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Chemical Syntheses of Oligodeoxyribonucleotides Containing Spore Photoproduct

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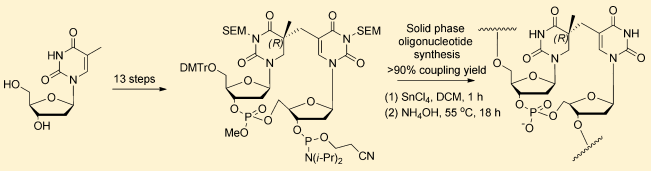
Supporting Information

ABSTRACT: 5-(α-Thyminyl)-5,6-dihydrothymine, also called spore photoproduct or SP, is commonly found in the genomic DNA of UV-irradiated bacterial endospores. Despite the fact that SP was discovered nearly 50 years ago, its biochemical impact is still largely unclear due to the difficulty of preparing SP-containing oligonucleotide in high purity. Here, we report the first synthesis of the phosphoramidite derivative of dinucleotide SP TpT, which enables successful incorporation of SP TpT into oligodeoxyribonucleotides with high efficiency via standard solid-phase synthesis. This result provides the scientific community a reliable means to prepare SP-containing oligonucleotides, laying the foundation for future SP biochemical studies. Thermal denaturation studies of the SP-containing oligonucleotide found that SP destabilizes the duplex by 10−20 kJ/mol, suggesting that its presence in the spore-genomic DNA may alter the DNA local conformation.



INTRODUCTION

Among the four nucleobases, thymine is most sensitive to UV irradiation.[1] In regular cells, the adjacent thymine residues in genomic DNA will dimerize, resulting in *cis-syn* cyclobutane dimer (CPD) and (6−4) photoproduct ((6−4) PD) as the most common products (Figure 1).[2−7] Further irradiation of (6−4) PD under ∼310 nm UV light triggers isomerization of the pyrimidinone ring to its Dewar valence isomer.[8,9] In addition to these three species, the fourth dimer, 5-(α-thyminyl)-5,6-dihydrothymine, which is also called spore photoproduct or SP, was identified as the sole photolesion in the genomic DNA of UVC-irradiated bacterial endospores.[10−13]

SPs are quickly repaired in germinating spores by a radical SAM enzyme−spore photoproduct lyase,[10,13−25] thus posing little threat to spores' survival. The unrepaired SPs, however, prove lethal to germinating spores.[26,27] It is currently unclear whether the lethality of SP is due to its induction of mutagenesis or due to its ability to halt polymerase. Moreover, although SP was generally considered to exist only in bacterial endospores, a recent study found it was the dominant DNA photolesion in UV-irradiated airborne *Mycobacterium parafortuitum* under 20−40% relative humidity (RH),[28] suggesting that SP may exist in other species as well. Its formation is likely to be responsible for the UV-killing effect in the air sterilization process. SP was also implied to be present in the UV-irradiated frozen *E. coli* cells, and *E. coli* cells were more sensitive to SP than to other dithymine photoproducts.[29] These findings suggest that SP may play a role in nature that is much bigger than we currently think.

Different from CPD and (6−4) PD whose formations are mediated by [2 + 2] photocyclization reaction, SP is formed via an intramolecular H atom-transfer mechanism.[30−32] Its formation requires A-DNA, which is induced by the low hydration level in endospores.[11,27,33] This, coupled with other key factors such as the presence of a group of DNA-binding proteins named small acid soluble proteins to solidify the A-conformation,[34−36] determines SP to be the sole photolesion in UV irradiated endospores. SP can be generated under in vitro conditions via solid phase (dry film or ice) DNA photoreaction;[15,31,37−39] however, its yield is very low (<1%).[31] This yield and the simultaneous formation of many other photolesions determine that it is very difficult to obtain enough SP-containing oligonucleotide with high purity for biological studies. As a consequence, although SP has been discovered for half a century,[40] little is known about its impact to the biological function of DNA.

The best means to prepare highly pure SP containing oligonucleotide is via chemical synthesis using traditional phosphoramidite chemistry, which requires SP phosphoramidite as a building block. Currently, an SP dinucleoside phosphoramidite, which does not contain the phosphodiester moiety, is available.[41] However, the lack of the phosphodiester linkage likely releases any distortion created by the methylene bridge between the two thymine bases in SP. Thus, research conducted using dinucleoside SP-containing oligomer may not be truly biologically relevant. In this report, we describe the first

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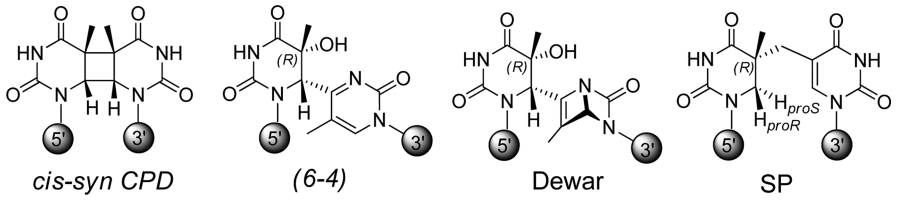
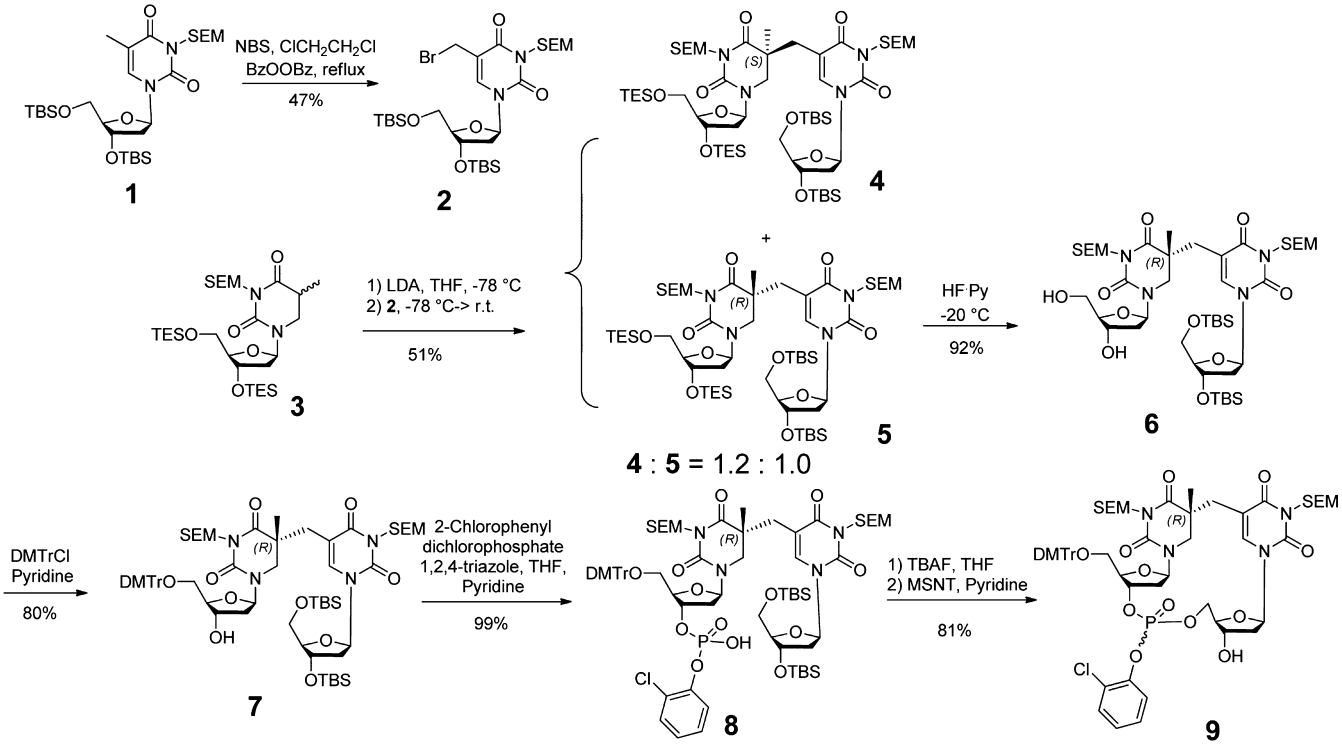


Figure 1. Structures of the major type of thymine photoproducts.

Scheme 1. Synthesis of the Protected *R* Isomer of SP TpT



synthesis of the phosphoramidite for dinucleotide SP TpT, which enables SP TpT incorporation into oligonucleotide with high efficiency, making SP biological studies possible.

