Solid-Phase Synthesis of Azaphthalocyanine−Oligonucleotide Conjugates and Their Evaluation As New Dark Quenchers of Fluorescence

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Hydrophobic nonaggregating metal-free azaphthalocyanines (AzaPc) of the tetrapyrazinoporphyrazine type were synthesized, characterized, and used for oligonucleotide labeling. Both 3′-end and 5′-end labeling methods using solid phase synthesis suitable for automatic processes in the DNA/RNA synthesizer were developed. The hydrophobic character of AzaPc enabled the anchoring of the conjugates on reverse phase of the oligonucleotide purification cartridge, thus enabling their simple purification. AzaPc did not show any fluorescence and extremely low singlet oxygen quantum yields (Φ_Δ = 0.015−0.018 in DMF) in a monomeric state due to ultrafast intramolecular charge transfer. That is why they were investigated as a new dark quencher structural type. They profit particularly from absorption in a wide range of wavelengths (300−740 nm) that covers all fluorophores used in hybridization assays nowadays. As an example, quenching efficiency was evaluated in a simple hybridization assay using monolabeled probes. AzaPc-based probes efficiently quenched both fluorescein and Cy5 fluorescence by both resonance energy transfer and contact quenching. The results were compared with three established dark quenchers, and the AzaPc exerted better (BHQ-1 and BHQ-2) or comparable (BBQ-650) quenching efficiencies for both fluorophores.

INTRODUCTION

Modification of oligonucleotides with dyes is a commonly used procedure in genomic assays, e.g., in the monitoring of real-time polymerase chain reaction (PCR[1]). Most of the hybridization assays are based on the combination of a fluorophore with a quencher leading to the decrease of the fluorophore emission after detection of the target (*1*). The dark quenchers are a modern type of quenchers with no intrinsic fluorescence, which substantially simplifies the hybridization assays and increases their sensitivity. However, the currently used dark quenchers are based on two or three structural types, and they are often suitable only for a restricted number of fluorescent labels due to a limited spectral overlap. Therefore, the development of new highly efficient and versatile dark quenchers is desirable.

Phthalocyanines (*Pc*) and their aza-analogues azaphthalocyanines (AzaPc) are long known organic dyes with application in various areas (*2*). Despite deep investigations in *Pc* and AzaPc chemistry and applications, *Pc* conjugates with oligonucleotides were described only recently (*3*−*5*); furthermore, those using AzaPc have not been reported at all. For example, strong fluorescent properties of *Pc* were appreciated in *Pc*-oligonucleotide conjugates, and several new *Pc*-based near-infrared emitting fluorophores for DNA-hybridizations assays were developed (*6, 7*). The use of *Pc*−oligonucleotide conjugates for a specific DNA catalytic or photosensitized modification was also described (*8, 9*). The conjugation of *Pc* with the oligonucleotide is usually performed in a solution that requires the water solubility of the *Pc* moiety and subsequent extensive purification involving HPLC. The *Pc*−oligonucleotide labeling approaches tested until this time cover isothiocyanate (*5*) or succinimidyl methods (*6, 10, 11*), Huisgen cycloaddition (*12*), and reductive amination (*12*).

During our studies of AzaPc, we systematically observed that alkylamino substituted derivatives even in the strictly monomeric form are lacking any fluorescent or photosensitizing properties typical for other AzaPc derivatives (*13*). Recently, we discovered and fully explained that ultrafast intramolecular charge transfer (ICT) is responsible for the quenching of the S_1 excited state of such AzaPc (*14*). This unique mechanism of S_1 state deactivation, not yet observed at *Pc* derivatives, is responsible for the lack of fluorescence. These properties, together with strong absorption over a wide range of wavelengths, make alkylamino AzaPc ideal candidates for the modern dark quenchers. In order to confirm the hypothesis, suitable AzaPc were synthesized, simple solid-phase labeling of oligonucleotide probe was developed, and the conjugates were subjected to hybridization assays with two commonly used fluorophores.

EXPERIMENTAL PROCEDURES

All organic solvents were of analytical grade. Anhydrous butanol was stored over magnesium and distilled prior to use. Anhydrous pyridine was distilled from potassium hydroxide and stored over molecular sieves. Anhydrous tetrahydrofuran (THF) was stored over sodium and distilled prior to use. All chemicals for synthesis were obtained from established suppliers (Aldrich, Acros, Merck) and used as received. TLC was performed on Merck aluminum sheets with silica gel 60 F254. Merck

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[1]Abbreviations: AzaPc, azaphthalocyanine; BBQ, BlackBerry Quencher; BHQ, Black Hole Quencher; CPG, controlled pore glass, DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethylsulfoxide, DMTr, 4,4′-dimethoxytrityl; DMTrCl, 4,4′-dimethoxytrityl chloride; FAM, fluorescein; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; ICT, intramolecular charge transfer; lcaa, long chain aminoalkyl; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MeOH, methanol; NHS, *N*-hydroxysuccinimide; OPC, oligonucleotide purification cartridge; *Pc*, phthalocyanine; PCR, polymerase chain reaction; RET, resonance energy transfer; TFA, trifluoroacetic acid; THF, tetrahydrofuran; ZnPc, zinc phthalocyanine.

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Kieselgel 60 (0.040−0.063 mm) was used for column chromatography. Long chain amino alkyl (lcaa) solid phase (500 Å controlled pore glass (CPG)) was obtained from ChemGenes Corporation (Wilmington, MA, USA). All HPLC chromatographic separations were performed on a Shimadzu chromatography system consisting of a communication bus module CBM 20A, a diode array detector SPD-M20A, two pumps LC-20AD, an autoinjector SIL-20AC, a column compartment CTO-20AC, a degasser DGU-20A_3, and a computer-based chromatographic software LC solution (Shimadzu, Tokyo, Japan). The infrared spectra were measured on IR-Spectrometer Nicolet Impact 400 in KBr pellets. The ^{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). The reported chemical shifts are relative to tetramethylsilane. The UV/vis spectra were recorded using a Shimadzu UV-2401PC spectrophotometer. The fluorescence spectra were obtained by an AMINCO-Bowman Series 2 luminescence spectrometer. The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra of compounds **1**, **2**, and **7**−**10** were collected by a Voyager-DE STR mass spectrometer (Applied Biosystems) in α-cyano-4-hydroxycinnamic acid or *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]-malononitrile as the matrix. Mass spectra of oligonucleotide probes were obtained using a MALDI-TOF Bruker Autoflex II mass spectrometer. Compound **3** was prepared according to Mørkved et al. (*15*), and compounds **1** (*16*) and **6** (*17*) have been published by our group earlier.

