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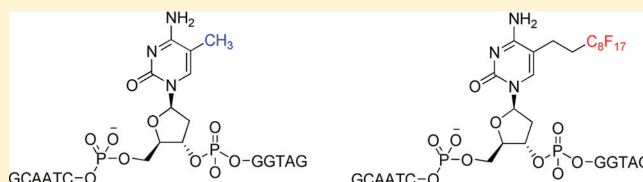
Design, Synthesis, and Incorporation of Fluorous 5-Methylcytosines into Oligonucleotides

Zhiquan Song and Qisheng Zhang*

Division of Chemical Biology and Medicinal Chemistry, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States

Supporting Information

ABSTRACT: A palladium-catalyzed Negishi coupling reaction has been developed to synthesize fluorous 5-methylcytosines. These fluorous nucleosides are incorporated into the oligonucleotides that correspond to part of the promoter region of Oct4, a master gene that undergoes dynamic DNA demethylation during cellular reprogramming. The separation of the fluorous oligonucleotides from its nonfluorous analogues has been achieved through solid-phase extraction over fluorous silica, suggesting its potential use in probing DNA demethylation.



Deoxyribonucleic acid (DNA) methylation and demethylation of cytosine at the C5 position play important roles in many fundamental biological processes including transcription regulation, stem cell pluripotency, and cancer.[1] In early embryogenesis, dynamic change of DNA methylation and demethylation is one key event in nuclear reprogramming and genomic imprinting. Aberrant regulation of this process leads to developmental defects and plays a role in carcinogenesis. Consequently, understanding the mechanisms of DNA methylation and demethylation is important in illustrating the epigenetic and genetic regulation of normal and disease development and could have potential therapeutic applications.

Various DNA methyltransferases have been identified, and their catalysis has been illustrated in detail.[2] In contrast, the enzymes that are responsible for the reverse process, DNA demethylation of 5-methylcytosine (5mC), have not been identified. Consequently, the mechanism of active DNA demethylation is not clear, and three possibilities have been proposed (Figure 1A). The demethylation process could occur through direct C−C bond cleavage in pathway A, the glycosate cleavage or deamination followed by a base excision repair in pathway B, or dinucleotide replacement in pathway C. Enzymes that are responsible for glycosate cleavage for 3-methylcytosine (3mC) and dinucleotide replacement have been identified in plants,[3] but such mechanisms are unlikely to be present in mammalian cells where analogous glycosylases do not exist. Furthermore, any mistake from genome-wide DNA repair in one-cell embryos in mammalian systems is likely detrimental. Recently, ten eleven translocation (Tet) proteins have been demonstrated to convert 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC).[4] However, it is still unclear how Tet proteins control the relative ratio of these species and how they are further converted to cytosine. To elucidate the demethylation events, it will be helpful to analyze the biochemistry of the demethylation reaction actually taking place in vivo. However, the complex nature of living cells makes it difficult to trace DNA demethylation reactions endogenously.

Small molecules with highly fluorinated domains tend to preferentially partition into the fluorocarbon-enriched phase of a biphase or triphase system, partially due to the fluorine−fluorine interactions.[5] This fact forms the foundation of the recently established fluorous chemistry. The word "fluorous" is coined to describe the phase formed from highly fluorinated molecules in analogy to an aqueous phase.[6] Small molecules with highly fluorinated domains are called fluorous compounds or molecules. Initially, highly fluorinated (fluorous) solvents are used to separate fluorous from nonfluorous compounds through fluorous−organic liquid−liquid extraction.[7] Later, fluorous-functionalized silica gel and the fluorous solid-phase extraction (FSPE) technique are used to further improve the efficiency of separation.[8] Typically, the reaction mixture is loaded on a column with fluorous silica. The organic products are eluted with organic solvents and the fluorous products with fluorous solvents. FSPE has since been employed for the recycling and reuse of catalysts,[9] removal of reaction intermediates,[10] and fluorous mixture synthesis of libraries of compounds.[11] Recently, fluorinated peptides were demonstrated to be efficiently separated from nonfluorinated peptides through FSPE.[12] The fluorous protecting groups have also aided in the purification of oligosaccarides and oligonucleotides. In addition, a single C_8F_{17} group renders the easy separation of up to 100-membered oligonucleotides from nonfluorinated nucleotides or reagents in the mixture.[13] These successes prompt us to explore the applications of fluorous unnatural DNAs in uncovering the mechanisms of DNA demethylation.