RESULTS AND DISCUSSION

The phosphoramidite derivatives of *cis-syn* CPD,[42−49] (6−4) PD,[50] and Dewar PD[51] have been available, which enabled successful preparations of thymine dimer containing oligonucleotides with high reaction efficiencies. All these derivatives were generated via hybrid approaches where the corresponding dimers were first produced by photochemistry using partially protected dinucleotide TpT before the phosphoramidite moiety was introduced to the 3′-OH group at the 3′-end of the dimer via organic synthesis. Such an approach, however, proves futile in preparing the SP phosphoramidite. As mentioned above, the unprotected dinucleotide TpT generates SP in ∼1% yield via solid-state photoreaction.[31] Once the phosphodiester moiety is protected by esterization with either $-CH_3$ or $-CH_2CH_2CN$, the resulting dinucleotide no longer supports SP formation. Such a result agrees with our previous finding that dinucleotide T($_{CH2}$)T which contains a neutral formacetal linkage did not support SP photochemistry,[52] suggesting the negative charge carried by the phosphodiester linker is essential for the two thymine residues to adopt the "right" stacking conformation to enable SP photochemistry. It is thus impractical to generate enough SP via photoreaction. To prepare SP phosphoramidite on a relatively large scale, new strategies have to be developed.

Different from the other thymine dimers where chemical syntheses are not available, synthesis of the dinucleotide SP TpT was successfully achieved via a 14-step reaction by Begley et al.[53] The synthesis employed C=C hydrogenation, methyl bromination, and nucleophilic substitution to yield two dinucleoside isomers and phosphodiester moiety insertion to obtain the dinucleotide SP TpT. However, in Begley's synthesis, the two dinucleoside diastereoisomers were used as intermediates and the *R* and *S* isomers were not separated by HPLC until the very last step. As only the *R* isomer is formed in endospores' genomic DNA,[54] it is desirable to separate these isomers before the phosphodiester moiety is introduced. Separation of the protected dinucleoside SP isomers was achieved by Broderick et al.[55] and Carell et al.,[41] respectively, using different deoxyribose protection reagents. Having these previous works in mind, we decided to adopt a combined approach to prepare the protected *R* isomer of SP TpT (Scheme 1).

Fully protected thymidine **1** and dihydrothymidine **3** were prepared from thymidine using Begley's procedure.[53] In that approach, the methyl bromination reaction in the production of **2** was conducted via a photochemical assay using elemental bromine. Carell et al. later employed N-bromosuccinimide

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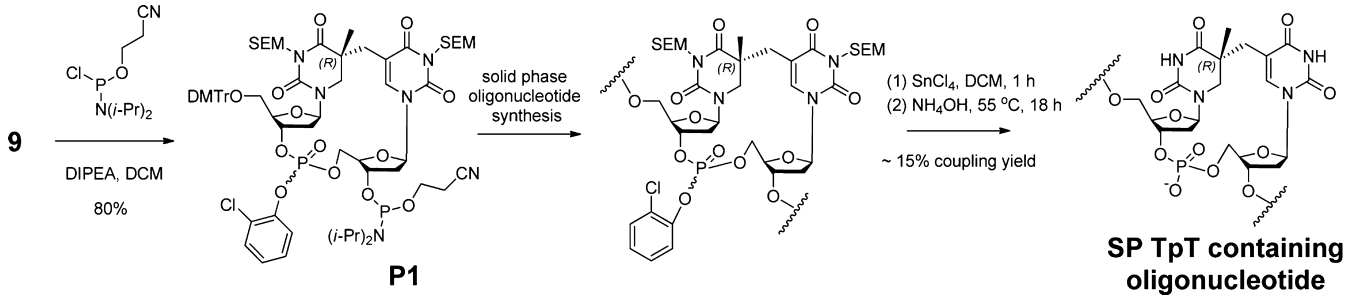
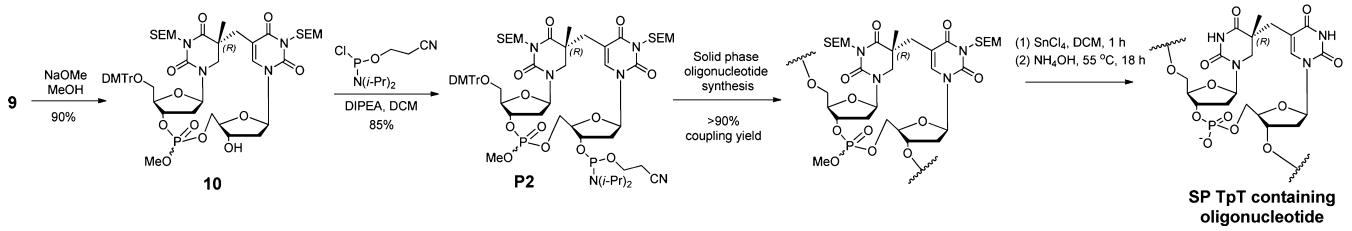
Scheme 2. Synthesis of the SP TpT-Containing Oligonucleotide via Phosphoramidite P1



Scheme 3. Synthesis of the SP TpT-Containing Oligonucleotide via Phosphoramidite P2



(NBS) as a substitute of Br_2 to brominate the methyl moiety because NBS is easier to handle and reacts more mildly.[56] We further modified Carell's method, using 1, 2-dichloroethane to replace CCl_4 as the solvent for the NBS reaction (Scheme 1). Such a solvent change resulted in a slightly reduced reaction yield (47% vs 60%); however, compared to CCl_4, 1, 2-dichloroethane is much cheaper and more environmentally friendly. The coupling reaction between the enolate of **3** and bromomethyl deoxyuridine **2** afforded **4** and **5** (1.2:1.0) in 53% total yield. After separation via flash chromatography using a literature procedure,[55] the resulting **5** was treated with either 4% HF in pyridine[53] or HCO_2H in methanol[55] to remove the triethylsilyl (TES) protecting groups, generating **6**. The yield of **6** is modest in either reaction due to the unexpected loss of the *tert*-butyldimethylsilyl (TBS) moiety. We found that treating **5** with 4% HF·pyridine in acetonitrile drastically improved the yield to 92%. TBS is very stable under this condition, and TES can thus be selectively removed. Subsequent dimethoxytritylation of the 5′-OH followed by phosphorylation of the 3′-OH afforded **8**. After desilylation, the MSNT-mediated formation of the phosphotriester yielded the protected SP **9** as a mixture of two diastereomers in a ratio of 1:1.7 (*Sp*/*Rp*), as indicated by LC−MS analysis.

Using **9** as precursor, an SP TpT phosphoramidite **P1** (Scheme 2) was readily synthesized. **P1** was stable enough to survive the column purification; an 80% yield was obtained for this reaction step. In another attempt, we removed the 2-(trimethylsilyl)ethoxymethyl (SEM) protecting groups attached to the N3 positions of the thymine ring in SP. Surprisingly, deletion of SEM greatly destabilizes the corresponding SP phosphoramidite. Although its formation was indicated by ESI-MS analysis, this phosphoramidite decomposed completely during purification via flash chromatography. Such a result is surprising as phosphoramidites of unprotected CPD and (6−4) PD species are all fairly stable.[42−44,46,50] We tentatively ascribe the instability of the unprotected SP phosphoramidite to its unique chemical structure. Moreover, in the previously synthesized dinucleoside SP TT phosphoramidite, the N3 positions were also protected by SEM.[41] The N3 protection thus appears essential for the production of a stable SP phosphoramidite, although it is unclear why alteration at the remote N3 site can impact the phosphoramidite moiety attached to the 3′-deoxyribose.

Subsequent oligonucleotide synthesis using **P1** as a building block afforded an SP TpT containing 10-mer d(CACC[SP TpT]CATC) in a proof-of-concept experiment. However, the coupling yield for the SP incorporation step was found to be merely ∼15%, even after we extended the coupling time to 2 h or repeated the coupling step twice. This result is in sharp contrast to the >95% yield obtained in the previous preparation of bent DNA, which used uracil alkylene cross-linked phosphoramidites containing the 2-chlorophenyl protecting group and a 20-min reaction time.[57] Such a low coupling yield determines that it is impractical to use **P1** to synthesize SP-containing oligonucleotide on a relatively large scale.

Analyzing the phosphoramidites of other thymine dimers reveals that their phosphodiester moieties were all protected by a small alkyl group,[42−44,50,51] suggesting that the bulky 2-chlorophenyl moiety in **P1** may create some steric hindrance, preventing an efficient coupling reaction from occurring during the solid-phase synthesis. We therefore incubated **9** with sodium methoxide to replace the 2-chlorophenyl moiety with a $-CH_3$ and prepared an SP phosphoramidite derivative **P2** (Scheme 3). As expected, **P2** fully supports the solid-state synthesis, exhibiting a >90% coupling efficiency during the preparation of the 10-mer d(CACC[SP TpT]CATC) (Figure 2A). During the synthesis, the reaction time was extended to 1 h for the SP incorporation step. In contrast, the coupling reaction for a regular deoxyribonucleotide usually finishes within 1 min. Such an elongated reaction time is necessary for an efficient SP incorporation; similar strategies were utilized previously to ensure the incorporation of other dithymine photoproducts into oligonucleotides with satisfactory yields.[42,43] Product formation was confirmed by ESI-MS analysis (Figure 2B). After purification by HPLC, the overall yield for the SP containing 10-mer was found to be 63%, based on the amount of resin used.

