than 1.5 and high peak symmetry enabled precise quantification of the synthesized compounds. A perfect linearity of calibration curves was obtained for each congener.

For the congeners' distribution analysis, several reactions were performed under the same conditions but with different molar ratios of compounds **1** and **2** (see Section 2.3.2) and the mixtures were analyzed by HPLC. No side-products from the reaction were eluted in the retention times of AzaPc congeners on HPLC. This was confirmed by comparing the absorption spectra of the chromatogram peaks of the pure standards with the analyzed mixtures (see Supporting Information). Furthermore, the chromatograms were analyzed at 510 nm where only the AzaPc macrocycle is expected to absorb.

The obtained results are summarized and best viewed in Fig. 3. Obviously, the yield of AAAB type (compound **4**) increased in the range 0–25% of **2** in the mixture. The maximum was reached at a 3:1 ratio of starting materials (**1**:**2**) and then slowly decreased in favor of other derivatives. A similar conclusion was made also for the reverse ABBB type (compound **6**). The highest yield of the A_2B_2 (**5**) type compound was found at approximately 1:1 ratio. All these results were in good agreement with theoretical predictions of the

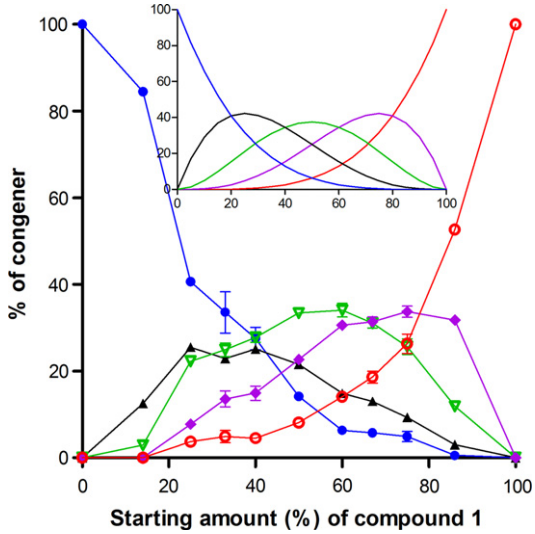

Fig. 3. Percentage distribution of congeners in the mixture after statistical condensation in dependence on the ratio of starting materials **1** and **2** (expressed as amount of **1** in the mixture). Compound **3** (○), **4** (◆) **5** (▽), **6** (▲), **7** (●). Inset: theoretical calculation of the distribution.

congeners' yield if no other effect influences the distribution (Fig. 3 – inset). In some cases, the percentage distribution may not follow the theoretical calculations. The results will differ when one or both precursors contain substituents causing steric hindrance between two adjacent units of the final macrocycle. The formation of some of the congeners is then unfavorable and their yield is decreased [41–43]. Also in the case of differing reactivities of both precursors, some of the congeners are reported to appear in the mixture in lower yields [44,45]. Results from the abovementioned distribution analysis of compounds **3**–**7** may lead to the conclusion that the reactivities of both precursors **1** and **2** are very similar and that no steric constraint occurs between two adjacent units in the final AzaPc macrocycle.

3.3. UV–vis absorption spectra

All congeners showed almost identical spectra with high energy B-bands around 363–370 nm, low energy Q-bands (split) around 653–684 nm and a band around 510–529 nm attributed to n–π* transitions from the lone pairs of peripheral amino groups (Fig. 4). The Q-bands of all AzaPc were split due to the lower symmetry

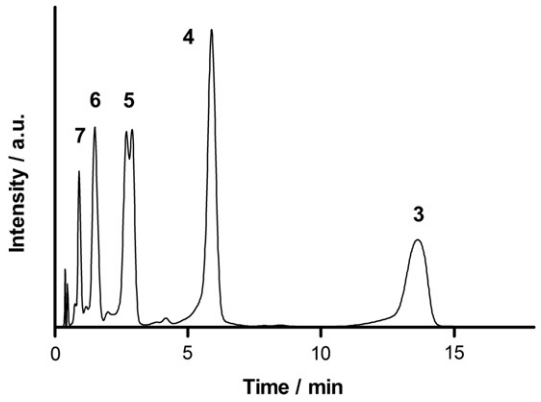

Fig. 2. HPLC chromatogram of compounds **3**–**7** under the optimal conditions (see text). Measured at $\lambda = 510$ nm.