The concept of using fluorous 5mC derivatives to probe DNA demethylaiton is illustrated in Figure 1B. Fluorinated 5-

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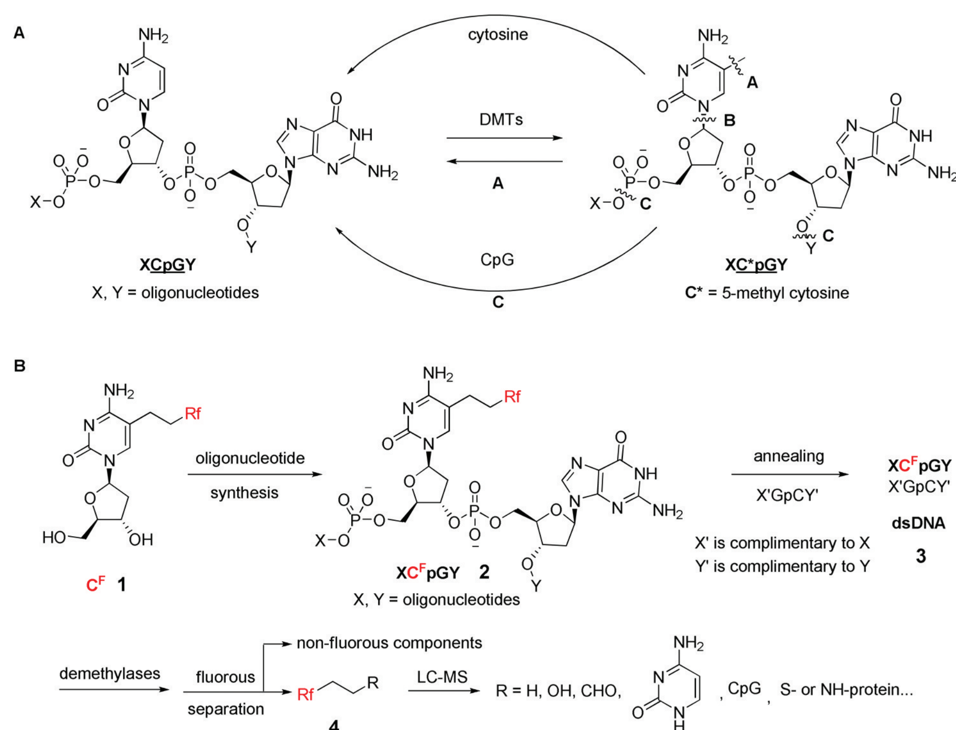
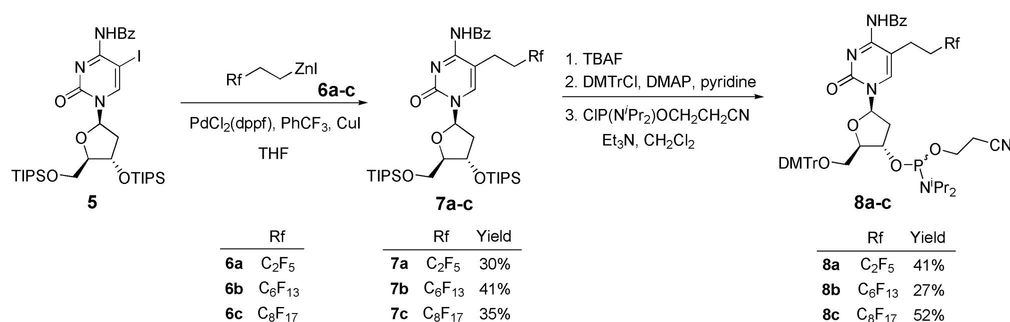


Figure 1. Fluorous approach to DNA demethylation. (A) Three possible mechanisms for DNA demethylation. (B) Schematic illustration of using fluorous 5mC derivatives in DNA demethylation.

