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Bioorganic & Medicinal Chemistry 13 (2005) 2413–2421
Bioorganic & Medicinal Chemistry

Porphyrin substituted phosphoramidites: new building blocks for porphyrin–oligonucleotide syntheses

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Received 11 December 2004; revised 22 January 2005; accepted 25 January 2005

The authors gratefully dedicate this paper to Professor Koji Nakanishi in honor of his 80th birthday

Abstract—Thymidine phosphoramidites containing trispyridylphenyl and tetraphenylporphyrin chromophores attached via a short amide linker in the 3′-position have been synthesized and used as building blocks in solid-phase synthesis of self-complementary 8-mer oligonucleotides 3′-T-5′-GCGCGCA-3′ and 5′-ACGCGCGT-3′. To our knowledge, these are the first porphyrin–oligonucleotide conjugates carrying the porphyrin chromophores in the 3′-position. Chain assembly was achieved by automated solid-phase synthesis and by inexpensive straightforward 'in flask' modification of commercially available solid supported oligonucleotides. This approach allows the synthesis of modified oligonucleotides without using costly instrumentation for automated DNA synthesis. Porphyrin-containing self-complementary oligonucleotides are expected to be a valuable model for drug binding studies and determination of conformational changes in DNA sequences using circular dichroism.

1. Introduction

The porphyrin structure is ubiquitous in nature with essential biochemical functions in plants and animals. The characteristic tetrapyrrole nucleus is involved in a wide range of activities, such as oxygen storage, transport, sensing, oxygen activation, electron transfer, light harvesting, and energy transfer.[1,2] Many of these biological processes depend on porphyrin–porphyrin interactions. Due to the unique spectroscopic aspects that derive from these interactions their monitoring is of fundamental importance.

Porphyrins are versatile and powerful reporter groups for structural studies by circular dichroism (CD) due to their chemical structure and electronic properties. The planar macrocyclic ring can incorporate a variety of metals and can be chemically functionalized in order to tune the solubility, chemical recognition and aggregation. The unique electronic structure[3] is related to the intense (extinction coefficients $\varepsilon \sim 450{,}000$–$550{,}000$), red-shifted Soret absorption band in the 400–450 nm region and the presence of two B transitions perpendicularly oriented. Porphyrins and metalloporphyrins, covalently attached to biopolymers and other biologically important molecules such as peptides,[4] oligosaccharides,[5] glycol lipids,[6] dimeric steroids, and the marine neurotoxin brevetoxin B (BTX-B),[7] have been employed for conformational and configurational analysis by exciton chirality CD.

In the last decade, porphyrin–DNA conjugates and their biomedical,[2] fluorescence,[8] oxidative cleaving,[9] and nuclease activity,[10] were extensively studied as well as their effect on DNA assembly, and thermal stability.[11]

This paper describes the syntheses, isolation and characterization of 3′-tetraarylporphyrin-thymidine-5′-phosphoramidites and their use as building blocks in the synthesis of oligonucleotides–porphyrin conjugates. We expect that the porphyrin chromophores will serve as CD reporter groups of minor conformational variations of DNA in the UV/vis region. The exciton coupled CD signal originating from the chiral twist between porphyrin chromophores at the end of oligonucleotide sequences has been found to be more sensitive than

Keywords: Porphyrin; Automated oligonucleotide synthesis; Modified oligonucleotide; DNA Synthesis; Purification.
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doi:10.1016/j.bmc.2005.01.045

monitoring at the conventional 200–300 nm region to investigate double strand to single strand transitions.[12]

2. Results and discussion

2.1. Molecular design

In order to maximize the potential for conformational studies by CD, the major design criteria for porphyrin–nucleotide conjugates include: (a) simple synthesis and purification of the porphyrin unit; (b) short linker between the porphyrin and the nucleobase; (c) easy modification of the nucleobase.

Considering that TPyrPhP-COOMe **1a** can be easily prepared in a one-step synthesis from inexpensive starting materials and TPhP-COOMe **1b** is commercially available, *meso*-trispyridylphenylporphyrin carboxylic acid (TPyrPhP-COOH, **2a**) and *meso*-tetraphenylporhyrin carboxylic acid (TPhP-COOH, **2b**) have been selected for these studies (Fig. 1). Their hydrophilicity and DNA intercalation properties can be easily modified by metalation of the macrocycle, and pyridyl-nitrogen methylation in the case of TPyrPhP-COOMe.

In search of a favorable design for the application of the exciton chirality method it was necessary to restrict the conformational flexibility at the site of attachment between the porphyrin and the nucleobase. A short and more rigid amide bond has been selected rather than an ester bond (Fig. 2). In our design each porphyrin rotates freely around one preferential axis (corresponding to the linkage to the rest of the molecule). The effective transition dipole moment, directed along that axis, in the 5–15 direction, correctly describes the coupling between the B transitions.[3] (Fig. 2).

Thymidine represents the ideal nucleobase for the introduction of an amide bond in the 3′-position. In contrast to the other nucleobases, it does not require the use of protecting groups. The amino-group can be introduced in the 3′-position with a good yield in four steps following well established literature procedures.[13]

2.2. Synthesis

2.2.1. Porphyrin-thymidine phosphoramidite (6)

2.2.1.1. Porphyrin-carboxylic acid 2a. The synthesis of *meso*-trispyridylphenylporphyrin **1a** was carried out by a modified version of the Adler–Longo method[14] (Scheme 1). The 2-pyridine-carboxaldehyde and the pyrrole were added successively to the methyl-4-formylbenzoate, and acetic anhydride dissolved in propionic acid at 110 °C. Porphyrin oxidation occurred spontaneously, and no oxidation reagent was necessary. The formation of several differently substituted *meso*-arylporphyrins was observed by TLC. The desired porphyrin, **1a**, was easily separated by flash chromatography as the last eluting band in silica gel chromatography and identified by mass spectroscopy and NMR. Not surprisingly, the yield of desired porphyrin **1a** was low (4–5%) as is usual in statistical syntheses. The methyl ester **1a** was hydrolyzed in basic condition using KOH in a mixture MeOH/water to obtain, after neutralization with diluted HCl, trispyridylphenylporphyrin carboxylic acid **2a** (TPyrPhP-COOH) in 87% yield. The tetraphenylporphyrin carboxylic acid (TPhP-COOH, **2b**) was prepared similarly from the commercially available methyl ester **1b**.