Synthesis. *Azaphthalocyanine (**2**).* AzaPc **1** (100 mg, 0.09 mmol) in anhydrous dichloromethane (3 mL) was added to 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (102 mg, 0.36 mmol) and diisopropylethylamine (126 μL, 0.72 mmol) in anhydrous dichloromethane (5 mL) and stirred for 2 h at rt under an argon atmosphere. Progress of the reaction was monitored on TLC (dichloromethane/acetone/methanol 50:1:1; product R_f = 0.56; compound **1** R_f = 0.12). Thereafter, the solution was washed with saturated $NaHCO_3$, dried by Na_2SO_4, and evaporated to dryness. Any possible purification on a silica column failed due to the decomposition of **2** on silica. That is why the product was used directly for oligonucleotide modification without any further purification. The similar instability of phosphoramidites on silica or alumina has been described also by other researchers (*18*). The MALDI-TOF spectra of **2** showed the mass at expected *m*/*z* = 1322.6, approximately 10% of the oxidized product at *m*/*z* = 1338.6, and a fragment at *m*/*z* = 1105.6 caused by laser ionization. Yield, 105 mg; 89% as a deep purple solid. MS (MALDI-TOF) *m*/*z* = 1105.6 (55) $[M\text{-}OPN(iPr)_2OCH_2CH_2CN]^+$, 1322.6 (100) $[M]^+$, 1338.6 (10) $[M + O]^+$.

*4-[(3-Chloro-5,6-dicyanopyrazin-2-yl)methylamino]benzoic Acid (**4**).* A mixture of compound **3** (400 mg, 2 mmol) and 4-(methylamino)benzoic acid (604 mg, 4 mmol) in THF (75 mL) was refluxed for 12 h. The reaction was cooled down and filtered, and the filtrate was purified on silica with chloroform/acetone/methanol 10:1:1 as the mobile phase. The product was recrystallized from ethanol/water. Yield, 520 mg; 83% as a yellow powder; mp 208.1−211.6 °C. ^{1}H NMR (300 MHz, [D_6] acetone): δ = 8.09 (d, ^3J(H,H) = 8.5 Hz, 2H, arom. CH); 7.48 (d, ^3J(H,H) = 8.5 Hz, 2H, arom. CH_2) and 3.66 (s, N−CH_3, 3H,) ppm. ^{13}C NMR (75 MHz, [D_6] acetone): δ = 166.7, 153.5, 149.6, 141.1, 131.9, 130.7, 130.0, 125.8, 121.9, 114.3, 114.2, and 42.6 ppm. IR (KBr): ν = 3066, 2957, 2663, 2546, 2360, 2343, 2234 (CN), 1694, 1607, 1537, 1494, 1406, and 1375 cm^{-1}.

*4-[(5,6-Dicyano-3-diethylaminopyrazin-2-yl)methylamino]benzoic Acid (**5**).* Compound **4** (312 mg, 0.89 mmol) was refluxed in THF (50 mL) with diethylamine (1.3 g 18 mmol) for 2 h. The reaction was cooled down, filtered, and the solvent evaporated. The crude product was purified on silica with a mobile phase of chloroform/methanol 10:1 and finally recrystallized from ethanol/water. Yield, 324 mg; 62% of yellow powder; mp 263.3−266.2 °C (decomp.). ^{1}H NMR (300 MHz, [D_6] DMSO−): δ = 7.79 (d, ^3J(H,H) = 8.8 Hz, 2H, arom. CH_2), 7.09 (d, ^3J(H,H) = 8.8 Hz, 2H,arom. CH), 3.48 (s, 3H, N−CH_3), 3.25 (q, ^3J(H,H) = 7 Hz, 4H, N−CH_2) and 0.78 (t, ^3J(H,H) = 7 Hz, 6H, CH_2−CH_3) ppm. ^{13}C NMR (75 MHz, [D_6] DMSO): δ = 167.0, 148.5, 147.6, 143.9, 130.9, 126.0, 124.1, 120.4, 118.3, 115.4, 115.2, 42.8, 39.2, and 12.5 ppm. IR (KBr): ν = 2976, 2936, 2876, 2360, 2229 (CN), 1686, 1607, 1533, 1501, 1404, and 1292 cm^{-1}.

*2-[(4-Carboxyphenyl)methylamino]-3,9,10,16,17,23,24-heptakis(diethylamino)-1,4,8,11,15,18,22,25-(octaaza)phthalocyanine (**7**).* Metal lithium (168 mg, 24 mmol) was added to the refluxing mixture of compounds **5** (300 mg, 0.85 mmol) and **6** (700 mg, 2.57 mmol) in anhydrous butanol (20 mL) and the reflux continued for the next 3 h. The solvent was removed under reduced pressure, dilute acetic acid (50%, 50 mL) was added, and the suspension was stirred for 30 min at rt. The crude product was filtered and washed thoroughly with water. The resulting mixture of congeners was separated on silica with dichloromethane/acetone/methanol 20:1:1 as the mobile phase. The second intense purple fraction (product) was purified again on silica with dichloromethane/acetone/methanol 30:1:1. Finally, pure compound **7** was dissolved in 5 mL of dichloromethane, dropped into 200 mL of hexane, and cooled to −30 °C overnight. The precipitated fine suspension was filtered and washed with hexane. Yield, 150 mg; 15% of deep purple solid. ^{1}H NMR (300 MHz, [D_5] pyridine): δ = 13.83 (br s, 2H; central NH), 8.48 (d, ^3J(H,H) = 9 Hz, 2H; arom. CH), 7.20 (d, overlap with solvent, 2H; arom. CH), 3.96−3.86 (m, 24H, N−CH_2), 3.85 (s, 3H; N−CH_3), 3.72 (q, ^3J(H,H) = 7 Hz, 4H; N−CH_2) and 1.27−1.06 ppm (m, 42H; CH_3). ^{13}C NMR (75 MHz, [D_5] pyridine): δ = 169.0, 153.2, 151.0, 150.9, 150.5, 146.7, 146.6, 143.0, 142.9, 141.6, 140.0, 139.9, 139.7, 139.7, 131.4, 117.3, 43.8, 43.1, 43.1, 39.1, 13.2, 13.1, and 13.1 ppm (some signals were overlapped with signals of solvent). IR (KBr): ν = 2969, 2931, 2873, 1710 (CO), 1639, 1605, and 1426 cm^{-1}. UV/vis (THF): λ_{max} (ε) = 680 (80000), 654 (66500), 622sh, 597sh, 518 (54300) and 369 nm (93200 M^{-1}cm^{-1}). MS (MALDI-TOF): *m*/*z* = 1168.6 $[M]^+$. Anal. ($C_{60}H_{80}N_{24}O_2$ + 2 H_2O) calcd. C 59.78, H 7.02, N 27.89; found, C 60.18, H 7.30, N 27.53.