Scheme 1. Synthesis of Fluorous 5-Methylcytosine Derivatives 7a−c and 8a−c



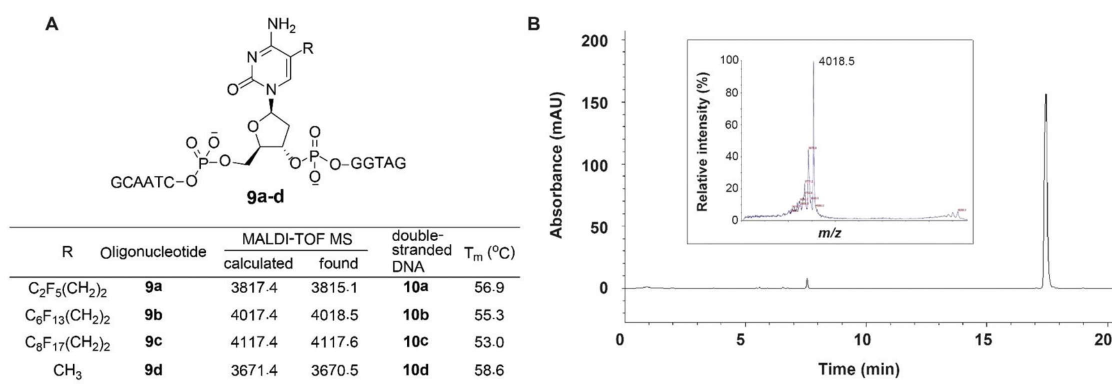
	Rf
6a	C_2F_5
6b	C_6F_{13}
6c	C_8F_{17}

	Rf	Yield
7a	C_2F_5	30%
7b	C_6F_{13}	41%
7c	C_8F_{17}	35%

	Rf	Yield
8a	C_2F_5	41%
8b	C_6F_{13}	27%
8c	C_8F_{17}	52%

methylcytosines **1** are synthesized and incorporated into a sequence in a selected promoter region containing the CpG unit of genes that are known to be regulated by DNA methylation/demethylation. The resulting oligonucleotides **2** (XC^{F}pGY) then are annealed with their complementary oligonucleotides to form double-stranded DNAs (dsDNAs) **3** that will be introduced into cells. The resulting mixture then will be applied to a fluorous chromatographic support to retain the fluorinated compounds selectively. Elution with more fluorophilic solvents affords the enriched fraction containing products **4** derived directly from fluorinated unnatural DNAs. Separation and analysis of these enriched products by HPLC-MS will provide novel insights into the mechanisms of DNA demethylation. Because the fluorous tags are chemically inert and highly stable in tandem mass spectrometric analysis, the DNA demethylation products of fluorous oligonucleotides will be analyzed without the interference of other molecules in the cells, thus representing a novel approach to understand the biochemistry of DNA demethylation in vivo. For example, if the demethylation reaction proceeds as in pathway A in Figure 1A, then fluorinated small molecules will be enriched. Identification of the enriched small molecules will provide insights into the mechanisms of DNA demethylation. Similarly, if pathway B dominates, then fluorinated cytosine will be recovered in the fluorous fraction. In this work, we will report the results on design, synthesis, and incorporation of fluorous 5-methylcytosines into oligonucleotides.

To selectively enrich the products derived from DNA demethylation, we envisioned that a fluorous tag (Rf) could be attached to the 5-methyl group. The optimal size of the Rf should be sufficient to separate the tagged components from the untagged ones, while the DNA demethylation process is minimally interfered. Accordingly, several 5mC analogues with different Rf groups were synthesized. The modification at the C5 position of cytosine typically starts with either 2′-deoxyuridine or 5-iodocytosine. When 2′-deoxyuridine is used as the starting material, multiple-step reactions[14] that include nucleophilic addition to aldehydes and conversion of deoxyuridine to cyctosine are applied. The nucleophilic addition reaction requires harsh conditions and generally provides low yields.[14] In addition, the hydroxyl group in the addition product has to be removed. Consequently, this



R	Oligonucleotide	MALDI-TOF MS calculated	MALDI-TOF MS found	double-stranded DNA	T_m (°C)
$C_2F_5(CH_2)_2$	**9a**	3817.4	3815.1	**10a**	56.9
$C_6F_{13}(CH_2)_2$	**9b**	4017.4	4018.5	**10b**	55.3
$C_8F_{17}(CH_2)_2$	**9c**	4117.4	4117.6	**10c**	53.0
CH_3	**9d**	3671.4	3670.5	**10d**	58.6

Figure 2. Incorporation of fluorous 5-methylcytosines into oligonucleotides. (A) Structure of synthetic oligonucleotides. (B) Representative MS and LC chromatogram of **9c**.