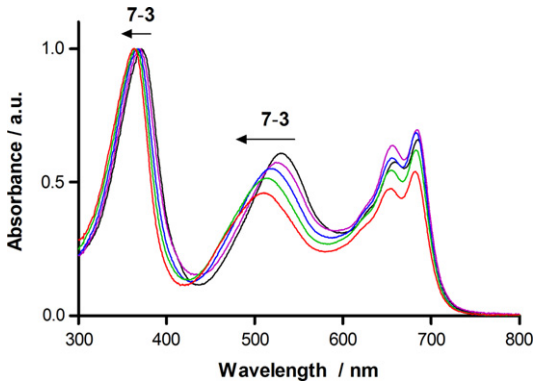

Fig. 4. Absorption spectra of AzaPc **3**–**7** in DMF. Spectra were normalized to the same absorption in B-band. Spectrum of compound **7** was taken with 1% of MeOH as soon as possible after mixing the MeOH stock solution with DMF.

(D_{2h}) caused by the presence of two central hydrogens. The symmetry of the macrocycle was not lowered further by differences in peripheral substitution because the electronic effects of both substituents were almost the same. It is well documented on the positions of the Q-band and B-band, that these were influenced only slightly by peripheral substitution. They shifted bathochromically by 3 nm and 7 nm (in DMF), respectively, with increasing number of peripheral tertiary amino groups in a series from **7** to **3** (Table 1, Fig. 4). Interestingly, the n–π* transition band shifted markedly (+19 nm) depending on the number of secondary and tertiary amines on the periphery of the AzaPc. This might be explained in terms of the high sensitivity of this band to the electron density of the lone pair of the amino substituent that was influenced by the electron-donating effect of the alkyl groups. The n–π* transition band is of particular significance for AzaPc dark quenchers because it extends absorption from the Q-band to lower wavelengths where a range of important fluorophores emits (e.g., fluorescein, λ_{em} = 517 nm). Unsubstituted or aryl, heteroaryl, alkylsufanyl or alkyloxy substituted AzaPc absorb only weakly in this region [5,15,46]. Efficient overlap of the absorption spectrum of the quencher and the emission spectrum of the fluorophore is crucial for the resonance energy transfer responsible for dynamic quenching in DNA hybridization probes [47].

Aggregation generally causes changes in the shapes of UV–vis spectra [48], in particular lowering the extinction coefficients in the Q-band at high concentrations. No changes in the absorption spectra were observed at different concentrations up to 50 μM that was the maximum concentration used during the experiments. Both the shapes of spectra and their extinction coefficients remained the same and obeyed the Lambert–Beer law within the range of concentration 0.1–50 μM. This fact confirmed the starting hypothesis that the diethylamino substituents efficiently inhibit the aggregation.

The presence of both basic (diethylamino) and acidic (carboxy) substituents in the AzaPc may theoretically lead to the appearance of inter- or intramolecular bridges based on formation of H-bonds. However, no spectral evidence of this behavior was observed during the study as a consequence of the extremely low basicity of the diethylamino substituents attached to the pyrazine ring (e.g. compound **1**, $pK_a = -0.75 \pm 0.10$, calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02).

It is known that metal-free AzaPc and Pc may form so called "proton-transfer complexes" when dissolved in basic organic solvents (pyridine, DMF, DMSO, amines) [49]. The proton-transfer complex changes the symmetry of the molecule from D_{2h} to D_{4h}, leading to unsplit Q-bands [8,9,50]. Formation of the proton-transfer complex depends on the basicity of the solvent and the acidity of the central NH of the AzaPc macrocycle. Only small changes that may be attributed to such behavior were observed for the investigated compounds in DMF. This is due to the very low basicity of the solvent as well as the decreased acidity of the central NH of the tested AzaPc due to the strong electron-donating effect of the peripheral substituents. Such changes were negligible and insignificant in the studies of AzaPc **3**–**6**. However, some formation of the proton-transfer complex was observed for compound **7** over time (see Supporting Information). For this reason the spectral, photophysical and photochemical data for **7** were collected as soon as possible after mixing the MeOH stock solution with DMF. Despite this, some parameters of **7** were most likely influenced by a small amount of the proton-transfer complex present at the moment of measurement (see also Section 3.4.).