*Azaphthalocyanine (**8**).* AzaPc **7** (290 mg, 248 μmol) was dissolved in anhydrous tetrahydrofuran (40 mL). *N*,*N*′-dicyclohexylcarbodiimide (DCC) (56.2 mg, 273 μmol) and subsequently *N*-hydroxysuccinimide (NHS) (31.4 mg, 273 μmol) were added. The reaction was stirred for 72 h at rt. The precipitated solid (*N*,*N*′-dicyclohexylurea) was filtered off and washed with THF. 3-Amino-1,2-propandiol (113 mg, 1.24 mmol) in methanol was added into the THF solution, and the reaction was stirred at rt. The reaction was protected from light by aluminum foil. After 36 h, the solvent was evaporated at reduced pressure, and the rest was suspended in water, filtered, and washed thoroughly with water. Purification was performed on silica with a dichloromethane/acetone/methanol gradient from 30:1:1 to 10:1:1. Yield, 150 mg; 49% as deep purple solid. ^{1}H NMR (300 MHz, [D_5] pyridine): δ = 13.81 (br s, 2H; central NH), 9.32 (t, ^3J(H,H) = 6 Hz, 1H; NHCO), 8.32 (d, ^3J(H,H) = 9 Hz, 2H; arom. CH), 7.17 (d, overlap with solvent, 2H; arom. CH), 4.53−4.42 (m, 1H; C*H*−OH), 4.32−4.00 (m, 4H; NH−CH_2, O−CH_2), 3.97−3.84 (m, 24H; N−CH_2), 3.83 (s, 3H, N−CH_3), 3.69 (q, ^3J(H,H) = 7 Hz; 4H, N−CH_2) 1.21 (t, ^3J(H,H) = 7 Hz, 30H; CH_3), 1.14 (t, ^3J(H,H) = 7 Hz, 6H; CH_3) and 1.06 ppm (t, ^3J(H,H) = 7 Hz, 6H; CH_3). ^{13}C NMR (75 MHz, [D_5] pyridine): δ = 168.4, 153.1, 151.0, 150.9, 150.9, 150.6, 150.5, 150.0, 147.0, 146.1, 142.8, 142.7, 141.5, 140.1, 139.9, 139.8, 139.7, 139.6, 129.4, 128.9, 128.3, 126.1, 122.9, 117.9, 116.7,

114.4, 72.2, 65.2, 43.7, 43.1, 43.0, 39.2, 30.2, 13.3, 13.1, and 13.1 ppm (some signals were overlapped with signals of solvent). IR (KBr): $\nu = 2969, 2931, 2873, 1636$ (CONH), 1607, 1506, and 1427 cm^{-1}. UV/vis (THF): λ_{max} (ε) = 681 (80000), 655 (65600), 622sh, 597sh, 518 (53500) and 370 nm (91100 $M^{-1}cm^{-1}$). MS (MALDI-TOF) m/z = 1241.3 $[M]^+$. Anal. ($C_{63}H_{87}N_{25}O_3 + 3\ H_2O$) calcd. C 58.36, H 7.23, N 27.01; found, C 58.50, H 7.33, N 26.97.

*Azaphthalocyanine (**9**).* AzaPc **8** (124 mg, 0.1 mmol), 4,4′-dimethoxytrityl chloride (DMTrCl) (44 mg, 0.13 mmol), and a catalytic amount of 4-*N*,*N*-dimethylaminopyridine were dissolved in anhydrous pyridine (20 mL) and stirred for 14 h at rt. After that time, more DMTrCl (169 mg, 0.5 mmol) was added, and the reaction was stirred further. The reaction vessel was protected from light by aluminum foil. After 24 h, TLC (dichloromethane/pyridine 40:1) indicated full conversion. The product was used directly for the subsequent reaction without any isolation. A sample was taken from the solution and analyzed using mass spectrometry. MS (MALDI-TOF) m/z 1543.7 $[M]^+$, after the addition of trifluoroacetic acid (TFA) into the matrix (deprotection of 4,4′-dimethoxytrityl (DMTr)): m/z 1241.6 $[M\text{-}DMTr]^+$.

*Azaphthalocyanine (**10**).* Succinic anhydride (100 mg, 1 mmol) was added into the previous reaction, and the mixture was heated for 12 h at 60 °C. Thereafter, the solvent was removed under reduced pressure, and the crude product was purified on silica with a step gradient: dichloromethane/pyridine 40:1, dichloromethane/pyridine 20:1, and dichloromethane/methanol/pyridine 20:1:1. Yield, 40 mg; 25% (after two steps) as a deep purple solid. ^1H NMR (300 MHz, [D_5] pyridine): δ = 13.86 (br s, 2H; central NH), 9.11 (t, $^3J(H,H)$ = 5 Hz, 1H; NHCO), 6.88−8.46 (m, 17H; arom. CH), 4.33−4.20 (m, 1H; C*H*-O), 3.98−3.79 (m, 26H; 1 × NH−CH_2, 12 × N−CH_2), 3.75 (s, 3H; N−CH_3), 3.73−3.56 (m, 12H; 2 × O−CH_3, 1 × O−CH_2, 2 × N−CH_2), 2.91−2.87 (m, 2H; CH_2−CO), 2.85−2.81 (m, 2H; CH_2−CO), 1.20 (t, $^3J(H,H)$ = 7 Hz, 30H; CH_3), 1.14 (t, $^3J(H,H)$ = 7 Hz, 6H; CH_3) and 1.06 ppm (t, $^3J(H,H)$ = 7 Hz, 6H; CH_3). ^{13}C NMR (75 MHz, [D_5] pyridine): δ = 174.4, 172.2, 167.1, 158.3, 152.4, 150.3, 150.2, 150.1, 148.5, 146.3, 145.1, 144.7, 135.7, 135.3, 134.1, 131.0, 129.9, 128.3, 127.9, 127.6, 126.4, 116.9, 113.0, 85.8, 72.4, 62.2, 54.5, 42.9, 42.4, 42.3, 38.5, 29.5, 29.3, 29.1, 12.4 ppm (some signals were overlapped with the signals of the solvent). IR (KBr): $\nu = 3428, 3305, 3050, 2966, 2930, 2871, 1734$ (CO-O), 1640(CO-NH), 1607, 1508, 1425, and 1250 (O−CH_3) cm^{-1}. MS (MALDI-TOF): m/z = 1643.7 $[M]^+$; after the addition of TFA into the matrix, m/z = 1341.7 $[M\text{-}DMTr]^+$.