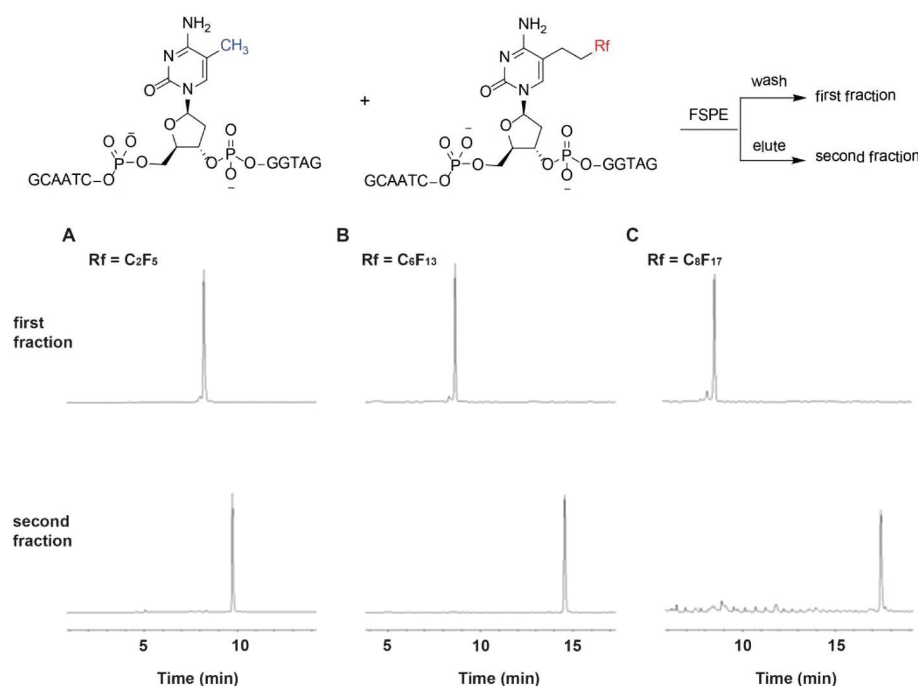


Figure 3. Separation of fluorous from nonfluorous oligonucleotides. A mixture of **9d** with **9a** (A), **9b** (B), or **9c** (C) was separated through FSPE into two fractions. Both fractions were analyzed by HPLC. Elution solvent for the first fraction was 10% MeCN in 0.1 M TEAA, while that for the second fraction was 20% MeCN in 0.1 M TEAA (A), 25% MeCN in 0.1 M TEAA (B), and 30% MeCN in 0.1 M TEAA (C). The flow rate was 1 mL/min.

strategy is not efficient for synthesis of fluorous 5mC derivatives. A more straightforward approach uses 5-iodocytosine as the starting material. Sonagashira reaction or Heck coupling with alkynes or alkenes has been used to synthesize the corresponding modified cytosines. Although not reported, further reduction of the coupling products with hydrogen should provide 5-alkylated cytosines. However, the fluorous terminal alkynes or alkenes are not readily available, making additional synthetic efforts necessary. In contrast, fluorous alkyliodides are commercially available and easy to handle. Consequently, we decided to develop a palladium-catalyzed coupling reaction between the 5-iodocytosine with organozinc reagents to facilitate the synthesis of the modified 5-methylcytosines.

The organozinc reagents derived from the fluorinated iodide **6a–c** were prepared according to the Knochel protocol.[15] We typically prepared fluorous zinc reagents in the concentration of 0.6 M as determined by titration with iodine.[16] Among several ligands and metal complexes tested, the use of [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium ($PdCl_2$(dppf)) and copper iodide (CuI) in the presence of 0.5 equiv of trifluoromethylbenzene ($PhCF_3$) gave 41% yield of the desired product when **6b** was used (Scheme 1). These conditions were also used for two other fluorous zinc reagents **6a** and **6c** to form the corresponding coupling products **7a** and **7c**, respectively. The triisopropylsilyl (TIPS) protective groups in **7a–c** were then removed by treatment with tetrabutylammonium fluoride (TBAF). In order to apply the standard oligonucleotide synthesis protocol for the commercial nucleic acid synthesizer,[17] the 5′-hydroxyl group is protected as the 4,4′-dimethoxytrityl (DMT) ether by reacting with DMT chloride, and the 3′-hydroxyl group is converted to the phosphoramidite monomer **8a–c** by reacting with 2-cyanoethyl *N*,*N*-diisopropyl chlorophosphine according to the literature protocol.[17a,c]

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Oct4 is one master gene that regulates the differentiation and pluripotency of embryonic stem cells.[18] In the cellular reprogramming in vivo and in vitro, the expression of Oct4 is turned on and DNA demethylations of the reprogrammed cell markers take place. For example, the CpG unit in the sequence 5′-GCAATCCGGTAG is predominantly in the demethylated state during cellular reprogrammng. The fluorous building blocks **8a−c** were thus incorporated into the CpG region of this sequence in Oct4 promoter according to the standard protocol. Briefly, all oligonucleotides are synthesized by standard phosphoramidite techniques and deprotected at 52 °C in saturated ammonia hydroxide for 5 h. The resulting mixtures are then purified by HPLC to provide oligonucleotides **9a−c** (Figure 2A). As a control, 5-methylcytosine was also incorporated into this sequence to generate oligonucleotide **9d**. The purity and identity of the oligonucleotide were determined by reverse-phase HPLC and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, respectively. The calculated and measured molecular weights of **9a−d** are listed in Figure 2A. One representative HPLC chromatogram of **9c** is shown in Figure 2B with the MS spectrum of **9c** in the inset. These results suggest that the desired oligonucleotides were obtained with high purity (>95%).

The fluorinated oligonucleotides **9a−c** were then annealed with the antisense pair 5′-CTACCGGATTGC to generate double-stranded DNAs **10a−c**. Similarly, the nonfluorinated oligonucleotide **9d** was also annealed to form double-stranded **10d** as a control. Next, the melting temperatures (T_m) of **10a−d** were measured (Figure 2A) to investigate the effect of fluorous tags. The T_m values decrease as the size of the fluorous tag increases, suggesting that the fluorous tags interfere with base pairing. Nonetheless, the T_m values of **10a−d** were all in the range of 53−58 °C, indicating that stable double helix structures were formed for all double-stranded DNAs.