Table 1
Spectral, photophysical and photochemical properties of studied AzaPc in DMF.

Compound	λ_{max} (nm) Q-band	λ_{max} (nm) n–π* transtition band	λ_{max} (nm) B-band	Φ_Δ[b]	Φ_F[b]
3	684, 657	529	370	0.0009	0
4	683, 657	522	369	0.0015	0
5	683, 656	517	368	0.0014	0
6	682, 654	514	364	0.0015	0
7[a]	681, 653	510	363	0.0043	0.005/0[c]

[a] Compound **7** measured in DMF with 1% of MeOH (v/v). Data were collected as soon as possible after mixing the solutions.
[b] Mean of three independent measurements, estimated error ± 25%.
[c] Measured in MeOH.

3.4. Fluorescence and singlet oxygen

AzaPc as well as Pc are well known to produce singlet oxygen efficiently and to exhibit fluorescence [51]. However, both these properties – highly appreciated in different applications (e.g., in photodynamic therapy [4]) – should be as low as possible in dark quenchers. Singlet oxygen is a highly reactive species and may destroy oligonucleotide probes [52], while intrinsic fluorescence of the quencher may interfere with the fluorescence of a reporter. For this reason, singlet oxygen (Φ_Δ) and fluorescence (Φ_F) quantum yields were determined in order to confirm the potential of these compounds as dark quenchers. DMF was chosen as suitable solvent for determination of photophysical and photochemical parameters because it was the only solvent in which all tested AzaPc showed reasonable solubility. Only in the case of **7**, the stock solution was prepared in MeOH and diluted with DMF to a maximum final MeOH concentration of 1% (v/v). This concentration was believed not to change the physical quenching of singlet oxygen by the solvent. Thus, the Φ_Δ value of 0.56 for the reference compound, ZnPc, in DMF [23] was also used in calculations for compound **7**.

Compounds **3**–**7** showed extremely low Φ_Δ values typically below 0.005 (Table 1). In comparison, fluorescein, a commonly-used dye in DNA hybridization assays, is reported to have Φ_Δ values in a range 0.03–0.10 [53]. Concerning fluorescence, no signal was detected for AzaPc **3**–**6**. Excitation at two wavelengths, both B- and Q-band, was tested. Compound **7** exhibited a very weak fluorescence signal ($\Phi_F = 0.005$) with an emission maximum at 656 nm. We assume that the signal originated from small amount of proton-transfer complex because the emission maximum is located at lower wavelengths than the absorption maximum of the metal-free form (Supporting Information). This would lead to negative Stokes shift that is not possible. This assumption is further supported by measurements in MeOH where no proton-transfer occurs and no fluorescence signal was detected. It must be noted that all measurements (fluorescence and singlet oxygen) were performed with AzaPc in monomeric form (see also Section 3.3 and Fig. 4). This means that aggregation may be excluded and ICT is the most likely relaxation pathway for the excited states. As a conclusion, the photochemical and photophysical properties of the tested AzaPc are highly suitable for efficient dark-quenching of fluorescence. The small fluorescence signal that was detected for **7** at DMF does not represent any obstacle for its use because it is attributed to the proton-transfer complex. It will not appear in the aqueous media required for final oligonucleotide-AzaPc conjugates in DNA hybridization assays.

4. Conclusion

In a conclusion, mono-, di-, tri- and tetracarboxy AzaPc suitable for dark quenchers were synthesized using statistical approach and isolated from the mixture of congeners. Analysis of the distribution of congeners in a statistical mixture using optimized HPLC method was performed. The analysis showed optimal ratios of starting materials to be 3:1 for AAAB, 1:3 for ABBB and 1:1 for AABB/ABAB types of

the congeners. All investigated AzaPc showed no fluorescence, extremely low singlet oxygen quantum yields in the monomeric form and intense absorption in a range from 300 nm to almost 700 nm. Such properties are highly promising for the future investigation of these compounds in DNA hybridization assays.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.dyepig.2011.03.016.