*Solid Phase Modified with Azaphthalocyanine (**11**).* AzaPc **10** (8 mg, 5 μmol), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (3 mg, 10 μmol), and anhydrous triethylamine (10 μL) were dissolved in anhydrous dimethylformamide (DMF) (2 mL), and 100 mg of lcaa CPG was added. The reaction vial was sealed, and the mixture was shaken for 24 h at rt on an orbital shaker protected from light. Thereafter, the solid phase was washed with DMF, acetonitrile, and diethylether and dried over the silica under reduced pressure. Unreacted amino groups were subsequently capped. A mixture of anhydrous THF, pyridine, and acetic anhydride (8:1:1 (v/v), 1 mL) was mixed with a 10% (m/m) solution of 1-methylimidazole in anhydrous THF (1 mL) and shaken with the solid phase **11** for 30 min at rt. Thereafter, the solid phase was washed with DMF, acetonitrile, and diethylether and dried over silica under reduced pressure. 4,4′-Dimethoxytrityl loading was determined as described in the following procedure. Approximately 2−3 mg of the support was accurately weighed out directly to the test tube, perchloric acid solution (10 mL, 51.4 mL of 70% $HClO_4$ + 46 mL of methanol) was added, and the test tube was sealed and shaken. The optical absorbance of the sample, diluted to fit an interval of 0.2−1.0 AU was measured at 498 nm in a 1 cm cuvette. The loading was calculated as given bellow:

$$\text{loading } (\mu\text{mol/g}) = (\text{absorbance at } 498) \times \text{dilution} \times 143/(\text{weight of support (mg)})$$

The loading of DMTr on solid phase **11** was determined to be 30.8 μmol/g.

Synthesis of Oligonucleotides. Oligonucleotides were synthesized on a Perkin-Elmer Applied Biosystems 394 DNA/RNA synthesizer. 3′-Labeling of the oligonucleotides was achieved by synthesis on modified solid phases (solid phase **11** and commercially obtained supports of Black Hole Quencher (BHQ) or BlackBerry Quencher (BBQ)). 5′-Labeling of the oligonucleotides was performed on the same synthesizer employing standard phosphoramidite chemistry and corresponding dye 2-cyanoethyl-(*N*,*N*-diisopropyl)-phosphoramidites (compound **2** and commercially obtained dyes). 3′-BHQ-1 CPG, 3′-BHQ-2 CPG, 5′-BHQ-1 phosphoramidite, 5′-BHQ-2 phosphoramidite, Cy5 phosphoramidite, and 5′-fluorescein phosphoramidite were supplied by Glen Research, Sterling, VA, USA. 3′-BBQ-650 CPG and 5′-BBQ-650 phosphoramidite were supplied by Berry & Associates, Inc., Dexter, MI, USA. The synthesis and purification of oligonucleotides labeled with commercially obtained dyes were performed according to the supplier's recommendations.

OPC Purification of Oligonucleotides S1−S6 Modified with AzaPc. Prepared oligonucleotides **S1−S6** with AzaPc were purified on oligonucleotide purification cartridges (OPCs), Puri-Pak cartridges, (ChemGenes Corp., Wilmington, MA, USA) directly after deprotection from the solid phase. The OPC was washed with acetonitrile (4 mL) and 2 M triethylammonium acetate (2 mL). A solution of oligonucleotide (2−3 mL) in deprotecting solution was loaded on the OPC repeatedly until all of the color was absorbed to the top of OPC. Thereafter, the OPC was washed with a diluted solution of ammonia (2.5%, 4 mL), then with 8% acetonitrile (4 mL) and 30% acetonitrile (2 mL). The desired sequence was obtained by freeze-drying the 30% acetonitrile fraction. Purified oligonucleotides were stored at −20 °C.

S1 (AzaPc): MS (MALDI-TOF), a cluster peaking at m/z 8805 $[M + H]^+$ and adducts with sodium and potassium. **S2** (AzaPc): MS (MALDI-TOF), a cluster peaking at m/z 10325 $[M + H]^+$ and adducts with sodium and potassium. **S3** (AzaPc): MS (MALDI-TOF), a cluster peaking at m/z 11846 $[M + H]^+$ and adducts with sodium and potassium. **S4** (AzaPc): MS (MALDI-TOF), a cluster peaking at m/z 8685 $[M + H]^+$ and adducts with sodium and potassium. **S5** (AzaPc): MS (MALDI-TOF), a cluster peaking at m/z 7400 $[M + H]^+$ and adducts with sodium and potassium. **S6** (AzaPc): MS (MALDI-TOF), a cluster peaking at m/z 5819 $[M + H]^+$ and adducts with sodium and potassium.

HPLC Analysis. A gradient elution was used to analyze **S1−S6** modified with AzaPc. The separation was performed on a Hypersil BDS C18 column (100 × 4.6 mm, 3 μm particle size) using a mobile phase consisting of acetonitrile and 50 mM triethylammonium acetate. Mobile phase A was 12% acetonitrile in 50 mM triethylammonium acetate, and mobile phase B was acetonitrile. The gradient time program was set as follows: 0−2 min 0%B; 2−18 min 0−85% B; 18−25 min 85% B; 25−25.5 min 85−0% B. The column was equilibrated for 4.5 min. The column temperature was maintained at 40 °C, and the flow rate was set at 0.7 mL/min. The compounds were analyzed by a diode array detector. The corresponding controls were analyzed separately to allow the determination of their elution patterns.

Unlabeled oligonucleotides were eluted with t_R of approximately 2 min and free dye (**1** or **8**) with t_R of approximately 23 min.