To demonstrate that long oligonucleotides can also be prepared by this method, d(CTCGACACG[SP TpT]-CGCATGCCA), a 20-mer, was synthesized. The SP TpT

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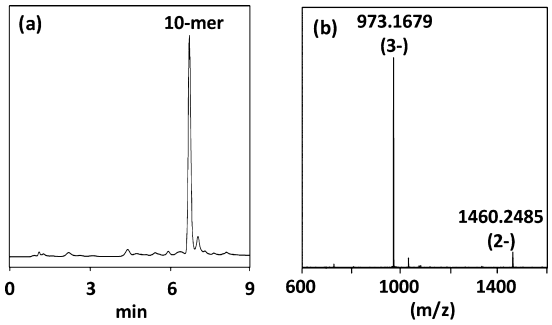

Figure 2. (a) HPLC chromatograph (260 nm) of the crude reaction mixture in the synthesis of 10-mer d(CACC[SP TpT]CATC). (b) ESI-MS analysis of the resulting SP containing 10-mer oligonucleotide. After deconvolution, the 10-mer exhibits a mass of 2921.51 (calcd 2921.53).

containing 20-mer was detected as the major product, as proved by the HPLC analysis and confirmed by ESI-MS spectrometry (Figure S3, Supporting Information). The overall yield of the 20-mer was determined to be 30% relative to the amount of resin used, again indicating **P2** is a good building block for SP incorporation. This method thus does not have sequence limitation and can be readily used to prepare the long oligonucleotides needed in typical biochemical studies such as the polymerase extension experiments.

The successful preparation of SP TpT-containing oligonucleotides provides us a good opportunity to study the influence of SP to the stability of duplex oligonucleotide. Such information is still unknown despite the fact that SP was discovered nearly 50 years ago. We thus compared the thermal denaturation curves of SP TpT-containing oligonucleotides with the corresponding undamaged parent strands. Using the 10-mer sequence at 2.4 μM concentration in 10 mM phosphate buffer at pH 7, which also contains 150 mM NaCl, 12 °C difference (27.8 °C vs 39.5 °C) between the oligonucleotide melting points (T_ms) was observed (Figure 3A). Using the same buffer and 1.6 μM oligomer, the presence of SP TpT in

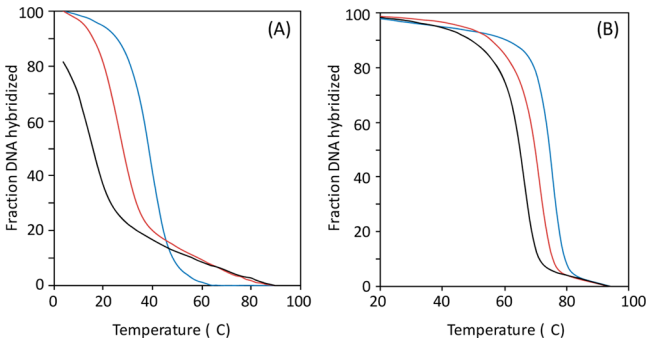

Figure 3. (A) Thermal denaturation curves of the 10-mers d(CACC**TT**CATC) (blue line), d(CACC[**SP TpT**]CATC (red line), and d(CACC[**SP TT**]CATC) (black line) annealed with the complementary strand d(GTGGAAGTAG), respectively. The buffer contained 10 mM phosphate at pH 7 and 150 mM NaCl; the oligomer concentration was 2.4 μM for all three samples. (B) Thermal denaturation curves of the 20-mers d(CTCGACACG**TT**CGCATGCCA) (blue line), d(CTCGACACG[**SP TpT**]CGCATGCCA) (red line), and d(CTCGACACG[**SP TT**]CGCATGCCA) (black line) annealed with the complementary strand, respectively. The buffer contained 10 mM phosphate at pH 7 and 150 mM NaCl; the oligomer concentration was 1.6 μM for all three samples.

the 20-mer decreases the T_m by 5 °C (69.5 °C vs 74.5 °C) (Figure 3B). Both measurements suggest that the presence of SP significantly destabilizes the duplex oligonucleotide. It is worth mentioning that the T_m difference between a modified and the corresponding unmodified duplex is sensible to the length of the oligonucleotide, and the difference is higher for a shorter duplex. Therefore, the T_m difference (12 °C vs 5 °C) observed between the 10- and the 20-mer is not surprising.

The crystal structure of the dinucleoside SP TT-containing oligonucleotide was recently solved by Carell et al.[41] The structure reveals that the two thymines in SP TT hydrogen bond with the two adenines on the complementary strand as if they were undamaged. As the lack of the phosphodiester linkage in dinucleoside SP TT (Scheme 4) could release the potential conformational distortion created by the methylene bridge between the two thymine bases of SP, we wonder if the structure observed truly reflects that of the SP containing spore genomic DNA.

We therefore synthesized the SP dinucleoside phosphoramidite **P3** using a small acetyl group to replace the bulky *tert*-butyldiphenylsilyl (TBDPS) moiety adopted previously to protect the 3′-OH group of the 5′-thymidine (Scheme 4). **P3** enables us to prepare the SP TT-containing 10-mer and 20-mer, respectively, using sequences identical to those above. A total yield of 60% (based on the amount of resin used) was obtained in the 10-mer synthesis,[30] which is comparable to the 63% yield found in the preparation of SP TpT containing 10-mer, but much higher than the 15% total yield obtained previously during a 12-mer preparation using the TBDPS-protected SP TT phosphoramidite.[41] This nearly 4-fold improvement in yield is significant considering the cost of the starting material and the 10+ synthetic steps to obtain the SP TT-containing oligomer. This finding is consistent with the observation above that **P2** is a far better reagent than its bulkier counterpart **P1** for SP TpT incorporation. These results indicate that the steric hindrance associated with the 3′-OH moiety at the 5′-end of SP has a big impact to the coupling reaction at the 3′-deoxyribose. Under conditions identical to those above, the SP TT containing 10-mer duplex exhibits ~25 °C T_m decrease compared to the undamaged parent 10-mer strand (Figure 3A). The 20-mer is relatively stable, exhibiting a T_m of 64.6 °C, which is still 10 °C lower than its parent strand (Figure 3B).

To further reveal the impact of the dinucleotide SP TpT and dinucleoside SP TT to the stability of the duplex oligonucleotide, we measured the melting points (T_m's) for the 10-mers and 20-mers containing TpT, SP TpT, and SP TT, respectively, as a function of concentration. For the 10-mer strands, using oligomer concentrations ranging from 0.6 to 4.8 μM, the thermodynamic parameters $\Delta H°$, $\Delta S°$, and $\Delta G°$ of the dissociation process at 310 K were derived from concentration dependent T_m investigations using van't Hoff plots.[58−60] Similarly, using oligomer concentrations ranging from 0.4 to 3.2 μM, the $\Delta H°$, $\Delta S°$, and $\Delta G°$ were determined for the corresponding 20-mer strands as well.

As shown in Table 1, the calculated free energy change ($\Delta\Delta G°$) for the dinucleotide SP TpT-containing duplex was found to be less negative than the native strand by 10 kJ/mol for the 10-mer and 21 kJ/mol for the 20-mer, respectively, at 37 °C. We tentatively ascribe the positive $\Delta\Delta G°$ for the SP TpT-containing duplexes to the poor H-bonding interaction with the complementary adenines and/or the weak stacking interaction between SP and its neighboring residues. This result suggests

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Scheme 4. Synthesis of the Dinucleoside SP TT-Containing Oligonucleotide via Phosphoramidite P3