References

[1] Dini D, Hanack M, Meneghetti M. Nonlinear optical properties of tetrapyrazinoporphyrazinato indium chloride complexes due to excited-state absorption processes. Journal of Physical Chemistry B 2005;109:12691–6.
[2] Dini D, Hanack M, Egelhaaf HJ, Sancho-Garcia JC, Cornil J. Synthesis of axially substituted tetrapyrazinoporphyrazinato metal complexes for optical limiting and study of their photophysical properties. Journal of Physical Chemistry B 2005;109:5425–32.
[3] Donzello MP, Viola E, Bergami C, Dini D, Ercolani C, Giustini M, et al. Tetra-2,3-pyrazinoporphyrazines with externally appended pyridine rings. 6. Chemical and redox properties and highly effective photosensitizing activity for singlet oxygen production of penta- and monopalladated complexes in dimethylformamide solution. Inorganic Chemistry 2008;47:8757–66.
[4] Zimcik P, Miletin M, Radilova H, Novakova V, Kopecky K, Svec J, et al. Synthesis, properties and *in vitro* photodynamic activity of water-soluble azaphthalocyanines and azanaphthalocyanines. Photochemistry and Photobiology 2010;86:168–75.
[5] Mørkved EH, Andreassen T, Novakova V, Zimcik P. Zinc azaphthalocyanines with thiophen-2-yl, 5-methylthiophen-2-yl and pyridin-3-yl peripheral substituents: additive substituent contributions to singlet oxygen production. Dyes and Pigments 2009;82:276–85.
[6] Jang CK, Byun SH, Kim SH, lee DK, Jaung JY. Synthesis and optical properties of tetrapyrazinoporphyrazines containing camphorquinone group. Journal of Porphyrins and Phthalocyanines 2009;13:794–7.
[7] Novakova V, Mørkved EH, Miletin M, Zimcik P. Influence of protonation of peripheral substituents on photophysical and photochemical properties of tetrapyrazinoporphyrazines. Journal of Porphyrins and Phthalocyanines 2010;14:582–91.
[8] Kokareva EA, Petrov OA, Khelevina OG. Acid and complex-forming properties of tetrapyrazinoporphyrazines. Russian Journal of General Chemistry 2009;79:2440–4.
[9] Petrik P, Zimcik P, Kopecky K, Musil Z, Miletin M, Loukotova V. Protonation and deprotonation of nitrogens in tetrapyrazinoporphyrazine macrocycles. Journal of Porphyrins and Phthalocyanines 2007;11:487–95.
[10] Bergami C, Donzello MP, Monacelli F, Ercolani C, Kadish KM. Tetra-2,3-pyrazinoporphyrazines with externally appended pyridine rings. 4. UV-visible spectral and electrochemical evidence of the remarkable electron-deficient properties of the new tetrakis-2,3-[5,6-di{2-(N-methyl) pyridiniumyl}pyrazino]porphyrazinatometal octacation. Inorganic Chemistry 2005;44:9862–73.
[11] Donzello MP, Ou Z, Dini D, Meneghetti M, Ercolani C, Kadish KM. Tetra-2,3-pyrazinoporphyrazines with externally appended pyridine rings. 2. Metal complexes of tetrakis-2,3-[5,6-di(2-pyridyl)pyrazino]porphyrazine: linear and nonlinear optical properties and electrochemical behavior. Inorganic Chemistry 2004;43:8637–48.
[12] Kobak RZU, Öztürk ES, Koca A, Gül A. The synthesis and cyclotetramerisation reactions of aryloxy-, arylalkyloxy-substituted pyrazine-2,3-dicarbonitriles and spectroelectrochemical properties of octakis(hexyloxy)-pyrazinoporphyrazine. Dyes and Pigments 2010;86:115–22.
[13] Donzello MP, Viola E, Cai XH, Mannina L, Ercolani C, Kadish KM. Tetra-2,3-pyrazinoporphyrazines with externally appended pyridine rings. 8. Central (Zn-II, Cu-II, Mg-II(H2O), Cd-II) and exocyclic (Pd-II) metal ion binding in heteropentametallic complexes from tetrakis-2,3-[5,6-di(2-pyridyl)pyrazino] porphyrazine. Inorganic Chemistry 2010;49:2447–56.
[14] Kobak RZU, Gül A. Synthesis and solution studies on azaphthalocyanines with quaternary aminoethyl substituents. Coloration Technology 2009;125:22–8.
[15] Makhseed S, Samuel J. The synthesis and characterization of zincphthalocyanines bearing functionalized bulky phenoxy substituents. Dyes and Pigments 2009;82:1–5.
[16] Makhseed S, Ibrahim F, Samuel J, Helliwell M, Warren JE, Bezzu CG, et al. Clathrate formation from octaazaphthalocyanines possessing bulky phenoxyl substituents: a new cubic crystal containing solvent-filled, nanoscale voids. Chemistry-A European Journal 2008;14:4810–5.
[17] Kopecky K, Novakova V, Miletin M, Kucera R, Zimcik P. Solid-phase synthesis of azaphthalocyanine-oligonucleotide conjugates and their evaluation as new dark quenchers of fluorescence. Bioconjugate Chemistry 2010;21:1872–9.
[18] Marras SAE. Interactive fluorophore and quencher pairs for labeling fluorescent nucleic acid hybridization probes. Molecular Biotechnology 2008;38:247–55.
