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Porphyrin conjugated to DNA by a 2′-amido-2′-deoxyuridine linkage

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Abstract—A porphyrin that contains a single carboxylic acid group was synthesized and coupled to 2′-amino-2′-deoxyuridine. The resultant product contained a free 3′ hydroxyl group and a 4,4′-dimethoxytrityl (DMT) protecting group on the 5′ hydroxyl of the uridine, making it suitable for use in oligonucleotide synthesis. The 3′ H-phosphonate derivative of this molecule was synthesized and used to form a conjugate with a 19 nucleotide sequence of DNA (5′-CCTCCAGTGGAAATCAAGG-3′). This was carried out with the DNA attached at the 3′ end to a control pore glass (CPG) substrate, allowing for rapid purification. After removal of the DMT group, an additional three nucleotides were added, leaving the porphyrin as an internal modification. This is the first report of porphyrin attached internally to an oligonucleotide using a hydrogen-bonding nucleoside analog. This allows oligonucleotides to be used as a scaffold for precise positioning of multiple porphyrins within biomimetic arrays.

Porphyrins perform a wide variety of functions in natural and synthetic systems. They have been used in light-activated cancer treatment,[1] are the functional element of light harvesting systems,[2] and have been used in sensor design due to their fluorescent and electrochemical properties.[3,4] Arrays that contain multiple porphyrins are also of interest in biological and synthetic systems since unique catalytic and photonic properties arise when porphyrins are held in close proximity to each other.[5–10] For example, the light-harvesting complex of photosystem II contains a number of porphyrinic chlorophyll and xanthophyll molecules spatially arranged to optimize light collection and energy transfer.[11,12]

Synthetic chemists strive to mimic the function of these complex biological systems and oligonucleotides can provide an organized scaffold for precise positioning of porphyrins within arrays. Two general strategies have been employed for conjugating porphyrins to oligonucleotides. In the first approach a porphyrin is coupled after the oligonucleotide has been synthesized and removed from a solid-phase support.[13] Typically this is accomplished by synthetic incorporation of a nucleophilic site at either the 3′ or 5′ end of the oligonucleotide that is subsequently coupled to an activated porphyrin. A second approach is to perform the porphyrin conjugation while the oligonucleotide remains on the solid-phase support. This approach simplifies purification and is advantageous in driving reactions to high yield. In one example, a 5′ porphyrin–oligonucleotide conjugate was synthesized on a control pore glass (CPG) support by an H-phosphonate method.[14] In another example, porphyrins have been conjugated to the 3′ and 5′ ends of oligonucleotides using phosphoramidite chemistry.[15,16] In each of these approaches, only a single porphyrin can be added to the oligonucleotide chain. To fully employ oligonucleotides as a scaffold for constructing arrays of multiple porphyrins, modification at internal positions without interrupting base pairing is necessary. Methods of internal modification have been reported, however, the modifiers that were used lacked hydrogen-bonding nucleosides and therefore are incapable of base pairing.[17]

A promising new route to internal modification of oligonucleotides is accomplished using a modified nucleoside that contains a 2′ amine group, a free 3′ hydroxyl group, and a 4,4′-dimethoxytrityl (DMT) protected 5′ hydroxyl group.[18] 2′-Amino-2′-deoxyuridine provides a convenient route for modification of the deoxyribose ring through coupling to a nucleophilic amine.[19] This approach avoids disruption of the hydrogen-bonding base

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by attachment to the 2′ position on the deoxyribose ring. In order to use aminouridine derivatives in an automated DNA synthesizer the 3′ hydroxyl is converted to an H-phosphonate or phosphoramidite derivative. 3′ Phosphoramidites of 2′ amido-modified uridine,[20,21] adenosine,[22] and guanine[23] have been previously reported. H-phosphonate chemistry has been used to synthesize DNA[24] and to modify oligonucleotides with porphyrins,[14] flavins,[25] and a benzo[*a*]pyrene derivative.[26]