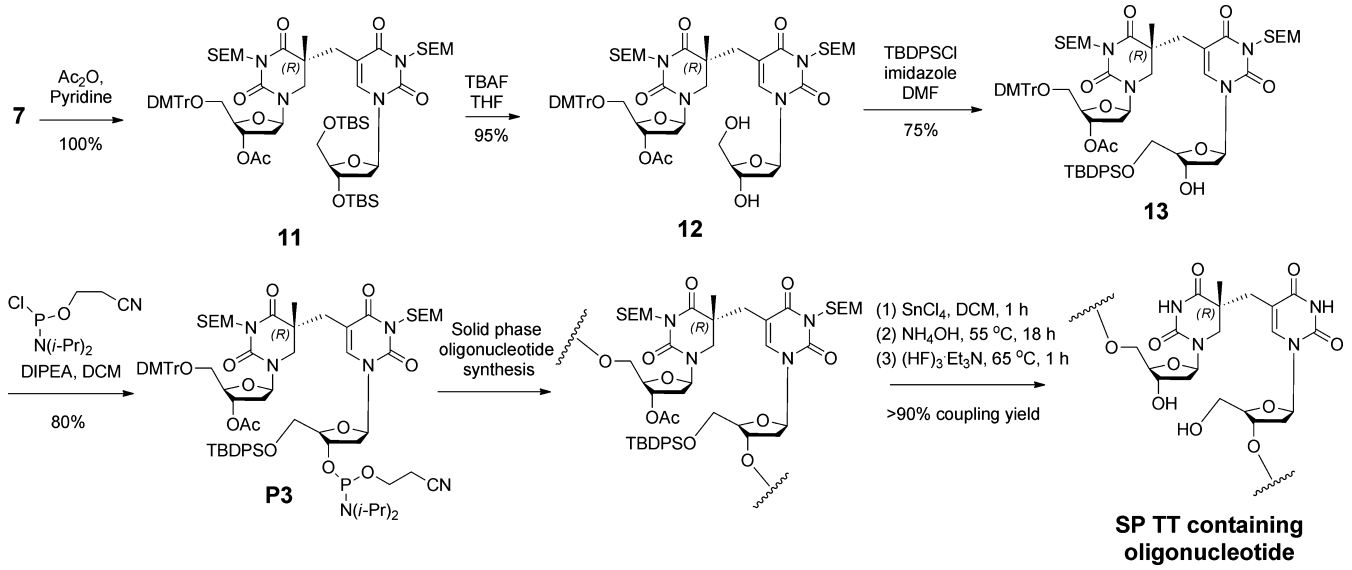

Table 1. Thermodynamic Parameters for Duplex Formation in Native and SP-Containing Oligonucleotides

		$\Delta H°$ (kJ/mol)	$\Delta S°$ (kJ/mol/K)	$\Delta G°$ (kJ/mol) (37 °C)	$\Delta\Delta G°$ (kJ/mol) (37 °C)
10-mer	native	−278	−0.763	−41	
	SP TpT	−259	−0.735	−31	+10
	SP TT	−240	−0.709	−20	+21
20-mer	native	−778	−2.11	−124	
	SP TpT	−670	−1.825	−103	+21
	SP TT	−618	−1.701	−90	+34

that similar to *cis-syn* CPD,[61,62] SP also destabilizes the duplex oligonucleotide. As shown by Taylor et al., the presence of *cis-syn* CPD destabilizes the duplex strands by 5.7−8.4 kJ/mol depending on the oligonucleotide sequence studied.[62] Barring the sequence difference, our data implies that the SP TpT may cause more local distortion to the duplex structure than the *cis-syn* CPD in aqueous solution. Moreover, the presence of dinucleoside SP TT destabilizes the duplex strands even further (Table 1). Although Carell's work implies that the presence of SP TT induces little conformational change; such a structure may only reflect the DNA conformation in the crystal state and is unlikely to represent that of the SP TpT containing oligomer in solution.

CONCLUSION

Here we report the first synthesis of SP TpT phosphoramidite, which allows us to prepare SP TpT-containing oligonucleotide with high purity and high efficiency. This work clears the major obstacle in studying the impact of SP to the biofunction of DNA.

EXPERIMENTAL SECTION

General Methods. dA-, dT-, dC-, and dG-phosphoramidites were purchased from Glen Research (Sterling, VA). CPG Nucleosides carriers were obtained from 3-Prime (3-prime, Aston, PA). All reactions were carried out using oven or flame-dried glassware under a nitrogen atmosphere in distilled solvents. Dichloromethane and pyridine were distilled over calcium hydride. Purification of reaction products was carried out by flash chromatography using silica gel (Dynamic Adsorbents, Inc., 32−63 μm). For TLC analysis, precoated plates (w/h F254, Dynamic Adsorbents Inc., 0.25 mm thick) were used. The ^{1}H, ^{13}C, and ^{31}P NMR spectra were obtained on a Bruker 500 MHz NMR Fourier transform spectrometer. NMR spectra were recorded in sample solutions in deuterated chloroform ($CDCl_3$), with residual chloroform (Δ 77.0 ppm for ^{13}C NMR) and TMS (Δ 0 ppm for ^{1}H NMR), deuterated methanol (Δ 3.31 ppm for 1H NMR and Δ 49.1 ppm for ^{13}C NMR), or deuterated methyl sulfoxide (DMSO-d_6), with residual methyl sulfoxide (Δ 2.50 ppm for ^{1}H NMR and Δ 39.5 ppm for ^{13}C NMR) taken as the standard. The chemical shifts in NMR spectra were reported in parts per million (ppm). Mass (MS) analysis was obtained via ESI with an ion-trap mass analyzer. The HR-MS was performed with Q-TOF LC/MS spectrometer; the data were acquired via Agilent MassHunter Workstation Data Acquisition (B.03.00) and analyzed via Qualitative Analysis of MassHunter Acquisition Data (B.03.00) software.

***α*-Bromo-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-3-[(trimethylsilylethoxy)methyl]thymidine (2).** Benzoyl peroxide (168 mg, 0.69 mmol) and NBS (8.71 g, 48.95 mmol) were added in turn to a solution of **1** (13.90 g, 23.13 mmol) in anhydrous 1,2-dichloroethane (168 mL). The reaction mixture was stirred at 80 °C for 1 h, and the solvent was removed by rotary evaporation. The resulting residue was purified via column chromatography (eluent: hexane/EtOAc = 7:1) to afford **2** as a pale yellow oil (8.18 g, 12.03 mmol, 52% yield): R_f = 0.60 (hexane/ethyl acetate = 4:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.01 (s, 9H), 0.08 (s, 3H), 0.09 (s, 3H), 0.137 (s, 3H), 0.144 (s, 3H), 0.87−1.08 (m, 20H), 1.92−2.04 (m, 1H), 2.30−2.39 (m, 1H), 3.64−3.72 (m, 2H), 3.74−3.81 (m, 1H), 3.86−3.92 (m, 1H), 3.95−4.01 (m, 1H), 4.24 (d, J = 10.4 Hz, 1H), 4.31 (d, J = 10.7 Hz, 1H), 4.36−4.41 (m, 1H), 5.38−5.45 (m, 2H), 6.28−6.34 (m, 1H), 7.89 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −5.35, −5.38, −4.9, −4.7, −1.5, 18.0, 18.1, 18.4, 25.7, 26.0, 26.2, 41.9, 63.0, 67.7, 70.2, 72.2, 86.2, 88.2, 110.9, 137.8, 150.4, 161.3.

(5*S*)- and (5*R*)-*α*-[3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-3-[(trimethylsilylethoxy)methyl]thymidyl]-5,6-dihydro-3′,5′-*O*-bis(triethylsilyl)-3-[(trimethylsilylethoxy)methyl]thymidine (4,5). LDA (5.40 mL, 10.80 mmol, 2 M solution in hexane/THF/ethylbenzene) was slowly added to a solution of **3**[53] (4.36 g, 7.23 mmol) in THF (60 mL) at −78 °C. The reaction mixture was stirred at the same temperature for 2 h before addition of **2** (6.14 g, 9.04 mmol) in THF (15 mL). The resulting mixture was stirred at −78 °C for 30 min and then slowly warmed to room temperature over 3 h. After being stirred at room temperature overnight, the mixture was diluted with EtOAc, washed with NH_4Cl, water, and brine successively, and dried over anhydrous Na_2SO_4. After the solvent was removed via rotary evaporation, the resulting residue was purified