Hybridization Assays. *Absorption Spectra of Duplexes.* Spectra of duplexes were measured in hybridization buffer. Stock solutions of oligonucleotides **S1**, **S3**, and **S4** labeled with AzaPc and **SF** and **SFX** labeled with Cy5 were prepared to a concentration 100 μM. Hybridization buffer (1 mL) was transferred to a cuvette, the stock solution of oligonucleotide probe with the quencher (10 μL) was added, and the absorption spectrum was measured. Thereafter, the stock solution of the oligonucleotide labeled with fluorophore (10 μL) was added, and the solution was heated to 50 °C for 1 min. The absorption spectrum of the duplex was measured after 30 min when the sample cooled down to laboratory temperature. The final concentration of both oligonucleotides was 1 μM. Absorption spectra of **SF** and **SFX** were measured also separately after the addition of the stock solution (10 μL) to hybridization buffer (1 mL).

Determination of Quenching Efficiency. The method was adopted from Marras et al. (*1*). Oligonucleotides **S1**−**S6** modified with AzaPc, BHQ-1, BHQ-2, and BBQ-650 or without any quencher were dissolved in hybridization buffer to a stock concentration 10 μM. Oligonucleotide **SF** was dissolved in hybridization buffer to a concentration 0.05 μM and transferred to a cuvette (800 μL), and the fluorescence was measured at 663 and 517 nm for Cy5 and fluorescein (FAM), respectively (excitation wavelengths 651 and 492 nm for Cy5 and FAM, respectively). Subsequently, the oligonucleotide with the quencher (20 μL of stock solution; final concentration 0.25 μM) was added, and the solution was heated to 70 °C for 5 min and allowed to cool to laboratory temperature for 30 min (**S6** was cooled to 4 °C). Thereafter, the fluorescence of the duplex was measured. A similar procedure was used for oligonucleotide **SFX**. The quenching efficiency (*QE*) was calculated according to the following equation (*1, 19*):

$$QE = (1 - F_X/F_0) \times 100$$

where F_0 is the fluorescence of fluorophore **SF** alone, and F_X is the fluorescence of the duplex.

Singlet Oxygen Quantum Yields and Fluorescence. Quantum yields of a singlet oxygen (Φ_Δ) of **1** and **7** were determined according to a previously published procedure (*20*) using the decomposition of 1,3-diphenylisobenzofuran in DMF. Zinc phthalocyanine (ZnPc) was used as a reference ($\Phi_\Delta = 0.56$ in DMF *21, 22*). The absorption of the dyes in the Q-band area was set approximately to 0.1. The presented data represent the mean of three measurements.

An attempt to detect any fluorescence signals from quenchers was also made. Compounds **1** and **7** in DMF, dimethylsulfoxide (DMSO), and THF or oligonucleotides **S1**−**S6** with AzaPc in hybridization buffer were excited at the B-band (365 nm) or Q-band (650 nm). Absorption at the excitation wavelength was set to approximately 0.1. Fluorescence was monitored in a range of 550−900 nm.

RESULTS AND DISCUSSION

Synthesis. All conjugation procedures between *Pc* and oligonucleotides until now were performed in water solution with an excess of *Pc* reagent. Isolation of the desired conjugate therefore always included several steps including the final HPLC separation. Solid-phase synthesis of the conjugates allows for the reduction of the side products, and the excess of the reagent can be easily washed out. In order to further simplify the purification steps, the AzaPc labels were designed to be highly hydrophobic, and that is why the conjugates may be anchored in a short OPC allowing easy purification without the need for

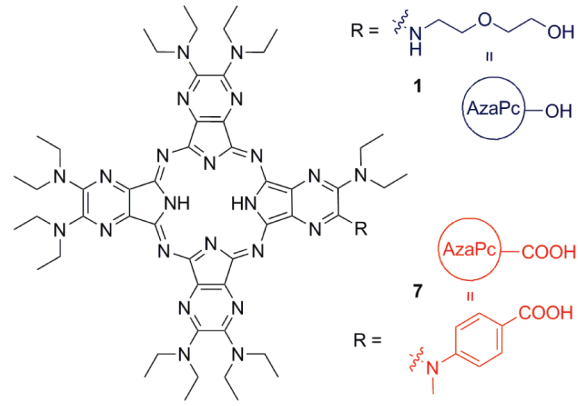


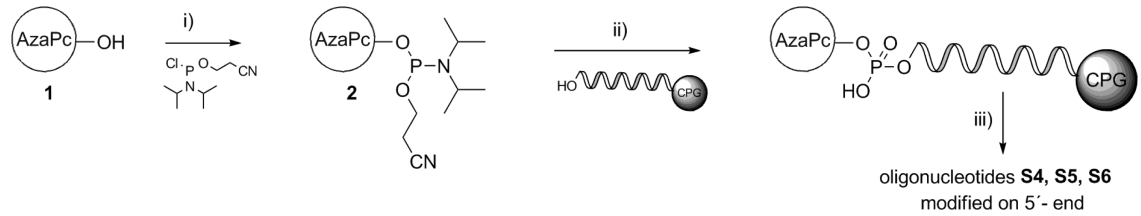
Figure 1. Structures of compounds **1** and **7**.

HPLC separation. The design of AzaPc labels was based on the following considerations: (1) the macrocycle must carry mostly hydrophobic substituents and one modifiable group; (2) the macrocycle must be substituted with alkylamino substituents in order to allow ICT to quench efficiently excited states; and (3) the hydrophobic substituents must be sufficiently bulky in order to reduce aggregation typical for planar AzaPc and *Pc* macrocycles. On the basis of these requirements, compounds **1** and **7** (Figure 1 and Schemes 1 and 2) were synthesized and further modified for both 3′- and 5′-end solid-phase labeling. Postsynthetic modification of AzaPc was easily performed because bulky *N*,*N*-diethylamino substituents reduced the aggregation to a minimum, and the typical problems with low solubility, silica binding (*22*), or tailing of AzaPc on TLC (*14*) were not observed (see Supporting Information, Figure S1).

5′-End modified oligonucleotides **S4**−**S6** were prepared from **1** (*16*) via phosphoramidite **2** (Scheme 1). Phosphoramidites are highly reactive intermediates (*23, 24*) finding their place in automatic DNA synthesis for both DNA elongation and labels attachment. Compound **2** was therefore introduced as the last step into the DNA/RNA synthesizer during solid-phase synthesis. Contrary to the in-solution conjugation methods, this approach simplifies the labeling and enables the general automatic processes to be used.