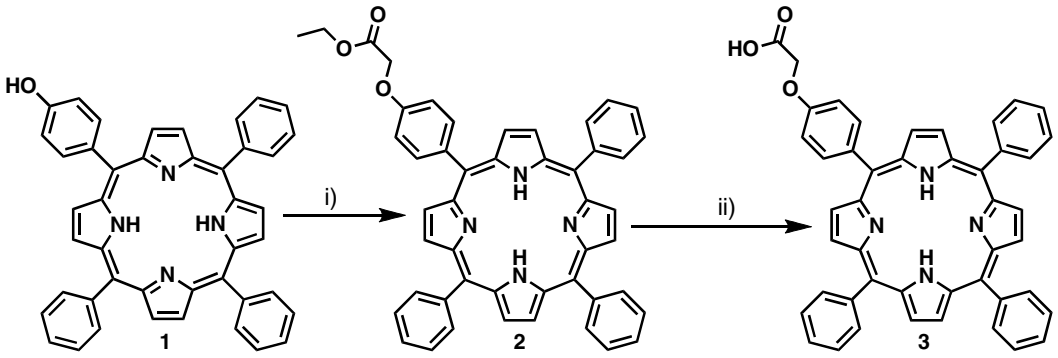
A novel route is described here to modifying DNA using a porphyrin coupled to the nucleoside, 2′-amino-2′-deoxyuridine. The synthesis of a 2′-amide linked porphyrin–uridine conjugate is reported and a method for activating it at the 3′ position using H-phosphonate chemistry is described.[27] Attachment of the porphyrin at the 2′ position leaves the 5′-DMT available for further reactions. Nucleotides modified at the 2′ position with aromatic groups tethered by appropriate length linkers have been shown to increase rather than disrupt base-pairing strength in DNA duplexes.[28] Furthermore, we demonstrate that this 3′ H-phosphonate can be used in solid-phase DNA synthesis by coupling it to a 19-mer DNA. Use of a 5′-DMT allowed for oligonucleotide extension after deprotection, leaving the porphyrin as an internal modification after continued synthesis. This approach allows for the inclusion of additional bases or additional modifiers (e.g., thiol, amine, or biotin) beyond the nucleotide that contains the porphyrin. Another benefit of this route is that it can accommodate porphyrins with different core structures,[1c] functional groups on the phenyl rings of the porphyrin, and linkers of various lengths to the oligonucleotide.[15,28]

Synthesis[29] of the 2′ porphyrin modified uridine started with preparation of porphyrin **1**, functionalized with a single hydroxyl group. Tetraphenyl porphyrin and its derivatives are commonly used as a starting point for the design of biomimetic porphyrin arrays[5] due to their synthetic tunability. By varying the functional groups on the peripheral phenyl rings of tetraphenyl porphyrin it is possible to alter the solubility and reactivity of the porphyrin without disrupting its interesting electronic and optical properties. A porphyrin functionalized with a single hydroxyl group, 5-(4-hydroxyphenyl)-10,15,20-triphenyl porphyrin, was synthesized by modification of literature procedures.[30,31] Compound **1** can be synthesized as described in Ref. 32 or by adapting a low-solvent, microwave method.[30] Compounds **2** and **3** were synthesized as shown in Scheme 1 by modification of a literature procedure for the conversion of tetra(hydroxyphenyl) porphyrin to tetra(carboxyphenyl) porphyrin.[32]