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via column chromatography (eluent: hexane/EtOAc = 7:1) to afford **4** (*S-isomer*, 2.43 g, 2.02 mmol, 28% yield) and **5** (*R-isomer*, 2.00 g, 1.66 mmol, 23% yield) as colorless oils. *S*-Isomer: R_f = 0.62 (hexane/ethyl acetate = 4:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.02 (s, 9H), −0.01 (s, 9H), 0.083 (s, 3H), 0.087 (s, 3H), 0.097 (s, 3H), 0.100 (s, 3H), 0.60 (q, *J* = 8.0 Hz, 6H), 0.63 (q, *J* = 7.9 Hz, 6H), 0.89 (s, 9H), 0.90 (s, 9H), 0.92−1.00 (m, 22H), 1.17 (s, 3H), 1.91−2.02 (m, 3H), 2.31 (ddd, *J* = 2.2, 5.7, 13.2 Hz, 1H), 2.53 (d, *J* = 14.2 Hz, 1H), 2.80 (d, *J* = 14.2 Hz, 1H), 3.05 (d, *J* = 13.1 Hz, 1H), 3.32 (d, *J* = 13.1 Hz, 1H), 3.54−3.61 (m, 3H), 3.61−3.66 (m, 2H), 3.68 (dd, *J* = 3.8, 10.9 Hz, 1H), 3.73 (dd, *J* = 5.0, 10.9 Hz, 1H), 3.78 (dd, *J* = 3.5, 10.9 Hz, 1H), 3.81−3.86 (m, 1H), 3.92−3.96 (m, 1H), 4.30−4.34 (m, 1H), 4.38 (td, *J* = 2.4, 5.5 Hz, 1H), 5.14 (d, *J* = 9.4 Hz, 1H), 5.21 (d, *J* = 9.6 Hz, 1H), 5.34 (s, 2H), 6.24 (dd, *J* = 5.6, 8.0 Hz, 1H), 6.37 (dd, *J* = 6.3, 8.1 Hz, 1H), 7.46 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) −5.5, −5.3, −4.9, −4.7, −1.5, −1.4, 4.3, 4.7, 6.7, 6.8, 17.9, 18.0, 18.1, 18.3, 20.7, 25.7, 25.9, 32.3, 37.0, 40.8, 42.2, 44.9, 63.2, 63.3, 66.7, 67.3, 70.0, 70.2, 72.67, 72.73, 84.9, 86.2, 86.7, 88.0, 108.4, 138.0, 150.6, 152.5, 163.3, 173.5. *R*-Isomer: R_f = 0.65 (hexane/ethyl acetate = 4:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.02 (s, 9H), 0.00 (s, 9H), 0.10 (s, 3H), 0.11 (s, 3H), 0.118 (s, 3H), 0.123 (s, 3H), 0.61 (q, *J* = 7.9 Hz, 6H), 0.65 (q, *J* = 7.9 Hz, 6H), 0.90 (s, 9H), 0.93 (s, 9H), 0.94−1.01 (m, 22H), 1.22 (s, 3H), 1.86−2.04 (m, 3H), 2.24 (ddd, *J* = 3.1, 6.1, 13.3 Hz, 1H), 2.65 (d, *J* = 13.5 Hz, 1H), 2.74 (d, *J* = 13.5 Hz, 1H), 3.09 (d, *J* = 13.1 Hz, 1H), 3.21 (d, *J* = 12.9 Hz, 1H), 3.53−3.71 (m, 6H), 3.75 (dd, *J* = 5.3, 11.9 Hz, 1H), 3.78−3.84 (m, 2H), 3.92 (dd, *J* = 3.9, 8.2 Hz, 1H), 4.28−4.35 (m, 1H), 4.41−4.46 (m, 1H), 5.13−5.23 (m, 2H), 5.30−5.40 (m, 2H), 6.33 (dd, *J* = 6.2, 7.5 Hz, 1H), 6.35 (dd, *J* = 6.1, 8.1 Hz, 1H), 7.52 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −5.4, −5.3, −4.9, −4.7, −1.45, −1.42, 4.2, 4.6, 6.7, 6.8, 17.9, 18.08, 18.11, 18.4, 22.0, 25.7, 25.9, 32.3, 36.8, 40.2, 42.9, 44.2, 62.9, 63.0, 66.7, 67.4, 69.9, 70.3, 72.0, 72.1, 84.4, 85.3, 86.3, 87.5, 108.5, 139.1, 150.6, 152.3, 163.5, 173.6.

(5*R*)-α-[3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-3-[(trimethylsilylethoxy)methyl]thymidyl]-5,6-dihydro-3-[(trimethylsilylethoxy)methyl]thymidine (6). HF·Py (28.7 mL, 1.7 M in acetonitrile) was slowly added to a solution of **5** (4.88 g, 4.06 mmol) in acetonitrile (80 mL) at −20 °C. After being stirred at the same temperature for 6 h, the reaction mixture was diluted with EtOAc, washed with $NaHCO_3$, water, and brine, and dried over anhydrous Na_2SO_4. After the solvent was removed via rotary evaporation, the resulting residue was purified via column chromatography (eluent: hexane/EtOAc/MeOH = 1:1:0.1) to afford **6** as a white solid (3.64 g, 3.74 mmol, 92% yield): R_f = 0.20 (hexane/ethyl acetate = 4:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.02 (s, 9H), 0.01 (s, 9H), 0.09 (s, 6H), 0.11 (s, 6H), 0.89 (s, 9H), 0.92 (s, 9H), 0.94−0.99 (m, 2H), 1.16 (s, 3H), 1.93−2.02 (m, 3H), 2.07 (ddd, *J* = 3.1, 6.3, 13.5 Hz, 1H), 2.19−2.28 (m, 2H), 2.61 (d, *J* = 3.9 Hz, 1H), 2.72 (d, *J* = 14.3 Hz, 1H), 2.79 (d, *J* = 14.5 Hz, 1H), 3.13 (d, *J* = 4.8 Hz, 1H), 3.19 (d, *J* = 13.1 Hz, 1H), 3.50 (d, *J* = 13.1 Hz, 1H), 3.57 (t, *J* = 8.2 Hz, 2H), 3.65−3.82 (m, 6H), 3.88 (dd, *J* = 3.1, 6.0 Hz, 1H), 3.95 (dd, *J* = 3.8, 10.0 Hz, 1H), 4.39−4.46 (m, 2H), 5.14 (d, *J* = 9.5 Hz, 1H), 5.23 (d, *J* = 9.4 Hz, 1H), 5.27 (d, *J* = 9.1 Hz, 1H), 5.41 (d, *J* = 9.2 Hz, 1H), 6.23 (t, *J* = 7.1 Hz, 1H), 6.32 (dd, *J* = 2.0, 8.0 Hz, 1H), 7.57 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −5.5, −5.3, −4.9, −4.7, −1.5, −1.4, 17.9, 18.0, 18.2, 18.4, 20.8, 25.7, 25.9, 31.9, 37.6, 40.7, 42.2, 45.0, 62.5, 63.1, 66.7, 67.8, 69.9, 70.2, 72.2, 72.3, 85.6, 85.7, 86.1, 87.9, 108.6, 139.0, 150.6, 152.3, 163.5, 174.3.

(5*R*)-α-[3′,5′-*O*-Di(*tert*-butyldimethylsilyl)-3-[(trimethylsilylethoxymethyl)]thymidyl]-5,6-dihydro-5′-*O*-(4,4′-dimethoxytrityl)-3′,5′-*O*-bis(triethylsilyl)-3-[(trimethylsilylethoxy)methyl]-thymidine (7). DMTrCl (937 mg, 2.77 mmol) was added to a solution of **6** (2.25 g, 2.31 mmol) in pyridine (13 mL). After being stirred at room temperature overnight, the solvent was removed under reduced pressure. The resulting residue was purified via column chromatography (eluent: hexane/EtOAc = 2:1) to afford **7** as a white solid (2.69 g, 2.11 mmol, 80% yield): R_f = 0.50 (hexane/ethyl acetate = 1:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.02 (s, 9H), 0.00 (s, 9H), 0.109 (s, 3H), 0.113 (s, 3H), 0.116 (s, 3H), 0.119 (s, 3H), 0.91 (s, 9H), 0.93 (s, 9H), 0.85−0.97 (m, 4H), 1.16 (s, 3H), 1.95−2.02 (m, 1H), 2.02−2.09 (m, 2 H), 2.11−2.19 (m, 1 H), 2.24 (ddd, *J* = 3.0, 6.1, 13.3 Hz, 1H), 2.61 (d, *J* = 14.0 Hz, 1H), 2.68 (d, *J* = 14.2 Hz, 1H), 3.16 (d, *J* = 13.1 Hz, 1H), 3.19 (d, *J* = 12.9 Hz, 1H), 3.32 (dd, *J* = 4.8, 10.7 Hz, 1H), 3.34 (dd, *J* = 4.6, 10.3 Hz, 1H), 3.56 (t, *J* = 8.1 Hz, 2H), 3.63−3.68 (m, 2H), 3.72−3.78 (m, 2H), 3.79 (s, 6H), 3.82 (dd, *J* = 3.9, 11.0 Hz, 1H), 3.92 (td, *J* = 3.5, 8.8 Hz, 1H), 4.36−4.42 (m, 1H), 4.43−4.47 (m, 1H), 5.16 (d, *J* = 9.7 Hz, 1H), 5.18 (d, *J* = 9.6 Hz, 1H), 5.30 (d, *J* = 9.8 Hz, 1H), 5.32 (d, *J* = 9.7 Hz, 1H), 6.33 (t, *J* = 7.1 Hz, 1H), 6.34 (t, *J* = 6.9 Hz, 1H), 6.83−6.87 (m, 4H), 7.19−7.24 (m, 1H), 7.28−7.32 (m, 2H), 7.32−7.37 (m, 4 H), 7.44−7.47 (m, 2H), 7.51 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −5.4, −5.3, −4.8, −4.7, −1.4, 17.9, 18.1, 18.2, 18.4, 22.3, 25.7, 25.9, 32.3, 36.5, 40.1, 43.0, 44.6, 55.2, 62.8, 63.5, 66.7, 67.5, 69.9, 70.4, 71.9, 72.0, 83.2, 83.5, 85.4, 86.4, 87.5, 108.4, 113.2, 126.8, 127.9, 128.1, 130.01, 130.03, 135.80, 135.84, 139.4, 144.7, 150.6, 152.3, 158.5, 163.5, 173.4; ESI-HRMS *m*/*z* calcd for $C_{65}H_{106}N_5O_{14}Si_4^+$ (M + NH_4^+) 1292.6813, found 1292.6811.