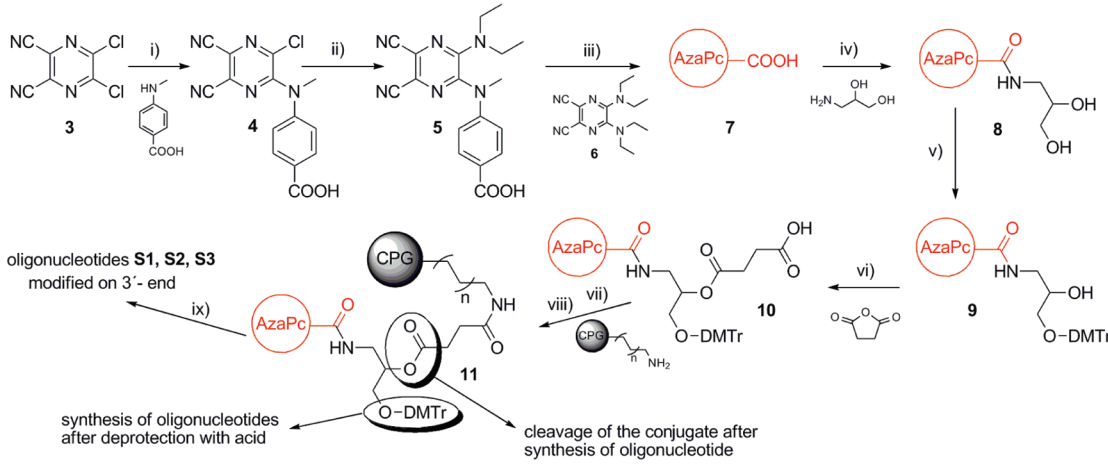
For the 3′-end labeling of oligonucleotides, the long chain aminoalkyl (lcaa) solid phase was modified by AzaPc **7** (Scheme 2) before the synthesis of the desired oligonucleotide chain (**S1**−**S3**). First, unsymmetrical AzaPc **7** was synthesized by statistical condensation of **5** and **6** and isolated from the mixture of six congeners (see Supporting Information, Figure S2) with a yield of 15%. Compound **5** was prepared by two step nucleophilic substitution of chlorine atoms in **3**. Although several selective strategies were developed for the synthesis of unsymmetrical *Pc* (*25*) including recent efforts in the improvement of solid-phase synthesis (*26*), the statistical condensation gave reasonable yields of **7** with very simple isolation of the desired congener. To verify the stability of **7** during the 3′-end labeling of oligonucleotides, the compound was subjected to the conditions occurring in the course of automated chemical oligonucleotide synthesis. It was shown to be stable in all the range of chemical conditions tested (see Supporting Information). Compound **7** was converted to dihydroxyamide **8** via an active succinimidyl ester. Protection of the primary hydroxy group with DMTrCl (**9**) allowed for selective modification of the secondary hydroxyl with succinate (**10**) and subsequent attachment of the AzaPc to lcaa solid phase (**11**). Unreacted amino groups in solid phase were capped with acetic anhydride. Solid phase **11** allows for the start of the synthesis of the DNA probe on hydroxyl after DMTr deprotection and simple cleavage of the synthesized conjugate from the solid phase by basic hydrolysis of the ester bond. Such a modified solid phase was

Scheme 1. Synthesis of 5′-End Modified Oligonucleotides Labeled with AzaPc[a]



[a] (i) anhydr. DCM, diisopropylethylamine, rt; (ii) 5-ethylthiotetrazole, anhydr. acetonitrile, DNA/RNA synthesizer; (iii) ammonia/methylamine, rt.

Scheme 2. Synthesis of 3′-End Modified Oligonucleotides Labeled with AzaPc[a]



[a] (i) THF, reflux; (ii) diethylamine, THF, reflux; (iii) anhydr. butanol, Li, reflux; (iv) DCC, NHS, anhydr. THF/methanol, rt; (v) DMTrCl, 4-(*N*,*N*-dimethylamino)pyridine, anhydr. pyridine, rt; (vi) anhydr. pyridine, 60°C; (vii) HBTU, triethylamine, anhydr. DMF, rt; (viii) acetic anhydride, 1-methylimidazole, THF/pyridine, rt; (ix) DNA synthesis in DNA/RNA synthesizer.

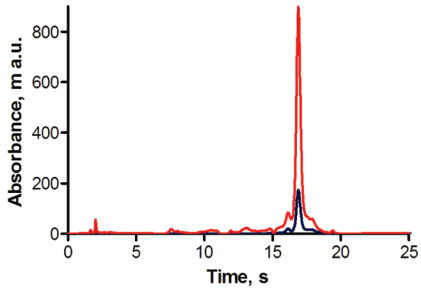


Figure 2. HPLC chromatogram of purified oligonucleotide **S3** labeled with AzaPc. Red line, 254 nm; blue line, 673 nm.

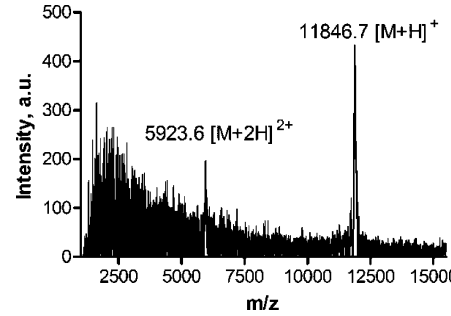


Figure 3. MALDI-TOF spectrum of oligonucleotide **S3** labeled with AzaPc.

used in a standard oligonucleotide synthesis in a DNA/RNA synthesizer. Also, this approach allows the general automatic processes in a DNA/RNA synthesizer to be used for both synthesis and cleavage of the conjugate from solid phase.

In all of the cases (**S1**−**S6**), the AzaPc conjugates cleaved from the solid phase were purified by passing through short OPC. The hydrophobic character of AzaPc labels allowed for the anchoring of the conjugates to reverse phase in OPC, and the unlabeled oligonucleotides were washed out with 8% acetonitrile. Subsequently, the increase of acetonitrile concentration to 30% released the AzaPc-oligonucleotide conjugates from OPC. The purified conjugates were analyzed by HPLC (see Figure 2), and two important peaks were detected in the chromatograms (typical retention times $t_R = 2$ min and $t_R = 17$ min). On the basis of an analysis of absorption spectra (see Supporting Information, Figure S5) and retention times of the controls, the base peak ($t_R = 17$ min) belongs to the oligonucleotide labeled with AzaPc. Unlabeled oligonucleotides were eluted in the second minute and did not exceed 4% in all cases (based on an area under the curve calculation at 254 nm). Nonconjugated AzaPc control eluted with $t_R = 23$ min and was not detected in chromatograms of AzaPc−oligonucleotide conjugates. In order to verify the composition of conjugates, MALDI-TOF spectra were recorded, and the expected mass to charge ratio was confirmed (Figure 3).