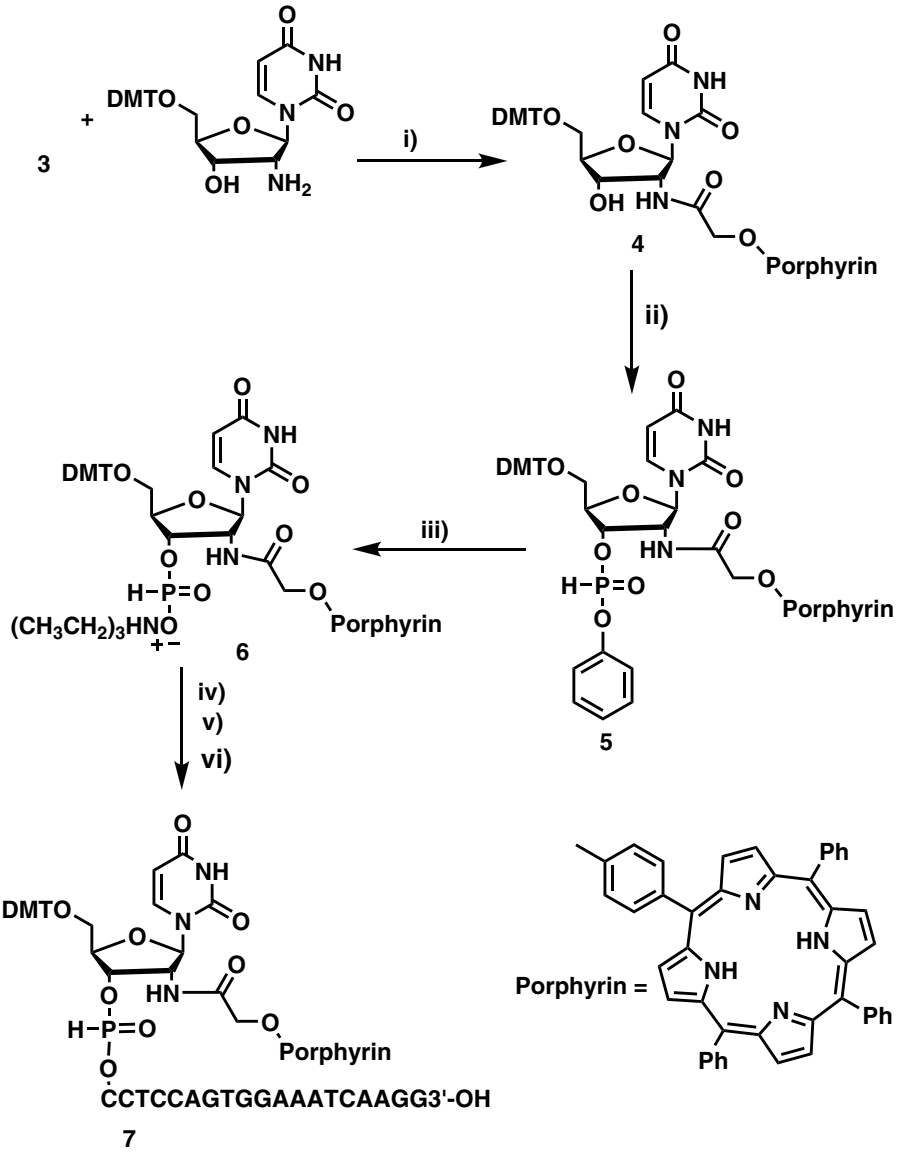
Compound **1** (30 mg, 0.047 mmol) was dried in vacuo in a 100-ml flask and flushed with nitrogen. Potassium carbonate (315 mg, 2.27 mmol) and potassium iodide (44 mg, 0.261 mmol) were added to the flask under nitrogen. Nitrogen-sparged DMF (0.5 ml) and CH_2Cl_2 (4 ml) were added. Ethylchloroacetate (100 μl, 0.704 mmol) was injected into the flask under nitrogen, the flask was fitted with a condenser and was refluxed for 5 h. Solvent was removed in vacuo. After removal of DMF the reaction mixture was extracted with CH_2Cl_2, washed with water (3× 20 ml), dried over magnesium sulfate, and the solvent was evaporated to yield ethyl-5-(4-carboxylatomethoxy),10,15,20-triphenylporphyrin **2** as a purple solid (32.3 mg, 95.8%). ^{1}H NMR and UV/vis spectra matched literature values.[31]

Conversion of the ethyl ester **2** to the carboxylic acid **3** was performed under basic conditions as shown in Scheme 1. Compound **2** (50 mg, 0.069 mmol) was added to a flask followed by 10% potassium hydroxide in 1:1 ethanol/water (30 ml) and refluxed for 18 h. The mixture was cooled to room temperature and diluted with water (10 ml). Afterward, HCl (0.1 M) was added dropwise until the solution became neutral. CH_2Cl_2 (50 ml) was added and the organic layer was separated, washed with water, and dried with magnesium sulfate. After removal of the solvent in vacuo, 5-(4-carboxylatomethoxy)-10,15,20-triphenyl-phenylporphyrin **3** was obtained as a purple solid (33 mg, 69.5%).[33]

The resultant carboxylic acid functionalized porphyrin was coupled to 2′-amino-2′-deoxyuridine to prepare **4** as shown in Scheme 2. 2′-Amino-2′-deoxyuridine was synthesized following a literature procedure.[20] EDCI (5.0 mg, 0.026 mmol), **3** (13 mg, 0.018 mmol), 2′-amino-2′-deoxyuridine (9 mg, 0.017 mmol), and DMAP (<1 mg) were added to a 25-ml flask. The flask was fitted



Scheme 1. Synthesis of porphyrin **3**. Reagents and condition: (i) $ClCH_2COOC_2H_5/KI/K_2CO_3$ in DMF/CH_2Cl_2; (ii) 10% KOH in ethanol/water (1:1)/reflux.