One key feature of using fluorous 5mC derivatives is to enrich the fluorous-tagged products. To test the separation efficiency of the fluorous 5mC derivatives, **9a** was mixed with **9d** and the resulting mixture was loaded onto a column with fluorous silica. Elution with the column with 10% CH_3CN in 0.1 M triethylammonium acetate (TEAA) buffer yielded the first fraction. The second fraction was obtained by eluting the column with 100% CH_3CN. Both fractions were then analyzed by HPLC (Figure 3) to assess the separation efficiency. The same sets of experiments were carried out for **9b** and **9c**. As expected, only the nonfluorous **9d** was eluted when 10% CH_3CN in 0.1 M TEAA buffer was applied. In contrast, elution with 100% CH_3CN provides **9a−c**. While the separation of **9a** (Rf = C_2F_5) from **9d** requires careful elution, **9b** (Rf = C_6F_{13}) and **9c** (Rf = C_8F_{17}) can be easily separated from the nonfluorous **9d**.

In summary, we have developed fluorous 5-methylcytosine derivatives and successfully incorporated them into one CpG region that exists in the Oct4 gene promoter. The modified oligonucleotides are effectively separated from the nonfluorous oligonucleotide with 5-methylcytosine incorporated. We acknowledge that these fluorous oligonucleotides have not been tested in DNA demethylation reactions in cells, which requires extensive studies on suitable oligonucleotide sequences and cellular systems. Nonetheless, the fact that fluorous 5mC derivatives can be incorporated into oligonucleotides and separated from nonfluorous species has laid the foundation to investigate the cellular mechanism of DNA demethylation through enriching and analyzing the reaction products.

■ EXPERIMENTAL SECTION

All of the solvents were purchased from suppliers as anhydrous grade. ^1H and ^{13}C NMR spectra were recorded at room temperature in $CDCl_3$ (containing 1% TMS) solutions on a 300 or 400 MHz spectrometer. HPLC analyses were performed on reverse-phase columns. Low- and high-resolution mass spectra were obtained from a Qh-FTICR mass spectrometer.

Synthesis of Compound 5. Imidazole (1.59 g, 23.32 mmol) and DMAP (64.8 mg, 0.53 mmol) were added to a solution of 5-iodo-2′-deoxycytidine (1.87 g, 5.30 mmol) in dry DMF (25 mL) at room temperature. Subsequently, triisopropylsilyl trifluoromethanesulfonate (3.57 g, 11.66 mmol) was added dropwise. After stirring for 16 h at room temperature, saturated NH_4Cl solution was added. The mixture was extracted with chloroform three times. The combined organic layers were dried over sodium sulfate and concentrated under vacuum to give a crude product of 5-iodo-3′,5′-*O*-bis(triisopropylsilyl)-2′-deoxycytidine, which was resuspended in dry pyridine (25 mL). DMAP (64.80 mg, 0.53 mmol) and benzoic anhydride (2.40 g, 10.60 mmol) were then added. After stirring at 35 °C for 5 h, the pyridine was removed under reduced pressure. The residue was purified through flash chromatography (hexanes/EtOAc = 9:1) to yield compound **5** as light yellow solid (3.26 g, 80%): ^1H NMR ($CDCl_3$, 300 MHz) δ 1.01−1.31 (m, 42H), 2.00−2.09 (m, 1H), 2.45 (dd, J = 12.0, 5.1 Hz, 1H), 3.89 (dd, J = 10.8, 2.7 Hz, 1H), 3.99 (dd, J = 10.8, 2.7 Hz, 1H), 4.09 (d, J = 1.5 Hz, 1H), 4.62 (d, J = 5.4 Hz, 1H), 6.30 (dd, J = 9.0, 5.1 Hz, 1H), 7.42−7.47 (m, 2H), 7.51−7.56 (m, 1H), 8.19 (s, 1H), 8.38 (d, J = 6.9 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 11.9, 12.0, 17.92, 17.94, 18.1, 42.6, 63.5, 69.4, 73.0, 86.3, 89.1, 128.1, 130.11, 132.6, 136.6, 145.2, 147.2, 156.7, 179.6; MS (ESI) m/z 770.3 $[M + H]^+$.