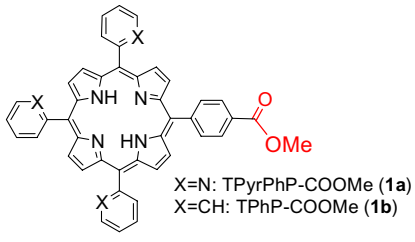


Figure 1. Selected tetraarylporphyrin carboxylic acid methyl esters.

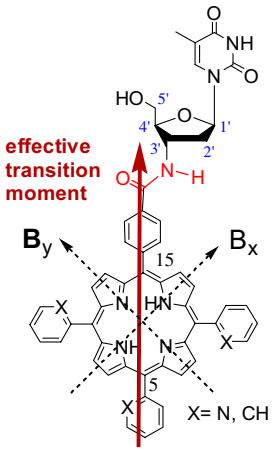
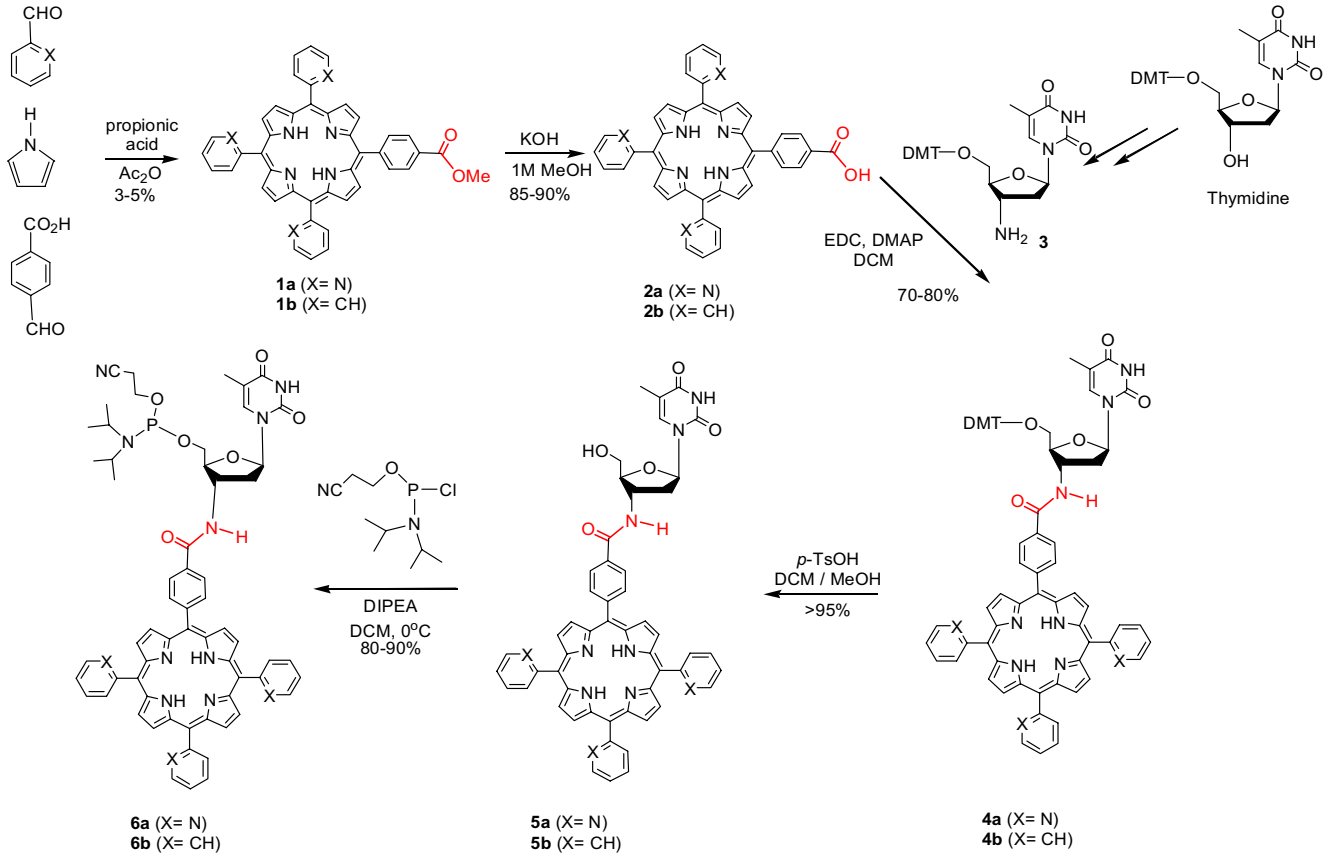


Figure 2. Tetraarylporphyrin-thymidine with shown effective transition moment.

2.2.1.2. Porphyrin-thymidine phosphoramidites 6. Porphyrin carboxylic acid **2a** and **2b** were coupled with 3′-amino-5′-dimethoxytritylthymidine **3**, which was synthesized in four steps from commercially available 3-hydroxy-5′-DMT-thymidine according to published methods,[13] using 1-[3-(dimethylamino)propyl]-3-ethylcarboimide hydroxide (EDC) and 4-dimethylaminopyridine (DMAP) in dry dichloromethane (DCM) under argon. Porphyrin-thymidine **4a** and **4b** were purified by column chromatography using DCM/MeOH/TEA.

The 5′-DMT protecting group was removed under acidic conditions with a 4% solution of *p*-TsOH in a DCM/MeOH at 0 °C in 15 min to yield **5a** and **5b**. Trispyridylphenylporphyrin-thymidine **5a** was purified by flash column chromatography on silica gel using a mixture DCM/MeOH/TEA. If triethylamine is not used, differently charged species with different retention times occur, which lead to several chromatographic bands on the silica gel column. Tetraphenylporphyrin-thymidine **5b** was chromatographed using a mixture DCM/MeOH.