***P*-(2-Chlorophenyl) (5*R*)-α-[3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-3-[(trimethylsilylethoxy)methyl]thymidyl]-5,6-dihydro-5′-*O*-(4,4′-dimethoxytrityl)-3′,5′-*O*-bis(triethylsilyl)-3-[(trimethylsilylethoxy)methyl]-3′-thymidylate (8).** 2-Chlorophenyl dichlorophosphate (0.93 mL, 5.74 mmol) was added dropwise to a solution of 1,2,4-triazole (792 mg, 11.47 mmol) and triethylamine (1.60 mL, 11.47 mmol) in THF (80 mL). The reaction was stirred for 40 min at room temperature. In a separate flask, dinucleotides **7** (1.46 g, 1.15 mmol) were dissolved in pyridine (80 mL). The solution of phosphoryl triazole was transferred into a flask by cannula through a sintered glass funnel. The reaction mixture was stirred for 1 h at room temperature and quenched by the addition of saturated $NaHCO_3$ and the solvent removed under reduced pressure. The crude product was dissolved in dichloromethane, extracted with saturated aq $NaHCO_3$, and dried over Na_2SO_4. After the solvent was removed via rotary evaporation, the resulting residue was purified via column chromatography (eluent: dichloromethane/methanol = 15:1) to afford **8** as a colorless oil (1.69 g, 1.14 mmol, 99% yield): R_f = 0.50 (dichloromethane/methanol = 9:1); ^{1}H NMR (500 MHz, acetone-d_6) δ −0.02 (s, 9H), −0.01 (s, 9H), 0.13 (s, 3H), 0.14 (s, 3H), 0.167 (s, 3H), 0.174 (s, 3H), 0.80−0.90 (m, 4H), 0.94 (s, 9H), 0.95 (s, 9H), 1.01 (s, 3H), 2.00−2.10 (m, 1 H), 2.14−2.25 (m, 2 H), 2.26−2.50 (m, 3 H), 2.67 (d, *J* = 13.7 Hz, 1H), 3.11−3.24 (m, 4 H), 3.57 (t, *J* = 7.9 Hz, 2H), 3.60−3.66 (m, 2H), 3.757 (s, 3 H), 3.761 (s, 3 H), 3.78−3.84 (m, 1 H), 3.90 (dd, *J* = 4.3, 10.9 Hz, 1H), 3.95−4.00 (m, 1H), 4.14 (s, 1H), 4.54−4.62 (m, 1H), 5.01 (s, 1H), 5.14 (d, *J* = 9.9 Hz, 1H), 5.16 (d, *J* = 10.0 Hz, 1H), 5.23 (d, *J* = 9.4 Hz, 1H), 5.28 (d, *J* = 9.8 Hz, 1H), 6.29 (t, *J* = 7.0 Hz, 1H), 6.32−6.38 (m, 1H), 6.83−6.93 (m, 4H), 7.01−7.03 (m, 1H), 7.17−7.26 (m, 2H), 7.27−7.37 (m, 6 H), 7.41−7.48 (m, 2H), 7.51 (s, 1H), 7.87 (s, 1H); ^{31}P NMR (202 MHz, acetone-d_6) δ −14.09 - −10.93 (broad multiple); ESI-HRMS *m*/*z* calcd for $C_{71}H_{105}ClN_4O_{17}PSi_4^-$ (M − H)$^-$ 1463.5978, found 1463.6009.

Synthesis of 9. A solution of the phosphorylated dinucleotides **8** (1.69 g, 1.14 mmol) and TBAF (11.4 mL, 1 M THF solution) in THF (115 mL) was stirred for 6 h at room temperature. The solvent was replaced with dichloromethane, and then the resulting solution was washed with $NaHCO_3$ and dried over anhydrous Na_2SO_4. After the solvent was removed via rotary evaporation, the resulting residue was purified via column chromatography (eluent: dichloromethane/methanol = 15:1) to afford a reaction intermediate as a yellow oil. The product was azotropically dried with pyridine and dissolved in anhydrous pyridine (330 mL), and then MSNT (1.69 g, 5.70 mmol) was added. The reaction mixture was stirred at room temperature overnight. After being quenched with water, the solvent was removed under reduced pressure and then extracted with dichloromethane. The extracts were dried over anhydrous Na_2SO_4. After concentration under reduced pressure, the residue was purified via column chromatography (eluent: dichloromethane/methanol/thiethylamine = 20:1:0.025) to afford **9**, a mixture of 2 diastereoisomers in a ratio of 1:1.7 (*Sp*/*Rp* mixtures), as indicated by LC−MS, as a white solid (1.13 g, 0.92 mmol, 81% yield for two steps): R_f = 0.66−0.67 (dichloromethane/methanol = 9:1); ^{1}H NMR (500 MHz, acetone-d_6) δ −0.07−0.05 (m, 18H), 0.84−0.97 (m, 4H), 1.14 (s, 1.11H), 1.23 (s, 1.89H), 1.94−2.02 (m, 0.37H), 2.16−2.37 (m, 1.63 H), 2.49−2.75 (m, 3 H), 2.77−2.92

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(m, 1 H), 3.14−3.55 (m, 4H), 3.56−3.69 (m, 4H), 3.71−3.81 (m, 6 H), 3.85−4.13 (m, 2H), 4.33−4.54 (m, 3H), 4.57−4.72 (m, 1H), 5.04−5.35 (m, 5H), 6.09 (dd, J = 5.5, 8.5 Hz, 0.37H), 6.28−6.44 (m, 1.63H), 6.71−6.88 (m, 4H), 7.14−7.53 (m, 13H), 7.71 (s, 0.37H), 7.94 (s, 0.63H); ^{13}C NMR (126 MHz, acetone-d_6) δ −2.1, −2.0, 17.62, 17.64, 17.7, 17.2, 21.5, 24.1, 34.0, 34.8, 37.2, 39.2, 40.1, 40.6, 41.2, 44.4, 44.6, 54.6, 62.2, 62.9, 66.0, 66.1, 68.59, 68.63, 69.5, 69.7, 70.13, 70.15, 70.25, 70.34, 75.4, 75.5, 77.5, 77.6, 80.6, 80.7, 83.4, 83.7, 83.9, 84.0, 84.3, 85.2, 86.1, 86.2, 108.8, 110.3, 112.96, 113.02, 121.44, 121.45, 121.50, 121.52, 124.77, 124.82, 125.02, 125.1, 126.5, 126.6, 126.7, 127.65, 127.69, 128.0, 128.1, 128.3, 128.6, 130.02, 130.07, 130.08, 130.6, 130.7, 135.57, 135.63, 135.69, 135.74, 137.2, 137.4, 145.10, 145.14, 146.15, 146.19, 146.39, 146.44, 150.6, 150.7, 153.4, 153.5, 158.7, 162.6, 162.9, 173.0, 173.3; ^{31}P NMR (202 MHz, acetone-d_6) δ −9.39, −6.44; ESI-HRMS m/z calcd for $C_{59}H_{80}ClN_5O_{16}PSi_2^+$ (M + NH_4^+) 1236.4565, found 1236.4556.

Synthesis of SP Phosphoramidite P1. To a solution of **9** (75 mg, 61 μmol)[1:1.7 (*Sp*/*Rp* mixtures)] in dry CH_2Cl_2 (4 mL) under argon atmosphere were added DIPEA (54 μL, 310 μmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (42 μL, 180 μmol). After 1 h of stirring, the solvent was removed by rotary evaporation, and the resulting residue was purified via column chromatography (eluent: dichloromethane/ethyl acetate/triethylamine = 5:5:1) to yield **P1**, a mixture of 4 diastereoisomers in a ratio of 1:1:1.7:1.7, as indicated by LC−-MS, as a white solid (69 mg, 49 μmol, 80% yield): R_f = 0.20−0.30 (dichloromethane/ethyl acetate/triethylamine = 4.5:4.5:1); ^{1}H NMR (500 MHz, acetone-d_6) δ −0.07−0.05 (m, 18H), 0.89−0.97 (m, 4H), 1.09−1.27 (m, 21H), 2.37−2.80 (m, 6H), 3.15−3.37 (m, 4H), 3.43−3.54 (m, 1H), 3.55−3.97 (m, 15H), 4.01−4.70 (m, 5H), 5.07−5.32 (m, 5H), 6.07−6.46 (m, 2H), 6.76−6.88 (m, 4H), 7.16−7.60 (m, 13H), 7.65−7.98 (m, 1H); ^{31}P NMR (acetone-d_6) δ −9.36, −9.23, −6.66, 148.72, 148.88, 149.31; ESI-HRMS m/z calcd for $C_{68}H_{97}ClN_7O_{17}P_2Si_2^+$ (M + NH_4^+) 1436.5643, found 1436.5628.