Scheme 2. Synthesis of a 2′ amido porphyrin–oligonucleotide conjugate **7**. Reagents and conditions: (i) EDCI/DMAP, rt/12 h; (ii) pyridine/diphenyl phosphite, rt, 20 min; (iii) triethyl amine/water (1:1), rt, 15 min; (iv) adamantyl chloride in acetonitrile/pyridine (1:1); (v) 5′-HO-CCTCCAGTGGAAA TCAAGG-CPG-3′; (vi) 30% ammonium hydroxide.

with a rubber septum and flushed with nitrogen. The reaction mixture was dissolved in CH_2Cl_2 and stirred at room temperature for 12 h. After consumption of 2′-amino-2′-deoxyuridine was complete (monitored by TLC), the reaction mixture was washed with water (3× 10 ml) and dried over magnesium sulfate. The crude product was purified on a silica column eluting with CH_2Cl_2/ether (1:4). After removal of solvent in vacuo, the second fraction was isolated as a purple solid (7.3 mg, 33.33%). Further purification was obtained by repeated recrystallization in ethyl acetate/hexanes. The compound was dissolved in benzene, dried in vacuo, and characterized by NMR spectroscopy and mass spectrometry.[34]

Prior to synthesis of the 3′ H-phosphonate derivative of **4**, an effort was made to utilize the phosphoramidite derivative for oligonucleotide conjugation. However, the phosphoramidite of **4** oxidized readily when the compound was dissolved in nitrogen-sparged solvent and added to a DNA synthesizer. TLC of the phosphoramidite solution after exposure to air showed that it contained a polar oxidized product. Based on reports of the improved stability of the H-phosphonate, we selected this alternative approach. In particular, the successful synthesis of oligonucleotides containing redox active flavins[25] and porphyrins[14] pointed to the suitability of this approach.

The 3′ H-phosphonate **6** (Scheme 2) was straightforward to synthesize. Compound **4** (20 mg, 0.016 mmol) was dissolved in dry pyridine (0.5 ml) and diphenyl phosphite (28 μl, 0.115 mmol) was added dropwise. The mixture was stirred at room temperature under nitrogen for 25 min. Intermediate **5** was not isolated during this reaction. The reaction was quenched by addition of

triethylamine/water (1:1, 1 ml) and stirring for 15 min. Solvent was removed in vacuo leaving a solid residue. CH_2Cl_2 (10 ml) was added and the solution was washed with 5% sodium bicarbonate (2 × 2 ml). The CH_2Cl_2 layer was separated and dried over magnesium sulfate. CH_2Cl_2 was evaporated to produce a solid that was dissolved in a minimal quantity of CH_2Cl_2 and precipitated with ether/hexanes (1:2). Excess diphenyl phosphite was effectively separated by this precipitation. The precipitate was dried in vacuo and the purple solid obtained (14 mg, 63.3%) was used for characterization by ^{1}H and ^{31}P NMR spectroscopy (Fig. 1) and mass spectrometry (Fig. 2).[35] Compound **6** was found to be more stable than the corresponding phosphoramidite, however, it was somewhat unstable on silica and silica column purification was avoided by use of precipitation. The proton coupled ^{31}P NMR spectrum of **6** (Fig. 1, top) showed two doublets at δ 4.68 ($J_{P,H}$ = 625 Hz) and δ 4.62 ($J_{P,H}$ = 625.3 Hz) with large coupling constant values in roughly equal proportions, indicating a non-stereoselective synthesis of two diastereomers. These well-split doublets are consistent with literature[25,26] reports of H-phosphonate doublets resulting from diastereomers. The proton decoupled (Fig. 1, bottom) spectrum showed only one singlet at δ 4.61 ppm with the two diastereomers indistinguishable.