Compound 7b. Trifluoromethyl benzene (4.3 μL, 35 μM) was added to a solution of **5** (54 mg, 70 μmol) in anhydrous THF (0.3 mL). The reaction solution was then heated to 50 °C. $PdCl_2$(dppf)-(CH_2Cl_2) (5.7 mg, 7 μmol) was added at the same temperature. After stirring for 5 min, 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl zinc iodide (0.18 mL, 0.6 M) was added dropwise, followed by the addition of CuI (13.3 mg, 70 μmol). The stirring was continued at the same temperature for 3 h. Another portion of the zinc reagent (0.18 mL, 0.6 M) was then added, and the reaction mixture was heated to 60 °C. After 14 h, the reaction mixture was cooled to room temperature and added into water, extracted with EtOAc three times. The combined organic fractions were dried over sodium sulfate. Evaporation of the solvent and flash chromatography (hexanes/EtOAc = 95:5) gave the compound **7b** as colorless oil (28 mg, 41%): ^1H NMR ($CDCl_3$, 300 MHz) δ 1.01−1.23 (m, 42H), 1.99−2.08 (m, 1H), 2.41 (dd, J = 12.9 Hz, 5.1 Hz, 1H), 2.44−2.56 (m, 2H), 2.82 (t, J = 8.1 Hz, 2H), 3.91 (dd, J = 11.1, 2.7 Hz, 1H), 3.41 (dd, J = 11.4, 2.1 Hz, 1H), 4.08 (d, J = 2.1 Hz, 1H), 4.63 (d, J = 5.4 Hz, 1H), 6.38 (dd, J = 8.7, 5.4 Hz, 1H), 7.41−7.46 (m, 2H), 7.51−7.56 (m, 1H), 7.79 (s, 1H), 8.25−8.29 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 11.8, 12.0, 17.9, 20.2, 30.2 (t, J = 21.6 Hz), 42.3, 63.6, 72.9, 85.6, 88.8, 112.2, 128.2, 129.8, 132.6, 136.9, 138.0, 147.6, 158.8, 179.6; ^{19}F NMR ($CDCl_3$, 282 MHz) δ −81.4 (t, J = 10.2 Hz), −115.6 (m), −122.2, −123.3, −124.1, −126.5; MS (ESI) m/z 990.3 $[M + H]^+$; HRMS (ESI) calcd for $C_{42}H_{61}F_{13}N_3O_5Si_2$ $[M + H]^+$ 990.3942, found 990.3938.

Compound 7a. In a similar manner to **7b**, **7a** (72 mg, 30%) was synthesized from **6a** (1.5 mL, 0.9 mmol) and **5** (233 mg, 0.3 mmol) as colorless oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88−1.23 (m, 42H), 1.98−2.07 (m, 1H), 2.38−2.52 (m, 3H), 2.80 (t, J = 8.1 Hz, 2H), 3.90 (dd, J = 11.4, 2.4 Hz, 1H), 3.99 (dd, J = 11.4, 2.4 Hz, 1H), 4.08 (d, J = 1.8 Hz, 1H), 4.62 (d, J = 5.4 Hz, 1H), 6.38 (dd, J = 8.7, 5.4 Hz, 1H), 7.42−7.47 (m, 2H), 7.51−7.56 (m, 1H), 7.78 (s, 1H), 8.25−8.29 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 11.8, 12.0, 17.91, 17.93, 20.3, 30.0 (t, J = 21.4 Hz), 42.3, 63.6, 72.9, 85.6, 88.8, 112.3, 128.2, 129.8, 132.6, 136.9, 137.9, 147.6, 158.8, 179.5; ^{19}F NMR ($CDCl_3$, 282 MHz) δ −85.9, −119.1 (t, J = 17.8 Hz); MS (ESI) m/z 790.3 $[M + H]^+$;

HRMS (ESI) calcd for $C_{38}H_{60}CsF_5N_3O_5Si_2$ [M + Cs]$^+$ 922.305, found 922.303.

Compound 7c. In a similar manner to **7b**, **7c** (116 mg, 35%) was synthesized from **6c** (1.5 mL, 0.9 mmol) and **5** (233, 0.3 mmol) as white solid: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.00−1.23 (m, 42H), 1.99−2.08 (m, 1H), 2.42 (dd, *J* = 12.6, 5.1 Hz, 1H), 2.44−2.56 (m, 2H), 2.82 (t, *J* = 8.1 Hz, 2H), 3.90 (dd, *J* = 11.4, 2.4 Hz, 1H), 4.00 (dd, *J* = 11.4, 2.4 Hz, 1H), 4.08 (d, *J* = 1.8 Hz, 1H), 4.63 (d, *J* = 5.1 Hz, 1H), 6.38 (dd, *J* = 8.7, 5.4 Hz, 1H), 7.41−7.46 (m, 2H), 7.51−7.56 (m, 1H), 7.79 (s, 1H), 8.25−8.28 (m, 2H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 11.8, 12.0, 17.89, 17.92, 17.94, 20.2, 30.2 (t, *J* = 21.7 Hz), 42.3, 63.6, 72.9, 85.6, 88.8, 112.3, 128.2, 129.8, 132.6, 136.9, 138.0, 147.6, 158.8, 179.6; ^{19}F NMR (CDCl$_3$, 282 MHz) δ −81.3 (t, *J* = 10.5 Hz), −115.3, −121.9, −122.1, −122.9, −123.9, −126.3; MS (ESI) *m/z* 1090.3 [M + H]$^+$; HRMS (ESI) calcd for $C_{44}H_{60}CsF_{17}N_3O_5Si_2$ [M + Cs]$^+$ 1222.285, found 1222.284.