Compound **5** did not react under standard phosphitylation conditions, even after using large excesses



Scheme 1. Synthesis of 3′-porphyrin-thymidine 5′-phosphoramidites.

(5–10 equiv) of the phosphoramidite reagent. We determined that **5** had to be washed with water prior to reaction in order to remove inorganic salts originating from using TEA and MeOH as eluents for the chromatography on silica gel. A triethylammonium salt impurity was identified by ^{1}H NMR (triplet, quartet and broad singlet at 1.20, 3.12, and 12.04 ppm, respectively, in the 9:6:1 ratio). The formation of this salt was verified by a blank chromatography (without any product loaded) using the same eluting system. The presence of this inorganic impurity inhibits the phosphitylation (the formation of products **6**). However, phosphitylation of the washed porphyrin-nucleotide **5a** and/or **5b** with 5 equiv of 2-cyanoethyl-*N*,*N*-diisopropylchloro phosphoramidite in the presence of diisopropylethylamine (DIPEA, 10 equiv) in dry DCM under dry argon provided the corresponding phosphoramidites **6a** and/or **6b** in good yields. These phosphoramidites have been purified under argon by flash chromatography over silica gel and were used immediately after the purification. These products were found to be highly unstable due to over-oxidation, which occurs much faster than in the cases of non-porphyrin-thymidine phosphoramidites. Therefore, these key building blocks were handled under argon at 0 °C and carefully characterized by ^{1}H, ^{31}P NMR, and ESI mass spectrometry. ^{31}P NMR spectroscopy was an unambiguous method of following the progression of phosphitylation and undesired over-oxidation. The ratio of oxidized to non-oxidized could not be determined by mass spectrometry because over-oxidation of the product occurs immediately under mass spectrometry condition.

A ^{31}P NMR spectrum of key phosphoramidite intermediate **6a** (as a mixture of two diastereomers) with small amount of over-oxidized phosphate is illustrated in Figure 3c.

2.2.2. Porphyrin-thymidine oligonucleotide. The self-complementary octamer sequences 5′-ACGCGCGT-3′ and 5′-TGCGCGCA-3′ has been extensively studied by Raman[15] and NMR[16] spectroscopy and thus provide an ideal platform for conformational studies of modified oligonucleotides. Therefore, the oligonucleotides sequences 5′-ACGCGCG-3′ and 5′-GCGCGCA-3′ were selected to incorporate 3′-*meso*-tetraarylporphyrin-thymidine-5′-phosphoramidites **6**.

2.2.3. Oligonucleotide synthesis. The porphyrin group blocks the 3′-position of the thymidine and the standard 3′→5′ synthesis cannot be applied. Thus, we pursued two different synthetic routes to prepare the desired oligonucleotides:

2.2.3.1. Reverse 5′ to 3′ automated DNA synthesis. The oligonucleotide 5′-ACGCGCG-3′ was synthesized in the reverse 5′→3′ direction via automated oligonucleotide synthesis using commercially available reverse 5′-2-cyanoethyl phosphoramidites.[17] The porphyrin derivatized thymidine is attached to a growing 7-mer

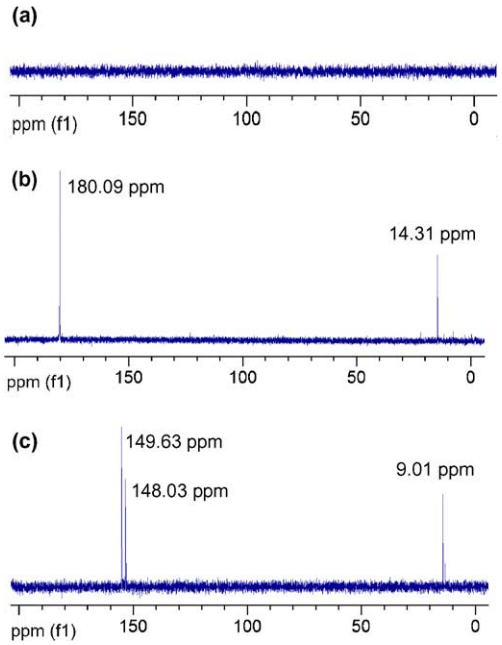


Figure 3. ^{31}P NMR spectrum taken at 121.5 MH: (a) free hydroxyl on 5′ of porphyrin–thymidine before conversion to the phosphoramidite, (b) 2-cyanoethyl-*N*,*N*-diisopropylchloro-phosphoramidite, (c) phosphoramidite on 5′ of porphyrin-thymidine **6a**.

chain 5′-ACGCGCG-3′ (**7ss**) on solid supported CPG resin via the phosphoramidite function on the 5′-position of the thymidine.[18] The chain assembly used standard DNA synthesis cycles, except that the coupling time was extended to 30 min for the phosphitylation with **6**. Due to the low solubility in pure acetonitrile, porphyrin–thymidine conjugates were dissolved in 25% DCM/acetonitrile. The stepwise coupling yields, monitored by the trityl cation essay, vary between 85–90% for the guanosine and 95–99% for cytosine. The key-coupling step is shown in Scheme 2.