The synthesis of oligonucleotides was performed on a Beckman Oligo 1000 DNA Synthesizer using cyanoethyl-phosphoramidite chemistry.[36] Conjugation of the 19-mer DNA (5′-HO-CCTCCAGTGGAAATCAA GG-CPG-3′) with **6** (Scheme 2) was carried out manually on a Beckman column. Compound **6** (100 µl, 0.01 M in acetonitrile/pyridine 1:1) was activated with adamantyl chloride (10 µl, 0.1 M in 1:1 acetonitrile/pyridine) under nitrogen prior to addition to the CPG. The mixture was immediately added by syringe to the CPG-bound 19-mer DNA in a Beckman column. The reaction mixture was circulated in the column for 20 min. The column was washed with pyridine/acetonitrile (1:1) (15 ml) until the washes were clear. The column was further rinsed with CH_2Cl_2 (10 ml). After washing and

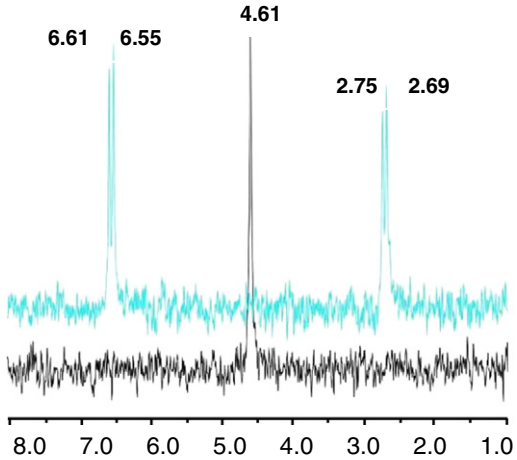


Figure 1. ^{31}P NMR spectrum of **6** (162 MHz, $CDCl_3$). Proton decoupled spectrum (black) and proton coupled spectrum (blue).

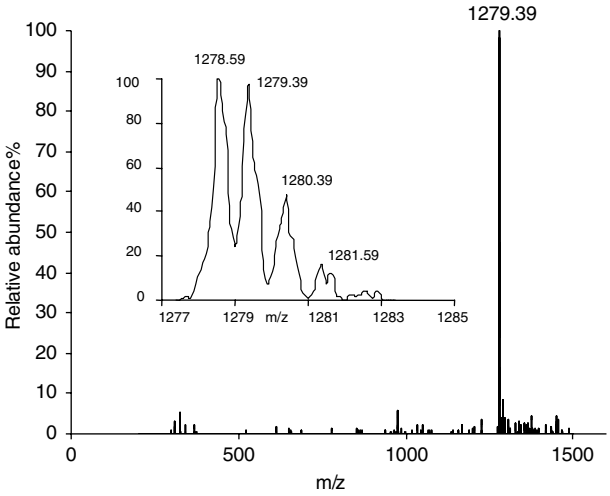


Figure 2. LCQ mass spectrum of **6** (positive ion trap); observed mass of anion ($C_{76}H_{61}N_7O_{11}P^-$) 1279.39 and calculated mass of anion 1279.31.

drying completely, ammonium hydroxide (30%) (1 ml) was injected into the column with a syringe and the sample was stored at 4 °C overnight. The DMT protecting group was left on the oligonucleotide to facilitate HPLC separation. The ammonium hydroxide solution was collected and examined by UV/vis spectroscopy. After cleavage from CPG, the conjugated product **7** in ammonium hydroxide solution showed a porphyrin Soret band with a λ_{max} at 415 nm. The ammonium hydroxide was heated at 37 °C for 16 h to remove the remaining nucleotide protecting groups and dried overnight in a speed vac.

The 20-mer conjugate **7** was re-suspended in water and analyzed by reverse phase HPLC. The material was injected onto the HPLC with a gradient from 80% acetonitrile/20% water for 20 min to 100% acetonitrile for 15 min at a flow rate of 0.4 ml/min.[37] The chromatogram of the product showed the porphyrin–DNA conjugate peak at 14.41 min and a trace amount of **6** at 24.78 min (Fig. 3). Pure **6** had a similar retention time near 25 min under similar conditions (not shown). In a trial experiment, the 5′ OH from a CPG-bound guanine was coupled to compound **6** following the same procedure as above. After cleavage from the CPG support with ammonium hydroxide, the solution did not show an absorbance for porphyrin indicating that the dinucleotide–porphyrin conjugate was insoluble in ammonium hydroxide. In contrast, the compound dissolved in methanol and this UV/vis spectrum showed the absorbance of the porphyrin Soret band at a λ_{max} of 415 nm, indicating that the GU dinucleotide–porphyrin conjugate was soluble in methanol.