Synthesis of 8b. TBAF (0.09 mL, 1.0 M in THF) was added to a solution of **7b** (40 mg, 40 μmol) in fresh distilled THF (1 mL). After stirring at room temperature for 30 min, the solvent was removed under reduced pressure. Flash chromatography (CH_2Cl_2/MeOH = 95:5) gave the deprotected product as white solid (20 mg, 75%): ^{1}H NMR (CD$_3$OD, 300 MHz) δ 2.04−2.37 (m, 2H), 2.58−2.73 (m, 2H), 2.86−2.91 (m, 2H), 3.77 (dd, *J* = 12.3 Hz, *J* = 3.3 Hz, 1H), 3.87 (dd, *J* = 12.3 Hz, *J* = 2.7 Hz, 1H), 3.95−3.99 (m, 1H), 4.41−4.45 (m, 1H), 6.29 (t, *J* = 6.3 Hz, 1H), 7.44 (t, *J* = 7.5 Hz, 2H), 7.56 (t, *J* = 7.5 Hz, 1H), 8.22−8.27 (m, 3H).

The deprotected product (42 mg, 63 μmol) was dried by first coevaporation twice with dry pyridine (0.5 mL) and then under vacuum for 3 h before redissolved in pyridine (0.5 mL) upon heating. DMAP (1.9 mg, 16 μmol) and DMTrCl (43 mg, 126 μmol) were added to the resulting solution at 0 °C. The reaction mixture was warmed to room temperature and stirred for 16 h. The solvent was then removed under reduced pressure, and the residue was purified by flash chromatography (CH_2Cl_2/MeOH = 95:5) to yield DMTr-on product as a white foam (38 mg, 62%). To a solution of the DMTr-on compound (38 mg, 39 μmol) in dry CH_2Cl_2 (0.5 mL) was added triethylamine (16 μL, 117 μmol) at room temperature. After stirring for 15 min, cyanoethyl(diisopropylamino)phosphino chloride (17 μL, 78 μmol) was added dropwise. The stirring was continued for another 2 h, and the solvent was then removed under reduced pressure. Flash chromatography (CH_2Cl_2/acetone = 95:5) gave the compound **8b** as a white foam (27 mg, 59%). As judged by ^{31}P NMR, **8b** was presented as a 3:5 mixture of two stereoisomers: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.06−1.08 (4H), 1.16−1.19 (m, 8H), 1.95−2.17 (m, 2H), 2.36−2.51 (m, 4H), 2.57−2.70 (m, 2H), 3.33−3.40 (m, 1H), 3.51−3.72 (m, 4H), 3.76−3.78 (m, 6H), 3.81−3.89 (m, 1H), 4.19−4.25 (m, 1H), 4.69−4.74 (m, 1H), 6.38−6.45 (m, 1H), 6.82−6.86 (m, 4H), 7.23−7.33 (m, 7H), 7.39−7.44 (m, 4H), 7.50−7.55 (m, 1H), 7.89−7.94 (1H), 8.23 (d, *J* = 6.9 Hz, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 19.4, 20.37, 20.47, 24.4, 24.5, 24.6, 29.8 (t, *J* = 25.2 Hz), 40.5, 43.1, 43.3, 43.4, 55.1, 58.1, 58.4, 62.9, 63.1, 73.8, 74.0, 85.5, 85.6, 85.7, 85.8, 86.9, 112.5, 112.6, 113.2, 117.3, 117.5, 127.2, 128.0, 128.1, 128.2, 129.8, 130.1, 132.6, 134.8, 135.06, 135.12, 136.9, 138.4, 144.0, 147.7, 158.8, 179.6; ^{19}F NMR (CDCl$_3$, 282 MHz) δ −81.3, −115.2, −122.1, −123.1, −123.9, −126.3; ^{31}P NMR (CDCl$_3$, 121 MHz) δ 149.6, 150.1; MS (ESI) *m/z* 1180.3 [M + H]$^+$, HRMS (ESI) calcd for $C_{54}H_{55}CsF_{13}N_5O_8P$ [M + Cs]$^+$ 1312.263, found 1312.261.