Synthesis of 10. NaOMe (118 mg, 2.12 mmol) was added to a solution of **9** (861 mg, 0.71 mmol) [1:1.7 (*Sp*/*Rp* mixtures)] in MeOH (10 mL). After being stirred at room temperature for 1 h, the reaction mixture was diluted with EtOAc, washed with NH_4Cl, water and brine successively, and dried over anhydrous Na_2SO_4. After the solvent was removed via rotary evaporation, the resulting residue was purified via column chromatography (eluent: dichloromethane/methanol/triethylamine = 20:1:0.025) to afford **10**, a mixture of two diastereoisomers in a ratio of 1:1.2 (*Sp*/*Rp* mixtures), as indicated by LC−MS, as a white solid (719 mg, 0.64 mmol, 90% yield): R_f = 0.25 (hexane/ethyl acetate/methanol = 5:5:1); ^{1}H NMR (500 MHz, acetone-d_6) δ −0.05−0.03 (m, 18H), 0.85−0.96 (m, 4H), 1.15 (s, 1.35H), 1.23 (s, 1.65H), 2.11−2.38 (m, 2H), 2.43−2.82 (m, 4H), 3.13−3.34 (m, 3H), 3.37−3.55 (m, 1H), 3.57−3.72 (m, 7H), 3.74−3.81 (m, 6H), 3.85−4.06 (m, 2H), 4.26−4.57 (m, 3H), 4.66−5.02 (m, 2H), 5.11−5.34 (m, 4H), 6.10−6.18 (m, 0.45H), 6.28−6.42 (m, 1.55H), 6.84−6.95 (m, 4H), 7.19−7.40 (m, 7H), 7.44−7.50 (m, 2H), 7.74 (s, 0.45H), 7.92 (s, 0.55H); ^{13}C NMR (126 MHz, acetone-d_6) δ −2.08, −2.06, −2.04, 17.6, 17.7, 21.9, 24.1, 34.1, 34.7, 37.3, 39.2, 39.8, 40.6, 41.0, 44.5, 44.6, 46.0, 53.7, 53.8, 53.9, 54.59, 54.62, 62.3, 62.8, 65.69, 65.73, 65.96, 66.01, 66.77, 66.83, 75.9, 76.0, 80.8, 80.9, 81.0, 83.4, 83.7, 84.0, 84.1, 84.7, 86.08, 86.11, 109.2, 110.3, 113.0, 126.6, 126.7, 127.7, 128.0, 128.1, 130.00, 130.01, 130.1, 135.65, 135.70, 135.75, 137.17, 137.23, 145.1, 145.2, 150.6, 150.7, 153.2, 153.5, 158.68, 158.74, 162.6, 162.8, 173.0, 173.3; ^{31}P NMR (202 MHz, acetone-d_6) δ −2.27, 0.03; ESI-HRMS m/z calcd for $C_{54}H_{79}N_5O_{16}PSi_2^+$ (M + NH_4^+) 1140.4798, found 1140.4785.

Synthesis of P2. To a solution of **10** (138 mg, 123 μmol) [1:1.2 (*Sp*/*Rp* mixtures)] in dry CH_2Cl_2 (9 mL) under argon atmosphere were added DIPEA (106 μL, 615 μmol) and 2-cyanoethyl-*N*,*N*-diisopropylchloro phosphoramidite (84 μL, 369 μmol). After the solution was stirred for 1 h, the solvent was removed via rotary evaporation, and the residue was purified via column chromatography (eluent: dichloromethane/ethyl acetate/triethylamine = 5:5:1) to afford **P2**, a mixture of four diastereoisomers in a ratio of 1:1:1.1:1.1, as indicated by LC−MS, as a white solid (138 mg, 104 μmol, 85% yield): R_f = 0.63−0.71 (dichloromethane/ethyl acetate/triethyl amine = 4.5:4.5:1); ^{1}H NMR (500 MHz, acetone-d_6) δ −0.06−0.08 (m, 18H), 0.82−0.99 (m, 4H), 1.08−1.43 (m, 21H), 2.23−2.65 (m, 4H), 2.65−2.81 (m, 2H), 3.18−3.54 (m, 4H), 3.55−3.73 (m, 9H), 3.74−4.01 (m, 9H), 4.07−4.46 (m, 3H), 4.53−5.06 (m, 2H), 5.09−5.35 (m, 4H), 6.11−6.47 (m, 2H), 6.81−6.97 (m, 4H), 7.16−7.54 (m, 9H), 7.69−7.75 (m, 0.5H), 7.88−7.96 (m, 0.5H); ^{31}P NMR (202 MHz, acetone-d_6) δ −2.14, −2.11, −0.10, 148.55, 148.66, 148.68, 148.72; ESI-HRMS m/z calcd for $C_{63}H_{96}N_7O_{17}P_2Si_2^+$ (M + NH_4^+) 1340.5877, found 1340.5861.

(5*R*)-3′-*O*-Acetyl-α-[3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-3-[(trimethylsilylethoxy)methyl]thymidyl]-5,6-dihydro-5′-*O*-(4,4′-dimethoxytrityl)-3-[(trimethylsilylethoxy)methyl]-thymidine (11). To a solution of **7** (861 mg, 0.67 mmol) in dry pyridine (8 mL) was added Ac_2O (684 mg, 6.70 mmol). After the reaction was complete (12 h), the solvent was removed via rotary evaporation and the resulting residue was purified via column chromatography (eluent: hexane/ethyl acetate = 4:1) to afford **11** as a white solid (883 mg, 0.67 mmol, 100% yield): R_f = 0.50 (hexane/ethyl acetate = 3:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.01 (s, 9H), 0.00 (s, 9H), 0.105 (s, 3H), 0.107 (s, 3H), 0.112 (s, 6H), 0.87−0.97 (m, 4H), 0.91 (s, 9H), 0.92 (s, 9H), 1.14 (s, 3H), 1.96−2.07 (m, 2H), 2.06 (s, 3H), 2.20−2.29 (m, 2H), 2.57 (d, J = 14.0 Hz, 1H), 2.66 (d, J = 13.8 Hz, 1H), 3.21 (d, J = 12.9 Hz, 1H), 3.27 (d, J = 13.2 Hz, 1H), 3.29 (dd, J = 4.1, 10.0 Hz, 1H), 3.35 (dd, J = 3.9, 10.2 Hz, 1H), 3.58 (t, J = 8.2 Hz, 2H), 3.61−3.67 (m, 2H), 3.73 (dd, J = 5.5, 10.7 Hz, 1H), 3.79 (s, 6H), 3.81 (dd, J = 4.2, 11.4 Hz, 1H), 3.92−3.95 (m, 1H), 3.97 (dd, J = 3.9, 7.0 Hz, 1H), 4.42−4.46 (m, 1H), 5.16 (d, J = 9.5 Hz, 1H), 5.20 (d, J = 9.4 Hz, 1H), 5.23−5.32 (m, 3H), 6.30 (dd, J = 6.2, 7.4 Hz, 1H), 6.34 (dd, J = 5.4, 9.5 Hz, 1H), 6.84−6.89 (m, 4H), 7.19−7.24 (m, 1H), 7.29−7.37 (m, 6H), 7.43−7.48 (m, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −5.4, −5.3 −4.8, −4.7, −1.42, −1.41, 17.9, 18.09, 18.14, 18.4, 21.0, 21.9, 25.7, 25.9, 32.5, 34.2, 40.2, 42.8, 44.8, 55.2(2C), 62.9, 63.5, 66.8, 67.4, 70.1, 70.3, 72.1, 74.5, 81.8, 83.9, 85.6, 86.3, 87.5, 108.3, 113.2, 126.8, 127.9, 128.1, 130.0, 130.1, 135.74, 135.75, 135.9, 139.0, 144.7, 150.6, 152.6, 158.5, 163.3, 170.3, 173.4; ESI-MS m/z calcd for $C_{67}H_{108}N_5O_{15}Si_4^+$ (M + NH_4^+) 1334.6919, found 1334.6914.