UV−Vis Spectral, Photophysical, and Photochemical Properties. Absorption spectra of AzaPc **1** and **7** were analyzed in different solvents (DMSO, THF, methanol (MeOH), DMF, and acetone). The spectra in all solvents showed the shape characteristic for monomeric species with two absorption bands typical for AzaPc or *Pc*: a high energy B-band at approximately 360 nm and a low energy Q-band at approximately 660 nm. Both compounds also showed another broad band at approximately 550 nm due to the conjugation of lone electron pairs of *N*,*N*-diethylamino substituents with a macrocyclic system (n-π^* transitions) (*14, 27*). The Q-bands of **1** and **7** were split due to the unsymmetrical composition of the whole macrocycle because of the presence of the central hydrogens (*16*). The splitting was sharp in THF but became broad and

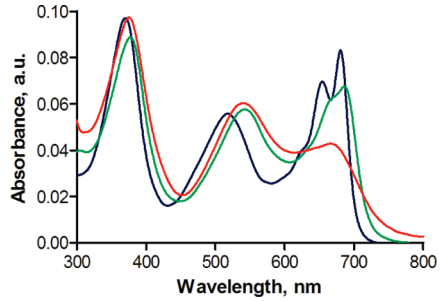


Figure 4. Absorption spectra of **7** in THF (blue), DMSO (green), and oligonucleotide **S1** with AzaPc in buffer (red) at a concentration of 1 μM.

Table 1. Spectral and Photochemical Properties of AzaPc 1 and 7

compound	λ (ε) in DMSO (nm (M^{-1} cm^{-1}))	λ (ε) in THF (nm (M^{-1} cm^{-1}))	λ (ε) in hybridization buffer (nm (M^{-1} cm^{-1}))[a]	Φ_Δ (DMF)
1	685 (57400)	677 (95300)	664 (45200)	0.015
	661 (50900)	647 (69500)	536 (66300)	
	530 (51900)	508 (58400)	373 (105900)	
	372 (84800)	365 (108000)		
7	686 (68000)	680 (86300)	668 (43000)	0.018
	542 (60000)	654 (72300)	537 (60300)	
	378 (91500)	516 (59600)	376 (97900)	
		370 (102700)		

[a] Conjugate **S1** (for **7**) or **S5** (for **1**).

featureless in solvents with increased polarity (e.g., DMSO), and completely disappeared when the conjugates where dissolved in hybridization buffer (Figure 4), indicating considerable effect of the solvent polarity on the Q-band shape. Such spectral changes in DMSO or buffer could also be attributed to aggregation, but neither the shape of the spectra nor the extinction coefficient changed in a very wide range of concentration (in DMSO 2.5×10^{-4} M-1.0×10^{-7} M). Also, no new bands at shorter or longer wavelengths corresponding to H- (*7, 28*) or J-dimer (*29*) formation were observed. Another support for the statement that the aggregation is not responsible for the broad Q-band is that the spectra of the conjugates did not change after hybridization with complementary oligonucleotides (see, e.g., the duplex **S4**$-$**SF** in Supporting Information, Figure S6-c).

Photophysical and photochemical parameters of AzaPc **1** and **7**, which are important for their prospective use as dark quenchers, were determined. No fluorescence of either AzaPc **1** and **7** in THF, DMF, DMSO, or the AzaPc labeled probes **S1**$-$**S6** in hybridization buffer was detected. Singlet oxygen, usually strongly produced by AzaPc after illumination (*30*), is a highly reactive species that may destroy oligonucleotide probes or the target sequence (*8, 31*), and thus, its formation is undesirable in hybridization assays. Importantly, AzaPc **1** and **7** reached very low singlet oxygen quantum yields (Φ_Δ), 0.015 and 0.018, respectively. For comparison, the Φ_Δ value for FAM, a routinely used fluorophore in hybridization assays, ranges from 0.03 to 0.1 (*32*). It must be noted that no aggregates were detected during measurements, and therefore, the lack of any detectable fluorescence can be attributed solely to the deactivation of the S_1 excited state by ICT (*14*). Spectral and photochemical data of compounds **1** and **7** are summarized in Table 1.

DNA Hybridization Assays and Quenching Efficiency. The absorption spectra of AzaPc$-$oligonucleotide conjugates **S1**$-$**S6** in hybridization buffer show a wide range of absorption from 300 to 740 nm due to the AzaPc moiety (Figure 5). Thus, it efficiently overlaps the emission spectra of all fluorophores currently used in hybridization assays. Such versatility cannot be found in any other structural type of dark quenchers and makes these compounds highly promising for future development. In order to prove the quenching ability of AzaPc, oligonucleotide **SF** labeled with two commonly used fluorophores was chosen for hybridization assays. For example, FAM (λ_{em} = 517 nm) emits at short wavelengths, while Cy5 (λ_{em} = 663 nm) emits at long wavelengths. For comparative purposes, the oligonucleotides **S1**$-$**S6** were labeled with the currently used dark quenchers BHQ-1, BHQ-2, and BBQ-650, which are highly efficient at the quenching of FAM or Cy5 (*33*).

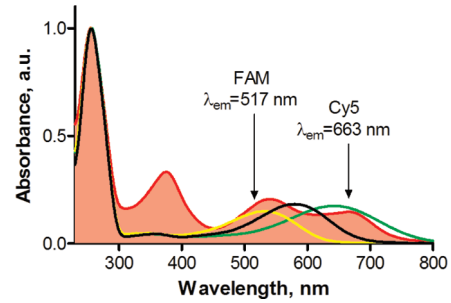


Figure 5. Absorption spectra of oligonucleotide **S1** labeled with AzaPc (red line, filled area), BHQ-1 (yellow), BHQ-2 (black), and BBQ-650 (green). The spectra were normalized to the same absorption at 254 nm (which is due to the oligonucleotides). The arrows indicate emission maxima of FAM and Cy5 fluorophores.

Figure 6. Sequences of modified oligonucleotides. F = fluorophore (FAM or Cy5), and Q = quencher (AzaPc, BHQ-1, BHQ-2, or BBQ-650).

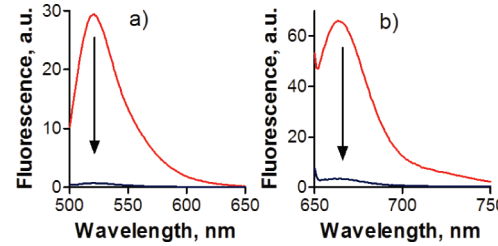


Figure 7. Fluorescence emission spectra of oligonucleotide **SF** with FAM (a) and Cy5 (b) before (red) and after (blue) the addition of the complementary oligonucleotide **S1** with AzaPc.