Compound 8a. In a similar manner to **8b**, **8a** (29 mg, 41%) was synthesized from **7a** (58 mg, 73 μmol) as colorless oil that existed as a 4:5 mixture of two stereoisomers: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.06−1.08 (3H), 1.16−1.19 (m, 9H), 1.93−2.17 (m, 2H), 2.35−2.49 (m, 4H), 2.61−2.66 (m, 2H), 3.33−3.40 (m, 1H), 3.51−3.66 (m, 4H), 3.79 (m, 6H), 3.81−3.85 (m, 1H), 4.19−4.26 (m, 1H), 4.68−4.74 (m, 1H), 6.41 (dd, *J* =13.8, 6.0 Hz, 1H), 6.82−6.86 (m, 4H), 7.25−7.34 (m, 7H), 7.39−7.45 (m, 4H), 7.49−7.55 (m, 1H), 7.89−7.93 (1H), 8.23 (d, *J* = 7.8 Hz, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 19.5, 20.1, 20.2, 20.3, 20.4, 24.38, 24.48, 24.54, 24.6, 29.5 (t, *J* = 21.2 Hz), 40.4, 40.5, 43.10, 43.18, 43.27, 43.34, 55.1, 58.0, 58.1, 58.2, 58.3, 62.9, 63.0, 73.4, 73.7, 74.0, 85.5, 85.6, 85.7, 85.8, 86.06, 86.11, 86.9, 112.5, 112.6, 113.2, 117.3, 117.5, 127.2, 128.0, 128.1, 129.8, 130.0, 130.1, 132.6, 135.05, 135.06, 135.12, 136.9, 138.2, 138.3, 144.0, 147.6, 158.8, 179.5; ^{19}F NMR (CDCl$_3$, 282 MHz) δ −86.0 (d, *J* = 5.4 Hz), −119.0 (m); ^{31}P NMR (CDCl$_3$, 121 MHz) δ 149.6, 150.0; MS (ESI) *m/z* 980.3 [M + H]$^+$, HRMS (ESI) calcd for $C_{50}H_{55}CsF_5N_5O_8P$ [M + Cs]$^+$ 1112.276, found 1112.289.

Compound 8c. In a similar manner to **8b**, **8c** (62 mg, 52%) was synthesized from **7c** (103 mg, 94 μmol) as a light yellow foam that existed as a 2:3 mixture of two stereoisomers: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.06−1.09 (2H), 1.14−1.25 (m, 10H), 1.84−2.17 (m, 2H), 2.37−2.51 (m, 4H), 2.57−2.67 (m, 2H), 3.34−3.40 (m, 1H), 3.53−3.72 (m, 4H), 3.76−3.78 (m, 6H), 3.83−3.87 (m, 1H), 4.20−4.26 (m, 1H), 4.70−4.75 (m, 1H), 6.42 (dd, *J* =13.2, 6.3 Hz, 1H), 6.82−6.86 (m, 4H), 7.24−7.31 (m, 7H), 7.35−7.44 (m, 4H), 7.49−7.54 (m, 1H), 7.91−7.95 (m, 1H), 8.23 (d, *J* = 7.8 Hz, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 19.4, 20.1, 20.2, 20.3, 20.4, 24.39, 24.49, 24.53, 24.61, 29.8 (t, *J* = 21.6 Hz), 40.5, 43.1, 43.2, 43.3, 43.4, 55.1, 58.0, 58.1, 58.2, 58.3, 62.9, 63.0, 73.4, 73.6, 73.7, 74.0, 85.5, 85.6, 85.8, 86.1, 86.9, 112.5, 112.6, 113.2, 117.3, 117.5, 127.3, 128.0, 128.1, 128.2, 129.8, 130.0, 130.1, 132.6, 135.0, 135.1, 136.8, 138.3, 138.4, 144.0, 147.62, 147.65, 158.8, 179.6; ^{19}F NMR (CDCl$_3$, 282 MHz) δ −81.3, −115.2, −121.9, −122.2, −122.9, −123.9, −126.3; ^{31}P NMR (CDCl$_3$, 121 MHz) δ 149.6, 150.0; MS (ESI) *m/z* 1280.3 [M + H]$^+$; HRMS (ESI) calcd for $C_{56}H_{55}CsF_{17}N_5O_8P$ [M + Cs]$^+$ 1412.257, found 1412.272.

Synthesis of Oligonucleotides. The oligonucleotides **9a−d** were synthesized with a DNA synthesizer and cleaved from the controlled pore glass (CPG) support with concentrated ammonium hydroxide (3 mL, 2 h). The deprotection was achieved by heating the resultant ammonium hydroxide solution at 52 °C for 5 h. Purification of the oligonucleotides was carried out by reverse-phase HPLC using a C18 column (21.2 mm × 150 mm). The molecular weights of the oligonucleotides were determined by MALDI-TOF mass spectroscopy.

■ ASSOCIATED CONTENT

Supporting Information

The experimental protocols and NMR spectra of key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: qszhang@unc.edu.

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