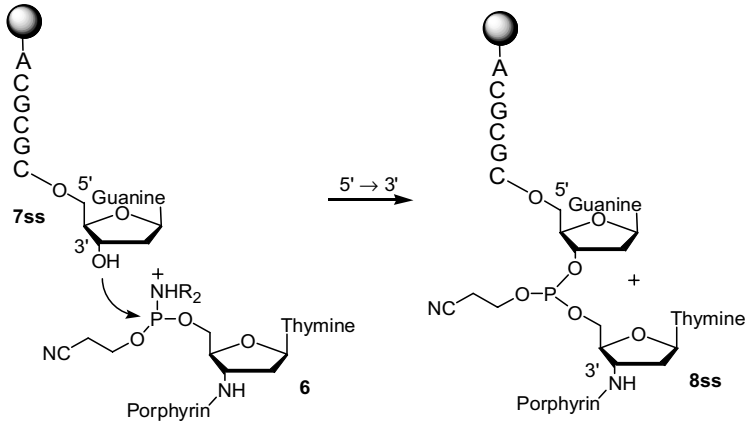
2.2.3.2. 'In flask' solid-phase synthesis by functionalization of the resin. The porphyrin thymidine was attached through a 5′ to 5′ coupling on a 7-mer 5′-GCGCGCA-solid support (**9ss**), which is commercially available and was prepared by regular 3′→5′ synthesis.[19] (Scheme 3) It has been shown previously that the 5′ to 5′ bond between two bases in an oligonucleotide chain does not cause any significant destabilization of the double helix.[17,20] Measured melting temperatures T_m of the standard sequence and the sequence with one 5′–5′ bond are shown in Table 1.

The sequence **9ss** is fully protected, except for the 5′-OH group, which is used for the coupling step with 3′-porphyrin-thymidine-5′-phosphoramidite **6**. This mixture was shaken in acetonitrile/tetrazole (an activation solution) at room temperature for 30 min to achieve good yield. After phosphitylation, the intermediate **10ss** was oxidized, and cleavage from the resin gave the crude porphyrin–oligonucleotide **11**.

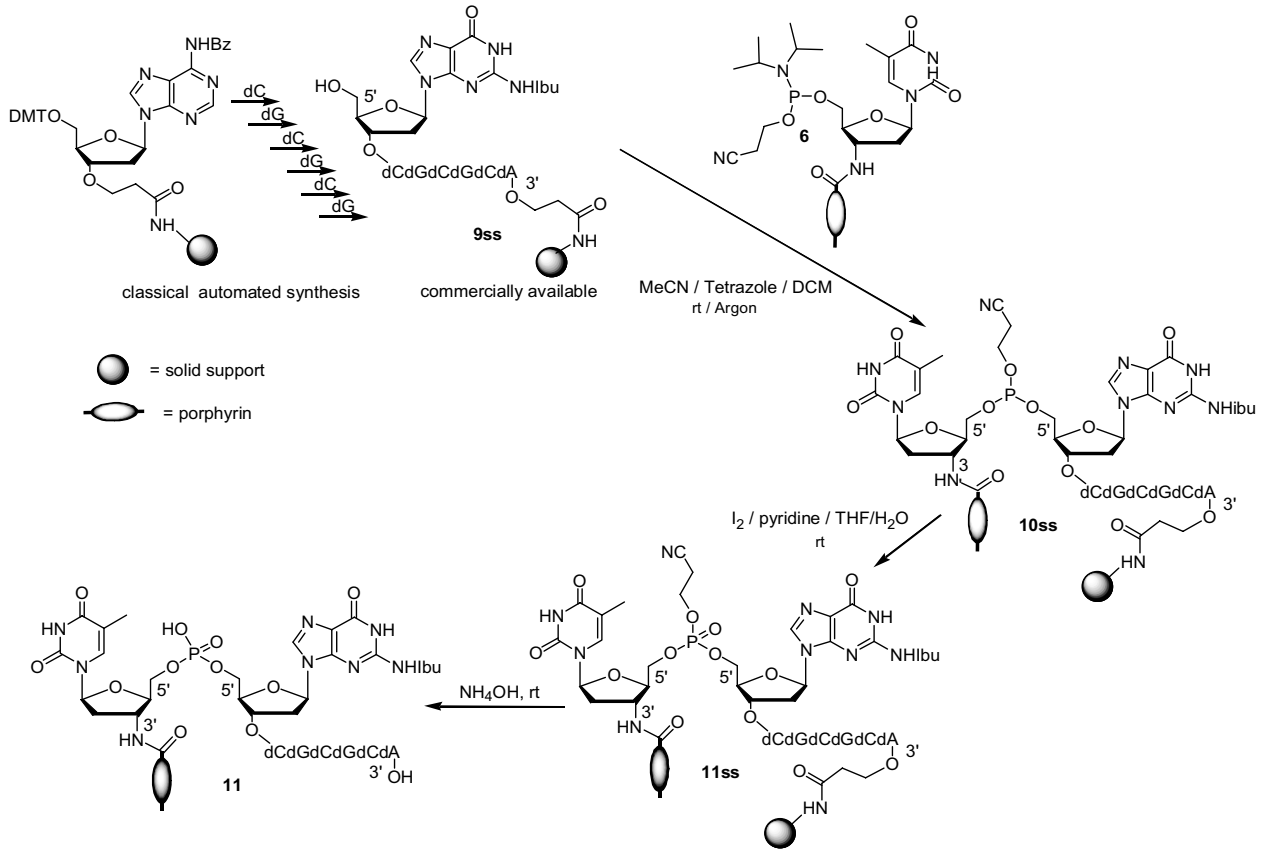
Although automated synthesis is widely used for oligonucleotide synthesis, we have sometimes observed unexpectedly low coupling yields of the porphyrin–thymidine to the oligonucleotide strand. On the other hand, 'in flask' synthesis always provided our desired product with good coupling yield. This is probably due to the fact that the reaction mixture can be stirred, which allows better contact between the sterically hindered phosphoramidite **6** and solid supported 7-mer **9ss**. Additionally the functionalization of the resin does not require any expensive instrumentation as for automated DNA synthesis, and the reactions can be done easily in laboratories that do not specialize in oligonucleotides synthesis.

2.3. Isolation and characterization of oligonucleotides

Release from solid support and deprotection of the synthesized 3′-porphyrin-modified oligonucleotides were achieved by concentrated aqueous ammonia solution (26 h, rt). These conditions did not cause cleavage of the porphyrin appendages. In order to remove failure sequences and residual byproducts, the crude oligonucleotide conjugates were pre-purified and desalted by using DNA purification cartridges from ChemGenes. It is known that the presence of the DMT group is important for this procedure since the separation relies upon the affinity of the resin for this hydrophobic group.