[19] Novakova V, Zimcik P, Miletin M, Vachova L, Kopecky K, Lang K, et al. Ultrafast intramolecular charge transfer in tetrapyrazinoporphyrazines controls the quantum yields of fluorescence and singlet oxygen. Physical Chemistry Chemical Physics 2010;12:2555–63.
[20] Ried W, Tsiotis G. New fused nitrogen-rich heterocycles from 5,6-dichloropyrazine-2,3-dicarbonitrile. Liebigs Annalen Der Chemie; 1988:1197–9.
[21] Zimcik P, Miletin M, Ponec J, Kostka M, Fiedler Z. Synthesis and studies on photodynamic activity of new water-soluble azaphthalocyanines. Journal of Photochemistry and Photobiology A: Chemistry 2003;155:127–31.
[22] Musil Z, Zimcik P, Miletin M, Kopecky K, Link M, Petrik P, et al. Synthesis and singlet oxygen production of azaphthalocyanines bearing functional derivatives of carboxylic acid. Journal of Porphyrins and Phthalocyanines 2006;10:122–31.
[23] Zimcik P, Miletin M, Musil Z, Kopecky K, Kubza L, Brault D. Cationic azaphthalocyanines bearing aliphatic tertiary amino substituents-synthesis, singlet oxygen production and spectroscopic studies. Journal of Photochemistry and Photobiology A: Chemistry 2006;183:59–69.
[24] Michelsen U, Kliesch H, Schnurpfeil G, Sobbi AK, Wöhrle D. Unsymmetrically substituted benzonaphthoporphyrazines: a new class of cationic photosensitizers for the photodynamic therapy of cancer. Photochemistry and Photobiology 1996;64:694–701.
[25] Lakowicz JR. Principles of fluorescence spectroscopy. 3rd ed. New York: Springer; 2006. pp. 54–55.
[26] Seybold PG, Gouterman M. Porphyrins .13. Fluorescence spectra and quantum yields. Journal of Molecular Spectroscopy 1969;31:1–13.
[27] Torres T. Perspectives in the selective synthesis of phthalocyanines and related compounds. Journal of Porphyrins and Phthalocyanines 2000;4:325–30.
[28] De La Torre G, Claessens CG, Torres T. Phthalocyanines: the need for selective synthetic approaches. European Journal of Organic Chemistry; 2000:2821–30.
[29] Leznoff CC, Hall TW. The synthesis of a soluble, unsymmetrical phthalocyanine on a polymer support. Tetrahedron Letters 1982;23:3023–6.
[30] Erdem SS, Nesterova IV, Soper SA, Hammer RP. Solid-phase synthesis of asymmetrically substituted "AB(3)-type" phthalocyanines. Journal of Organic Chemistry 2008;73:5003–7.
[31] Kobayashi N, Kondo R, Nakajima S, Osa T. New route to unsymmetrical phthalocyanine analogs by the use of structurally distorted subphthalocyanines. Journal of the American Chemical Society 1990;112:9640–1.
[32] Maizlish VE, Kulinich VP, Shaposhnikov GP. Unsymmetrical azaporphyrins. synthesis, structure and properties. Russian Journal of General Chemistry 2004;74:1801–17.
[33] Nolan KJM, Hu M, Leznoff CC. "Adjacent" substituted phthalocyanines. Synlett; 1997:593–4.
[34] Fukuda T, Kobayashi N. Efficient synthesis of a donor-acceptor phthalocyanine having adjacently-fused pyrazine rings. Chemistry Letters; 2002:866–7.
[35] Leznoff CC, Greenberg S, Khouw B, Lever ABP. The syntheses of monosubstituted and disubstituted phthalocyanines using a dithioimide. Canadian Journal of Chemistry 1987;65:1705–13.
[36] Young JG, Onyebuagu W. Synthesis and characterization of di-Disubstituted phthalocyanines. Journal of Organic Chemistry 1990;55:2155–9.
[37] Vagin S, Frickenschmidt A, Kammerer B, Hanack M. Synthesis and properties of an unsymmetrical triazole-functionalized (phthalocyaninato)zinc complex. European Journal of Organic Chemistry; 2005:3271–8.
[38] Youssef TE, Hanack M. Synthesis and characterization of unsymmetrically substituted dienophilic nickel phthalocyanines for Diels-Alder reactions. Journal of Porphyrins and Phthalocyanines 2002;6:571–7.
[39] Giuntini F, Nistri D, Chiti G, Fantetti L, Jori G, Roncucci G. Synthesis of trimethylammoniumphenylthio-substituted phthalocyanines with different pattern of substitution. Tetrahedron Letters 2003;44:515–7.
[40] Hammer RP, Owens CV, Hwang SH, Sayes CM, Soper SA. Asymmetrical, water-soluble phthalocyanine dyes for covalent labeling of oligonucleotides. Bioconjugate Chemistry 2002;13:1244–52.
[41] Linssen TG, Hanack M. Synthesis, separation and characterization of unsymmetrically substituted phthalocyanines. Chemische Berrichte 1994;127:2051–7.
[42] Polley R, Linßen TG, Stihler P, Hanack M. Synthesis, separation and characterization of naphthobenzo-condensed porphyrazinato nickel(II) complexes. Journal of Porphyrins and Phthalocyanines 1997;1:169–79.
[43] Subbotin NB, Nemykin VN, Voloshin YZ. A synthesis of novel nonsymmetrical phthalocyanines. Mendeleev Communications; 1993:121–2.
[44] Musil Z, Zimcik P, Miletin M, Kopecky K, Petrik P, Lenco J. Influence of electron-withdrawing and electron-donating substituents on photophysical