Oligonucleotides **S1**$-$**S6** (Figure 6) were designed to investigate both mechanisms of quenching utilized in hybridization assays. Resonance energy transfer (RET) requires the emission spectrum of a fluorophore and the absorption spectrum of a quencher to overlap with the effective distance between labeling dyes being 20$-$100 Å (*19*). During contact quenching, the fluorophore and the quencher must be very close to each other in order to form a ground-state complex that does not emit any light. Overlap of the spectra is not required in this mechanism (*19, 34*). According to Marras et al. (*1*), the oligonucleotide **S1** can be used for the detection of the contact quenching after hybridization with **SF** because the fluorophore and the quencher may form the nonemitting heterodimer. **S3** is designed to allow purely RET quenching, and **S2** may involve both mechanisms. However, the absorption spectra of the duplex of AzaPc-labeled **S3** with Cy5-labeled **SF** revealed the presence of a certain level

Table 2. Quenching Efficiency (%)[a]

fluorophore (**SF**)	quencher	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S1 + SFX**
FAM	AzaPc	97.0 ± 0.12	92.3 ± 0.23	84.5 ± 0.74	35.8 ± 1.19	34.9 ± 2.11	54.9 ± 0.44	
	BHQ-1	98.5 ± 0.05	94.9 ± 0.08	88.4 ± 0.45	21.4 ± 3.73	28.0 ± 2.38	48.5 ± 0.69	
	BHQ-2	98.6 ± 0.02	95.7 ± 0.03	89.3 ± 0.10	9.9 ± 1.30	18.5 ± 1.61	48.5 ± 0.35	
	BBQ-650	97.6 ± 0.02	90.3 ± 0.11	78.9 ± 0.13	30.7 ± 0.32	36.6 ± 0.52	49.9 ± 0.03	
	no quencher	15.2 ± 0.71	5.5 ± 1.31	9.6 ± 2.15				
Cy5	AzaPc	93.8 ± 0.15	95.1 ± 0.11	96.9 ± 0.04	27.5 ± 1.55	25.1 ± 0.87	45.1 ± 0.42	−9.5 ± 10.2
	BHQ-1	96.9 ± 0.05	95.7 ± 0.08	86.9 ± 0.51	−0.5 ± 3.65	3.5 ± 5.36	4.1 ± 1.00	−4.5 ± 8.45
	BHQ-2	92.5 ± 0.28	96.3 ± 0.07	96.7 ± 0.08	5.5 ± 1.49	11.7 ± 0.95	33.1 ± 0.57	−9.1 ± 10.8
	BBQ-650	98.2 ± 0.04	96.3 ± 0.14	82.2 ± 0.06	18.6 ± 0.76	26.1 ± 0.19	46.5 ± 0.38	6.05 ± 1.01
	no quencher	3.0 ± 1.42	−35.0 ± 1.07	−38.1 ± 1.76				4.6 ± 15.5

[a] The mean of three measurements ± SD. The mathematical symbol minus means that fluorescence increased after hybridization.

of the heterodimer suggesting a looping of freely rotating 10T overhang (see Supporting Information, Figure S6). The heterodimer has a different absorption spectrum than a simple sum of the fluorophore and the quencher absorption spectra. Thus, **S3** cannot be used in this case for the detection of pure RET-based quenching.

In order to overcome this problem, oligonucleotides **S4**−**S6** were investigated in hybridization assays as well. The rigid double strand of their duplexes with **SF** prevents looping and thus permits the determination of pure RET quenching. At the same time, it enables the investigation of the quenching efficiency at a longer distance. The absorption spectra of the duplexes **S4**−**SF** corresponded well with a simple sum of spectra of **S4** and **SF** indicating the absence of the heterodimer (Supporting Information, Figure S6).

After hybridization, all of the prepared AzaPc-labeled oligonucleotides **S1**−**S6** efficiently decreased the fluorescence signal of **SF**, either Cy5 or FAM-containing (Figure 7). The quenching efficiency (*1, 19*) is shown in Table 2. While all tested quenchers were highly efficient and comparable at a shorter distance (**S1**−**S3**), marked differences appeared at a longer distance between the fluorophore and the quencher (**S4**−**S6**). BHQ-1 quenched only FAM emission, BHQ-2 quenched both FAM and Cy5 emission, but its efficiency considerably decreased with distance. The AzaPc quencher was able to quench the fluorescence of both Cy5 and FAM with higher efficiency than the above-mentioned BHQ dyes and with efficiency comparable to that of the BBQ-650 quencher. This is most likely a consequence of better overlap of the AzaPc quencher absorption and fluorophore emission spectra (Figure 5).

In order to exclude nonselective quenching, two kinds of controls were involved. The decrease of **SF** fluorescence emission by the **S1**−**S3** sequence without any attached quencher was negligible (Table 2). Nonspecific quenching of fluorescence was excluded using Cy5-labeled oligonucleotide **SFX** that was not complementary to **S1**. No quenching occurred for any of the quenchers tested (Table 2).

CONCLUSIONS

In conclusion, novel highly efficient structural type of dark quenchers from the group of AzaPc with advantageous properties was described. A solid-phase method for labeling oligonucleotides by AzaPc quenchers and a simple and fast purification method without a need of HPLC separation were developed. Of particular notice is that the solid-phase conjugation presented here allows for the utilization of automatic processes in DNA/RNA synthesizers for both 3′- and 5′-end labeling by AzaPc.

The prepared AzaPc-based probes efficiently quench both FAM and Cy5 fluorescence in DNA hybridization probes by both RET and contact quenching. The AzaPc quencher profits from absorption in a wide range of wavelengths that covers all fluorophores used in hybridization assays nowadays. Thus, AzaPc may become the quenchers of choice for any kind of routinely used fluorophore and any hybridization assay. Finally, AzaPc represents flexible macrocycles with the possibility of shifting their absorption maximum over 750 nm (*17*). Therefore, they appear to have a great potential in genetic analysis, especially for the imaging of gene expression in living cells, where excitation and emission at long wavelengths is highly desired.

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Supporting Information Available: TLC of the AzaPc **7** in a mixture before isolation, structures of the AzaPc congeners from statistical condensation, stability tests of compound **7**, absorption spectra from HPLC analysis, and absorption spectra of duplexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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