Scheme 2. The key-coupling step of reverse phase synthesis between 7-mer sequence 5′-ACGCGCG-3′ **7ss** and 3′-*meso*-tetraarylporphyrin-thymidine-5′-phosphoramidite **6**.



Scheme 3. 'In flask' solid-phase synthesis by functionalization of the resin. The porphyrin thymidine attached through a 5′ to 5′ coupling on a 7-mer 5′-GCGCGCA-solid support (**9ss**).

Table 1. Melting temperatures of studied oligonucleotides (50 mM phosphate buffer, pH = 7.0)

Duplex	T_m (°C)
5′-TGCGCGCA-3′	49
3′-T-5′-GCGCGCA-3′	48

Tetraarylporphyrin can mimic the role of the DMT group as a hydrophobic 'handle' for the resin to separate the modified oligonucleotide from the unmodified one. The failure strands do not contain porphyrins, and thus do not bind to the column and were easily washed off.

The pre-purified and desalted product was analyzed using MALDI-TOF mass spectroscopy. MALDI-TOF spectra of TPyrP-3′-T-5′-GCGCGCA **11a** and TPhP-3′-T-5′-GCGCGCA-3′ **11b** using a 2,4,6-trihydroxy acetophenone (THAP) plus ammonium citrate matrix gave mass peaks at m/z = 3047 and 3058, respectively. These values were within experimental error of the calculated molecular weights of **11a** (3053) and **11b** (3047). A lower intensity peak (2105), which corresponded to the sequence 5′-GCGCGCA-3′ without the incorporation of the thymidine–porphyrin, was observed only in the case of the synthesis of TPhP-3′-T-5′-GCGCGCA-3′ **11b** (Fig. 4).

Purification was achieved by semi-preparative reverse phase HPLC by taking advantage of the hydrophobicity

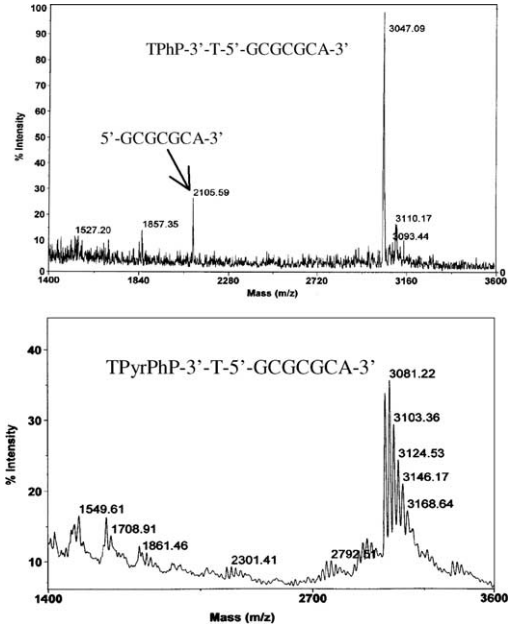


Figure 4. MALDI MS spectra of prepared *meso*-tetraarylporphyrin-modified oligonucleotides **11b** (above) and **11a** (below).

of the porphyrin group. During the chromatographic separation, the profiles were monitored by a two-channel array detector from JASCO using 260 nm for the

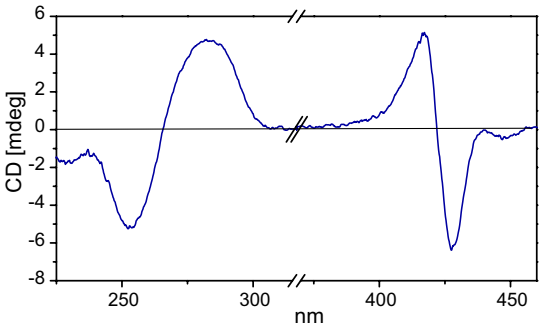


Figure 5. CD spectrum of prepared *meso*-tetraarylporphyrin-modified oligonucleotides **11a**.

oligonucleotides and 420 nm for the porphyrins. The desired product was eluted with a retention time of 59.5–61 min. The UV/vis spectra demonstrated two absorption maxima, one at 419 nm (Soret band of the *meso*-tetraarylporphyrin) and the other at 260 nm (oligonucleotide).

CD measurements (Fig. 5) indicated that incorporation of *meso*-tetraarylporphyrins at the 3′-position of the studied oligonucleotides prepared by 5′ to 3′ coupling (octamer **8**) or by 5′ to 5′ coupling (octamer **11**) did not cause any significant modification of the B-DNA profile observed by CD spectroscopy between the modified and unmodified sequences.

3. Conclusion

In this paper, we described the synthesis of new 3′-porphyrin-5′-phosphoramidites, which have been used as building blocks for the synthesis of porphyrin–oligonucleotide conjugates. These oligomers have been obtained by using reverse 5′ to 3′ automated DNA synthesis and by simple and efficient solid-phase methodology. The so called 'in flask' synthesis, is less expensive and more convenient when working with limited amounts of highly sensitive porphyrin–phosphoramidites. The CD spectra of the 3′-porphyrin-modified and unmodified sequences below 300 nm exhibited the same CD profile, typical of a right handed B-DNA. It is anticipated that the porphyrin chromophores at the end of the DNA sequences can serve as sensitive reporter groups for long-range exciton chirality CD studies in the visible region: thus, the conformational variations induced in the DNA backbone can be detected by CD changes within the porphyrin Soret region far from typical DNA absorption bands. Up to now, this approach has been successfully applied to detect the double strand to single strand transitions.

4. Experimental

4.1. General

Reagents and materials were obtained from commercial suppliers and used as received. Dichloromethane (DCM) was dried using activated 4 Å molecular sieves under argon. If it was necessary, the reactions were performed in vacuum dried glassware under argon. Purification was performed by flash column chromatography using ICN silica gel (32–63 mesh).