A small sample (3 mg) of CPG-bound **7** was used to ascertain that oligonucleotide synthesis could be continued beyond the porphyrin-containing base. The DMT was removed from the 5′ uridine (position #20) using 0.5 ml of 3% trichloroacetic acid in CH_2Cl_2 and a series of three deoxythymidines were added to the sequence

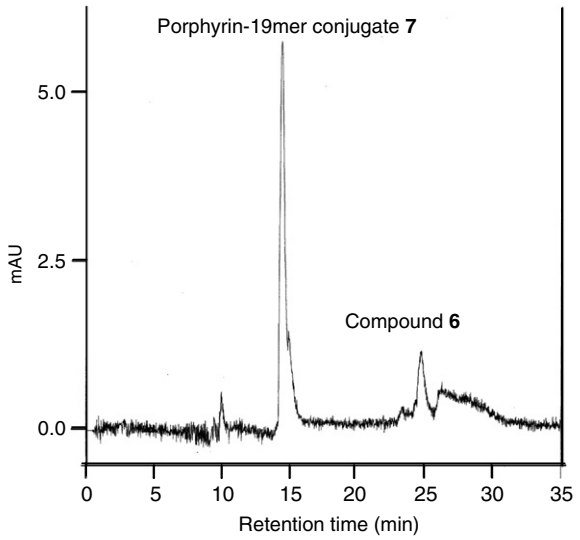


Figure 3. HPLC chromatogram of the coupled porphyrin–DNA conjugate **7** with residual, uncoupled compound **6** at λ_{max} 415 nm.

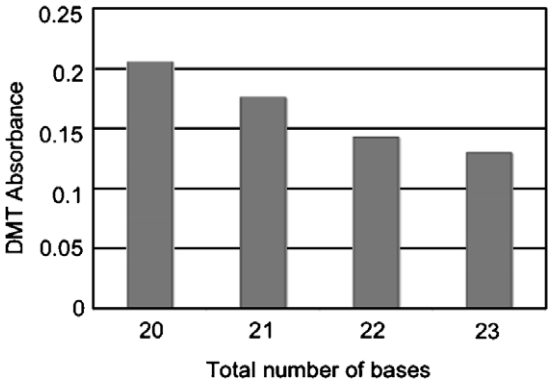


Figure 4. Base coupling efficiencies for extension of oligonucleotide after incorporation of porphyrin-uridine **6** (at position 20) measured by absorbance (504 nm) of DMT removed by trichloroacetic acid.

resulting in a 23-mer with a porphyrin at position 20. Each base was added using 3′ H-phosphonate thymidine (Glen Research) (0.2 ml, 0.05 M) activated with adamantyl chloride (0.01 ml, 0.05 M) and injected manually onto the CPG containing column. After each coupling, an automated capping and oxidation procedure (on a Beckman Oligo 1000) was followed by manual deprotection with 3% trichloroacetic acid in CH_2Cl_2. The trichloroacetic acid was collected, diluted to 1 ml with CH_2Cl_2 and the quantity of the removed DMT was measured by UV/vis spectroscopy (monitored at 504 nm with an Ocean Optics USB-2000). The average yield for base additions was 86% (Fig. 4), comparable to that obtained without the porphyrin present, indicating that the porphyrin does not interfere with additional base couplings using the H-phosphonate method.

Porphyrin–DNA conjugates are useful in photomedicine, biological chemistry, the study of DNA structure, and the design of light harvesting arrays. The 2′ amido-linked porphyrin reported here demonstrates a new method of synthesizing porphyrin–oligonucleotide conjugates. This route makes possible the construction of multi-porphyrin arrays built on a DNA scaffold. After converting the 2′ porphyrin modified uridine into a 3′ H-phosphonate derivative, the compound was coupled to the free hydroxyl of a CPG-bound oligonucleotide to form a porphyrin–oligonucleotide conjugate. 3′ H-phosphonate derivatives of **4** were found to be more stable than the corresponding phosphoramidite derivatives. This is the first report of porphyrin conjugation to a solid-support linked oligonucleotide with a base pairing porphyrin located at an internal position. In this route, the addition of porphyrin does not act as a capping agent, terminating the synthesis of DNA. Rather, the addition of nucleotides was continued on the DNA strand after DMT removal. This approach can be used to synthesize arrays of porphyrin using solid-phase automated DNA synthesis. This also allows for the combination of porphyrin modification with other modifiers. Previous methods for internal modification are not capable of nucleotide base pairing.[17] Efforts are underway to expand this route to include longer wavelength absorbing porphyrins.[1c]