properties of azaphthalocyanines. Journal of Photochemistry and Photobiology A: Chemistry 2007;186:316–22.
[45] Cook MJ, Jafari-Fini A. Phthalocyanine-related macrocycles: cross cyclotetramerisation products from 3,4-dicyanothiophenes, 2,3-dicyanothiophene and 3,6-dialkylphthalonitriles. Tetrahedron 2000;56:4085–94.
[46] Mørkved EH, Andreassen T, Fröhlich R, Mo F, Bruheim P. Unsymmetrical zinc azaphthalocyanines, peripherally substituted with thiophen-2-yl and 2-functionalized phenoxy groups. Polyhedron 2010;29:3229–37.
[47] Marras SA, Kramer FR, Tyagi S. Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes. Nucleic Acids Research; 2002:30.
[48] Kostka M, Zimcik P, Miletin M, Klemera P, Kopecky K, Musil Z. Comparison of aggregation properties and photodynamic activity of phthalocyanines and azaphthalocyanines. Journal of Photochemistry and Photobiology A: Chemistry 2006;178:16–25.
[49] Stuzhin PA. Azaporphyrins and phthalocyanines as multicentre conjugated ampholites. Journal of Porphyrins and Phthalocyanines 1999;3:500–13.
[50] Kopecky K, Zimcik P, Novakova V, Miletin M, Musil Z, Stribna J. The synthesis and characterization of metal-free, unsymmetrical azaphthalocyanines with hydroxy groups and their complex formation with pyridine. Dyes and Pigments 2008;78:231–8.
[51] Zimcik P, Miletin M, Novakova V, Kopecky K, Nejedla M, Stara V, et al. Effective monofunctional azaphthalocyanine photosensitizers for photodynamic therapy. Australian Journal of Chemistry 2009;62:425–33.
[52] Boutorine AS, Brault D, Takasugi M, Delgado O, Helene C. Chlorin-oligonucleotide conjugates: synthesis, properties, and red light-induced photochemical sequence-specific DNA cleavage in duplexes and triplexes. Journal of the American Chemical Society 1996;118:9469–76.
[53] Redmond RW, Gamlin JN. A compilation of singlet oxygen yields from biologically relevant molecules. Photochemistry and Photobiology 1999;70:391–475.