Automated DNA synthesis was performed on Expedite 8900 nucleic acid synthesis system. Oligonucleotides attached to solid support are commercially available from AlphaDNA. DNA purification cartridges (Puri-Pak 0.2 μM), activation and oxidation reagents were purchased from ChemGenes Corporation. The porphyrin–oligonucleotides were purified on JASCO or/and Waters HPLC system using Waters reverse phase XTerra column MS C_{18} 2.5 μm 10×50 mm equipped with column heater Fenomenex Thermasphere TS-130.

400 MHz ^{1}H NMR and 121.5 MHz ^{31}P NMR spectra were obtained on a Bruker Advance spectrometer and are reported in parts per million (δ) with coupling constants in hertz (Hz). ^{1}H NMR spectra were referenced to an internal TMS standard. ^{31}P spectra were referenced to an external 85% aqueous H_3PO_4 standard. FAB Mass spectra were measured on a JEOL JMS-DX303 HF mass spectrometer using a glycerol matrix and Xe ionizing gas. MALDI-TOF MS were performed on a Voyager Applied Biosystems spectrometer using a 2,4,6-trihydroxy acetophenone (THAP) plus ammonium citrate matrix. UV–vis spectra were recorded on Shimadzu UV-1601 spectrophotometer or/and on JASCO V-530 spectrophotometer.

4.2. 4-(10,15,20-Trispyridyl-porphyrin-5-yl)-benzoic acid methyl ester, TPyrPhP-COOMe (1a)

Methyl-4-formylbenzoate (2.0 g, 12.18 mmol) was dissolved in 90 mL of propionic acid. To this solution, 7.5 mL of acetic anhydride were added. The solution was stirred and heated under argon to a temperature of 115 °C. Once this temperature was reached, 2.2 mL (22.84 mmol) of 2-pyridine-carboxaldehyde and 2.1 mL (30.45 mmol) of pyrrole were added drop wise and successively. The solution was then heated, in the dark, at 120 °C for 1.5 h. The solution was allowed to cool to room temperature, evaporated, and placed under vacuum. Three distinct spots were observed in the TLC when using ethyl acetate as eluent. The product with $R_f = 0.12$ was isolated. A total of 240 mg of TPyrPhP-COOMe were obtained with a 4.6% yield.

UV–vis (CH_2Cl_2) 417.4 (3.256×10^5), 512.7 (1.59×10^4), 587.5 (4.96×10^3), 643.8 (2.15×10^3). ^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 9.11 (d, 3H, CH), 8.85–8.79 (m, 8H, pyrrole CH), 8.42 (d, $^{3}J = 8$ Hz, 2H, CH), 8.28 (d, $^{3}J = 8$ Hz, 2H, CH), 8.25 (m, 3H, CH), 8.09 (3H, CH), 7.70 (m, 3H, CH), −2.80 (s, 2H, NH). FAB-HRMS calcd for $C_{43}H_{29}N_7O_2$ (MH^+) 675.24, found 675.2468.

4.3. 4-(10,15,20-Triphenyl-porphyrin-5-yl)-benzoic acid methyl ester, TPhP-COOMe (1b)

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 8.83 (m, 6H, CH), 8.76 (d, 2H, CH), 8.41 (d, 2H, CH), 8.28 (d, 2H, CH),

8.19 (m, 6H, CH), 7.74 (m, 9H, CH), 4.10 (s, 3h, Me), −2.76 (s, 2H, NH).

4.4. 4-(10,15,20-Tri-pyridin-2-yl-porphyrin-5-yl)-benzoic acid, TPyrPhP-COOH (2a)

The methyl ester from the previous reaction (140 mg, 0.21 mmol) was dissolved in 5.6 mL of ethanol and 11 mL of 2 M NaOH. The solution was refluxed for 2 h. The reaction was then allowed to cool to room temperature. Once cooled, an equivalent amount of 1 M HCl (22 mL) was added. The resulting solution (pH = 5) was then extracted several times with ethyl acetate. The product was pure according to TLC and was used in the next reaction without further purification. A total of 120 mg of TPyrPhP-COOH were obtained with an 87% yield.

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 8.95 (d, ^3J = 4 Hz, 3H, pyrrole CH), 8.75 (broad band, 8H, pyrrole CH), 8.36 (d, ^3J = 8 Hz, 2H, CH), 8.20 (m, 3H, CH), 8.16 (d, ^3J = 7.9 Hz, 2H, CH), 8.13 (m, 3H, CH), 7.82 (m, 3H, CH), −2.91 (s, 2H, NH). FAB-HRMS calcd for $C_{42}H_{27}N_7O_2$ (MH^+) 661.2226, found 661.2262.

4.5. 4-(10,15,20-Triphenyl-porphyrin-5-yl)-benzoic acid (2b)

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 8.83 (m, 6H, CH), 8.76 (d, 2H, CH), 8.41 (d, 2H, CH), 8.28 (d, 2H, CH), 8.19 (m, 6H, CH), 7.74 (m, 9H, CH), −2.76 (s, 2H, NH). FAB-HRMS calcd for $C_{45}H_{30}N_4O_2$ (MH^+) 658.2369, found 658.2367.

4.6. *N*-[2-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-4-(10,15,20-trispyridin-2-yl-porphyrin-5-yl)-benzamide, TPyrPhP-CONHThyDMT (4a)

Porphyrin acid **2** (46 mg) was dissolved, under argon, in 2 mL of dry DCM. To the red solution, DMAP (9.34 mg, 0.0765 mmol), EDC (39 mg, 0.073 mmol), and 3′-aminothymidine **3** (39 mg, 0.073 mmol) were added successively. The solution was stirred at room temperature overnight. The crude reaction mixture was loaded directly onto a silica gel column and eluted using a mixture of DCM/MeOH = 95/5 with 0.5% of triethyl amine (TEA). A total of 60 mg of TPyrPhP-CONHThyDMT (R_f = 0.22, DCM/MeOH = 9/1) were obtained with a 73% yield.