Acknowledgments

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29. ^{1}H NMR spectra were obtained at 500 MHz on a Tecmag Nicolet referenced to an internal TMS standard. 100 MHz ^{13}C NMR spectra referenced to $CDCl_3$ and 162 MHz ^{31}P NMR spectra referenced to external H_3PO_4 were obtained on a BZH 400/52 Bruker spectrometer. Mass spectra were obtained on a JEOL MS and Thermo Finnigan LCQ positive ion trap. CH_2Cl_2 (VWR), pyridine (EMD), and ethyl acetate (Mallinkcrodt) were distilled over calcium hydride and stored over 4 Å molecular sieves. Acetonitrile (Transgenomic) and triethylamine (Sigma) were used as received. Silica gel TLC grade 7749 (Aldrich) was used for chromatotron purifications. Silica gel (60–200 mesh) was used for column chromatography. All other reagents were from Aldrich and used as received.
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33. ^{1}H NMR (500 MHz, $CDCl_3$) for compound **3** δ_H (ppm) −2.78 (s, 2H), 5.0 (s, 1H), 7.33–7.35 (m, 10H), 7.36 (s, 2H), 7.73–7.76 (m, 7H), 8.15 (d, 2H, J = 5 Hz), 8.82 (d, 4H, J = 5 Hz), 8.84 (s, 6H).
34. ^{1}H NMR (500 MHz, $CDCl_3$) for compound **4**: δ_H (ppm) −2.79 (s, 2H), 2.33 (s, 1H), 3.47 (s, 2H), 3.79 (s, 6H), 4.25 (s, 1H), 4.63 (d, 1H, J = 5 Hz), 4.83–4.81 (m, 2H), 5.53 (d, 1H, J = 5 Hz), 6.25 (d, 1H, J = 10 Hz), 6.87 (d, 4H, J = 10 Hz), 7.33–7.31 (m, 8H), 7.43 (d, 2H, J = 10 Hz), 7.62 (d, 1H, J = 5 Hz), 7.78–7.71 (m, 10H), 8.07 (s, 1H), 8.18 (d, 1H, J = 5 Hz), 8.21 (d, 8H, J = 10 Hz), 8.85 (s, 8H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 169.22, 162.53, 158.79, 156.94, 150.82, 144.07, 142.14, 139.94, 136.30, 135.76, 135.20, 135.07, 134.55, 130.12, 129.34, 128.17, 128.11, 127.73, 127.24, 126.69, 120.19, 119.23, 113.41, 113.15, 103.96, 102.97, 102.92, 87.31, 86.79, 85.58, 77.33, 77.01, 76.69, 67.57, 63.59, 56.35, 55.27, 55.23, 53.40. Mass calculated for ($C_{76}H_{61}N_7O_9$) 1216.34 and mass observed 1216.98.
35. ^{1}H NMR (500 MHz, $CDCl_3$) for compound **6**: δ_H (ppm); −2.79 (s, 2H), 1.33 (t, 9H), 3.05 (q, 6H), 3.75 (s, 6H), 4.44 (s, 1H), 4.77 (s, 2H), 5.11–5.12 (m, 1H), 5.30 (s, 1H), 5.39 (d, 1H, J = 5 Hz), 6.42 (d, 1H, J = 10 Hz) 6.85 (d, 4H, J = 10 Hz), 7.20–7.22 (m, 1H), 7.30–7.35 (m, 10H), 7.43 (d, 2H, J = 5 Hz), 7.73–7.75 (m, 10H), 7.83 (d, 1H, J = 5 Hz), 8.12–8.14 (d, 2H, J = 10 Hz), 8.19–8.21 (m, 7H), 8.84–8.86 (m, 8H), 12.14 (s, 1H). ^{31}P NMR (162 MHz, $CDCl_3$) δ_P δ 4.68 ($J_{P,H}$ = 625 Hz) and δ 4.62 ($J_{P,H}$ = 625.3 Hz) and proton decoupled δ 4.61. Calculated mass for anion ($C_{76}H_{61}N_7O_{11}P^-$) 1279.31 and observed mass for anion 1279.4.
36. The phosphoramidites of all four bases (ibu dG-CE, dmf dG-CE, bz dA-CE, and bz dC-CE were obtained from Transgenomic) were dissolved in anhydrous acetonitrile (10 ml) with 3 Å molecular sieves (Beckman) and attached directly to the synthesizer. The synthesizer was operated under an atmosphere of helium (99.99%).
37. HPLC analysis was performed on a Beckman Gold system with photodiode array detector using a reverse phase C-18 column (5 μm, 150 × 4.6 mm, 300 Å) (Phenomenex).