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 9.07 (m, 3H, CH), 8.88–8.71 (m, 8H, pyrrole CH), 8.12–8.19 (m, 6H, CH), 8.00 (m, 3H, CH), 7.92 (s, 1H, NH), 7.64 (m, 4H, CH), 7.40 (d, 2H, CH), 7.28 (m, 4H, CH), 7.20 (m, 2H, CH), 7.09 (s, 1H, CH), 6.73 (m, 4H, CH), 5.00 (s, 1H, NH), 4.25 (s, 1H, CH), 3.59 (s, 3H, CH_3), 3.55 (s, 3H, CH_3), 3.59 (s, 3H, CH_3), 3.20 (q, 1H, CH), 2.54 (s, 2H, CH_2), −2.81 (s, 2H, NH). FAB-HRMS calcd for $C_{73}H_{58}N_{10}O_7$ (MH^+) 1186.4490, found 1186.4586.

4.7. *N*-[2-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-4-(10,15,20-triphenyl-porphyrin-5-yl)-benzamide (4b)

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 8.84 (m, 6H, CH), 8.75 (d, 2H, CH), 8.21 (m, 10H, CH), 7.74 (m, 10H, CH), 7.43 (m, 3H, CH), 7.33 (m, 3H, CH), 7.25 (m, 4H, CH), 7.13 (m, 1H, CH), 6.78 (m, 3H, CH), 6.61 (m, 1H, CH), 5.05 (m, 1H, CH), 4,31 (br s, 1H, NH), 3.69 (m, 1H, CH), 3.62 (s, 3H, CH_3), 3.58 (m, 1H, CH), 3.56 (s, 3H, CH_3), 2.68 (m, 2H, CH), 1.50 (s, 3H, CH_3), −2.78 (s, 2H, NH). FAB-HRMS calcd for $C_{76}H_{61}N_7O_7$ (MH^+) 1183.4632, found 1183.4644.

4.8. *N*-[2-Hydroxymethyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-4-(10,15,20-trispyridin-2-yl-porphyrin-5-yl)-benzamide, TPyrPhP-CONHThyOH (5a)

5′-DMT–porphyrin–thymidine conjugate (60 mg) was dissolved in 1.3 mL of 60/40 (by volume) mixture of DCM/MeOH. The solution was placed in an ice bath and a 4% solution of *p*-TsOH in an 80/20 solution of DCM/MeOH was added drop wise. The solution turned from dark red to dark green in color. Once the reaction was complete (followed by TLC), 2.5 mL of a saturated solution of Na_2CO_3 were added. The product was extracted using DCM. The crude product was purified via column chromatography over silica gel using a 90/9/1 mixture of DCM/MeOH/TEA (R_f = 0.35). The obtained product was dissolved in DCM and was washed three times with water (to remove inorganic impurities). After evaporation, 42 mg of TPyrPhP-COOThyOH was obtained with a 95% yield.

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 9.10 (m, 3H, CH), 8.84 (s, 4H, CH), 8.79 (d, 2H, CH), 8.68 (d, 2H, CH), 8.23 (m, 3H, CH), 8.06 (m, 6H, CH), 8.18 (d, 1H, CH), 7.88 (s, 1H, NH), 7.64 (s, 1H, OH), 7.59 (s, 1H, CH), 6.96 (s, 1H, CH), 5.80 (s, 1H, CH), 4.30 (s, 1H, CH), 3.65 (m, 2H, CH), 3.54 (s, 1H, NH), 1.89 (s, 3H, CH_3), −2.88 (s, 2H, NH). FAB-HRMS calcd for $C_{52}H_{49}N_{10}O_5$ (MH^+) 884.3183, found 884.3220.

4.9. *N*-[2-Hydroxymethyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-4-(10,15,20-triphenyl-porphyrin-5-yl)-benzamide, TPhP-CONHThy-OH (5b)

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 8.82 (br s, 6H, CH), 8.74 (m, 2H, CH), 8.26 (s, 4H, CH), 8.17 (m, 6H, CH), 7.65–7.7.80 (m, 11H, CH), 6.39 (t, ^3J = 6 Hz, 1H, H^1), 5.40 (br s, 1H, NH), 4.83 (m, 1H, CH), 4.13 (m, 1H, CH), 4,02 (m, 1H, CH), 2.70 (m, 1H, CH), 2.46 (m, 1H, CH), 1.98 (s, 3H, CH_3), 1.24 (t, 1H, OH), −2.79 (s, 2H, NH). FAB-HRMS calcd for $C_{55}H_{43}N_7O_5$ (MH^+) 881.3326, found 881.3422.

4.10. TPyrPhP-CONHThy-Phosphoramidite (6a)

TPyrPhP-CONHThyOH (8.9 mg, 0.01017 mmol) was dissolved in anhydrous DCM under argon and at room

temperature. Diisopropylethylamine (DIPEA, 17.7 μL, 0.1017 mmol) was added and the resulting solution was placed in an ice bath. 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite was added via a syringe (11.4 μL, 0.05085 mmol). The reaction mixture was stirred for 70 min at 0 °C; it was then directly loaded, under argon, onto a short silica gel column (7 cm) and eluted with a 96/2/2 mixture of DCM/MeOH/TEA. After evaporation, the pure product was used *immediately* in the next reaction. The prepared amount was used for a 1 μM ODN solid-phase synthesis (using 1 μM scale solid support).

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 9.09 (m, 3H, CH), 8.83 (d, 8H, CH), 8.42 (m, 2H, CH), 8.22 (m, 6H, CH), 8.10 (m, 3H, CH), 7.70 (m, 3H, CH), 6.47 (m, 1H, CH), 5.82 (s, 1H, CH), 4.34 (s, 1H, CH), 3.64 (m, 2H, CH), 3.54 (br s, 1H), 1.87 (d, 3H, Me), 1.15 (m, 12H, Me), −2.83 (s, 2H, NH). MS: M^+ = 1084.10.

4.11. TPhP-CONHThy-Phosphoramidite (6b)

^{1}H NMR (400 MHz, $CDCl_3$) ppm (δ) 8.82 (br s, 6H, CH), 8.76 (d, ^3J = 6 Hz, 2H, CH), 8.34 (dd, ^3J = 8 Hz, J = 2.5 Hz, 2H, CH), 8.26 (d, ^3J = 8 Hz, 2H, CH), 8.18 (dd, ^3J = 8 Hz, J = 1.5 Hz, 6H, CH), 8.06 (dd, ^3J = 6 Hz, J = 1.5 Hz, 1H, CH), 7.72 (m, 11H, CH), 6.64 (dd, ^3J = 8 Hz, ^3J = 6 Hz, 0.5H, H^1), 6.55 (dd, ^3J = 8 Hz, ^3J = 6 Hz, 0.5H, H^1), 4.88 (m, 0.5H, first diastereomer), 4.79 (m, 0.5H, second diastereomer), 4.50 (m, 0.5H, CH, first diastereomer), 4.46 (m, 0.5H, CH, second diastereomer), 4.20 (m, 2H), 4.13 (m, 1H, CH), 4.02 (m, 3H, CH), 2.70 (m, 1H, CH), 2.46 (m, 1H, CH), 2.00 (d, J = 4 Hz, 3H, Me), 1.13 (m, 12H, Me), −2.77 (s, 2H, NH). MS: M^+ = 1081.48.

4.12. Automated 5′ to 3′ DNA synthesis

The oligonucleotide **8** was synthesized on 1.0 μM scale on 500-Å CPG solid support in the opposite sense (5′→3′) from standard synthesis (3′→5′) using 5′-phosphoramidites on an Expedite 8900 synthesizer with extended time (30 min) for porphyrin substituted thymidine. The porphyrin-thymidine phosphoramidite **6** was dissolved in 25% DCM/activation reagent (acetonitrile/tetrazole) due to low solubility in pure acetonitrile. The stepwise coupling yields, determined from monitoring the trityl cation formation by UV/vis spectroscopy, vary between 85% and 90% for the guanosine and 95–99% for cytosine. The porphyrin modified thymidine **6** was attached in the final step.

4.13. In flask' 5′ to 5′ DNA synthesis

TPyrPhP-CONHThyPA **6** from the previous reaction (0.01017 mmol) was dissolved in 1 mL of a 25/75 mixture of DCM/activation reagent (acetonitrile/tetrazole) under dry argon at room temperature. The 7-mer 5′-GCGCGCA-*solid support* (**9ss**, amount of one 1 μM cartridge dried in vacuum) was added in one portion against positive argon pressure. The reaction mixture was stirred (without using a stirring bar) for 30 min at room temperature in the dark. After filtration and washing (MeCN, DCM, and MeOH), 1 mL of the oxidizing reagent was added (I_2 in water/THF/pyridine). The resulting solution was stirred for 5 min. The solution was filtered off and a red colored resin was obtained after repeated exhaustive washing (MeCN, DCM, and MeOH). The prepared ODN–porphyrin conjugates **11** were purified as described below.

4.14. Cleavage from resin and deprotection

Highly graded concentrated ammonia solution was added to the resin containing prepared ODN–porphyrin conjugate (800 μL for 1 M cartridge) in screw-cap vial. After incubation for 2 h with occasional shaking, the red supernatant containing cleaved oligonucleotides were carefully transferred (without the beads) to a new screw-cap tube. The complete deprotection was achieved in 24 h at room temperature with moderate shaking. Higher temperatures (~50 °C) could cause the cleavage of the porphyrin–oligonucleotide's amide bond.

4.15. Purification

The crude oligonucleotide was pre-purified using DNA purification cartridges Puri-Pak from ChemGenes. The ammonia solution was loaded onto the pre-equilibrated cartridge (800 μL per one 0.2 μM cartridge). The cartridge was washed with deionized water and ammonium acetate solution in order to remove any failure sequences. The desired porphyrin–oligonucleotide conjugate was eluted using a 50/50 mixture of water/acetonitrile, concentrated under reduced pressure and finally dried under vacuum.

The final purification was done on a JASCO HPLC system using Waters semi-preparative reverse phase XTerra column MS C_{18} 2.5 μm 10 × 50 mm equipped with Phenomenex Thermasphere TS-130 column heater. Chromatographic condition: T = 50 °C, flow = 1 mL/min, linear gradient solvent system: MeOH/buffer A (100 mM hexafluoroisopropanol/8.6 mM TEA in water Table 2).[21] The crude oligonucleotide from one cartridge purification (the amount originating from 1 μM oligonucleotide synthesis) was dissolved in 200 μL HPLC water. 100 μL of this sample was injected for each run.

During the chromatographic separation, the profiles were monitored by a multiwavelength detector from JASCO (MD-1510) using 260 nm channel for the oligonucleotides and 420 nm channel for the porphyrins. The desired product was eluted with a retention time of 59.5–61 min.

Table 2. Solvent gradient used for HPLC purification

Time (min)	Buffer A	MeOH
0	88%	12%
30	64.5%	35.5%
90	20%	80%
120	5%	95%

4.16. Melting temperature experiments

A 50 mM phosphate buffer solution (pH 7.0) was used for the melting experiments. Melting curves were measured using 1 mL of the tested solution in Teflon capped quartz cuvettes of 1 cm optical path length. After thermal equilibration at 20 °C, UV absorption was monitored as a function of the temperature at 260 nm, increased by 1 °C/4 min, typically in the range 20–80 °C. The temperatures relative to the duplex melting, reported in Table 1, were determined as the maxima of the first derivative of absorbance versus temperature plots.

Acknowledgements

Funding of this work was provided by the National Institute of Health, GM 34509 (to N.B.) and GM 065716-01 (to G.P.). We are thankful to Professor J. Ju, Dr. Z. Li, Dr. Y. Itagaki, Professor A. McDermott, and K. Varga from Columbia University for extensive use of their facilities. We are also grateful to Dr. V. Otto, AlphaDNA for extensive discussions and help. We acknowledge Pamela C. Rodriguez, an NSF REU summer student, for her contribution in the synthesis of 3′-aminothymidine.

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