(5*R*)-3′-*O*-Acetyl-α-[3-[(trimethylsilylethoxy)methyl]-thymidyl]-5,6-dihydro-5′-*O*-(4,4′-dimethoxytrityl)-3-[(trimethylsilylethoxy)methyl]thymidine (12). To a solution of **11** (907 mg, 0.69 mmol) in THF (10 mL) was added TBAF (2.1 mL, 2.10 mmol). After the reaction was complete (2 h), the reaction mixture was diluted with EtOAc, washed with saturated aq $NaHCO_3$, and dried over anhydrous Na_2SO_4. After removal of the solvent via rotary evaporation, the resulting residue was purified via column chromatography (eluent: dichloromethane/methanol = 15:1) to afford **12** as a white solid (708 mg, 0.65 mmol, 95% yield): R_f = 0.25 (dichloromethane/methanol = 9:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.003 (s, 9H), 0.003 (s, 9H), 0.88−0.99 (m, 4H), 1.11 (s, 3H), 2.07 (s, 3H), 2.07−2.12 (m, 1H), 2.22−2.32 (m, 2H), 2.42 (dd, J = 3.4, 6.3 Hz, 1H), 2.45 (dd, J = 3.3, 6.2 Hz, 1H), 2.58 (d, J = 14.3 Hz, 1H), 2.64 (d, J = 14.3 Hz, 1H), 3.22 (d, J = 13.2 Hz, 1H), 3.29 (d, J = 13.4 Hz, 1H), 3.32−3.39 (m, 3 H), 3.59−3.69 (m, 4 H), 3.80 (s, 6H), 3.82−3.85 (m, 1H), 3.98−4.04 (m, 2H), 4.09 (dd, J = 2.9, 5.6 Hz, 1H), 4.52−4.58 (m, 1H), 5.14 (d, J = 9.8 Hz, 1H), 5.23 (d, J = 9.6 Hz, 1H), 5.28−5.31 (m, 1H), 5.32 (d, J = 9.4 Hz, 1H), 5.36 (d, J = 9.4 Hz, 1H), 6.22 (t, J = 6.5 Hz, 1H), 6.35 (dd, J = 5.4, 9.5 Hz, 1H), 6.85−6.90 (m, 4H), 7.20−7.25 (m, 1H), 7.29−7.38 (m, 6H), 7.43−7.47 (m, 2H), 7.84 (s, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −1.5, −1.4, 18.05, 18.13, 21.0, 21.4, 32.2, 34.3, 41.5, 42.6, 45.1, 55.2 (2C), 62.1, 63.5, 67.2, 67.5, 70.2, 71.8, 74.6, 82.0, 84.0, 86.4, 87.3, 87.6, 107.7, 113.2, 126.9, 127.9, 128.2, 130.01, 130.03, 135.9, 139.3, 144.4, 150.6, 152.6, 158.5, 163.4, 170.4, 174.0; ESI-HRMS m/z calcd for $C_{55}H_{80}N_5O_{15}Si_2^+$ (M + NH_4^+) 1106.5190, found 1106.5195.

(5*R*)-3′-*O*-Acetyl-α-[5′-*O*-(*tert*-butyldiphenylsilyl)-3-[(trimethylsilylethoxy)methyl]thymidyl]-5,6-dihydro-5′-*O*-(4,4′-dimethoxytrityl)-3-[(trimethylsilylethoxy)methyl]-thymidine (13). Imidazole (93 mg, 1.36 mmol) and TBDPSCl (0.23 mL, 0.88 mmol) were added in turn to a solution of **12** (746 mg, 0.68 mmol) in dry DMF (3 mL) at 0 °C. The reaction mixture was stirred at room temperature overnight before being quenched with aq

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$NaHCO_3$. The mixture was diluted with EtOAc, washed with water and brine successively, and dried over anhydrous Na_2SO_4. After the solvent was removed via rotary evaporation, the resulting residue was purified via column chromatography (eluent: hexane/EtOAc = 1:1) to afford **13** as a white solid (677 mg, 0.51 mmol, 75% yield): R_f = 0.33 (hexane/ethyl acetate =1:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.01 (s, 9H), 0.00 (s, 9H), 0.84−0.98 (m, 4H), 1.03 (s, 3H), 1.09 (s, 9H), 2.01−2.05 (m, 1H), 2.06 (s, 3H), 2.11 (d, J = 3.5 Hz, 1H), 2.12−2.25 (m, 2H), 2.33−2.40 (m, 2H), 2.60 (d, J = 14.4 Hz, 1H), 3.13 (d, J = 13.1 Hz, 1H), 3.23 (d, J = 13.4 Hz, 1H), 3.31 (dd, J = 4.2, 10.3 Hz, 1H), 3.35 (dd, J = 4.0, 10.5 Hz, 1H), 3.58 (t, J = 8.3 Hz, 2 H), 3.62−3.68 (m, 2 H), 3.79 (s, 6H), 3.88−4.03 (m, 4H), 4.47−4.57 (m, 1H), 5.14 (d, J = 9.7 Hz, 1H), 5.19 (d, J = 9.6 Hz, 1H), 5.25−5.32 (m, 3H), 6.27 (t, J = 6.4 Hz, 1H), 6.34 (dd, J = 5.4, 9.5 Hz, 1H), 6.83−6.89 (m, 4H), 7.15−7.24 (m, 1H), 7.26−7.48 (m, 15H), 7.67−7.73 (m, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ −1.4 (2 C), 18.10, 18.13, 19.2, 21.0, 21.6, 26.9, 32.3, 34.2, 39.8, 42.7, 44.6, 55.2 (2 C), 63.5, 64.0, 66.9, 67.5, 70.1, 70.3, 71.7, 74.5, 81.8, 83.9, 85.2, 85.7, 86.4, 108.4, 113.2, 126.8, 127.8, 127.9, 128.1, 129.89, 129.94, 130.0, 130.1, 132.92, 132.93, 135.50, 135.53, 135.7, 135.8, 138.8, 144.7, 150.5, 152.5, 158.5, 163.2, 170.3, 173.3; ESI-HRMS m/z calcd for $C_{71}H_{98}N_5O_{15}Si_3^+$ (M + NH_4^+) 1344.6367, found 1344.6360.

(5*R*)-3′-*O*-Acetyl-α-[3′-O-(2-cyanoethyl-*N*,*N*diisopropyl)-phosphoramidite (5′-*O*-(*tert*-butyldiphenylsilyl)-3-[(trimethylsilylethoxy)methyl]thymidyl]-5,6-dihydro-5′-*O*-(4,4′-dimethoxytrityl)-3-[(trimethylsilylethoxy)methyl]-thymidine, (P3). To a solution of **13** (102 mg, 77 μmol) in dry CH_2Cl_2 (7 mL) under argon atmosphere were added DIPEA (67 μL, 384 μmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (51 μL, 230 μmol). After 1 h of stirring, the solvent was removed via rotary evaporation, and the residue was purified via column chromatography (eluent: hexane/ethyl acetate/triethylamine = 2:1:0.01) to afford **P3**, a mixture of two diastereoisomers in a ratio of 1.3:1 (*Sp*/*Rp* mixtures, by ^{31}P NMR), as a white solid (94 mg, 62 μmol, 81% yield): R_f = 0.50 (hexane/ethyl acetate = 2:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.01−0.03 (m, 18H), 0.87−1.06 (m, 8H), 1.07−1.12 (m, 9H), 1.17 (s, 1.5H), 1.18 (s, 1.5H), 1.19−1.24 (m, 9H), 2.02−2.09 (m, 4H), 2.10−2.30 (m, 3H), 2.43−2.57 (m, 2H), 2.59−2.67 (m, 2H), 3.13 (d, J = 13.0 Hz, 1H), 3.19−3.27 (m, 1H), 3.29−3.38 (m, 2H), 3.55−3.67 (m, 2H), 3.61−3.68 (m, 4H), 3.80 (s, 6H), 3.69−3.87 (m, 2H), 3.88−3.95 (m, 2H), 3.97−4.01 (m, 1H), 4.11−4.18 (m, 1H), 4.60−4.71 (m, 1H), 5.11−5.20 (m, 2H), 5.25−5.33 (m, 3H), 6.28−6.41 (m, 2H), 6.82−6.90 (m, 4H), 7.19−7.24 (m, 1H), 7.27−7.49 (m, 15H), 7.65−7.73 (m, 4H); ^{31}P NMR (202 MHz, $CDCl_3$) δ 148.76, 149.25; ESI-HRMS m/z calcd for $C_{80}H_{112}N_6O_{16}PSi_3^+$ (M + H^+) 1527.7180, found 1527.7172.

***P*-Methylthymidylyl-(5′→3′)-thymidine (14) and *P*-(2-Cyanoethyl)thymidylyl-(5′→3′)-thymidine (15).** Syntheses of these compounds were conducted according to previously published procedures, respectively.[43,63]

Solid-State Photoreaction of 14 and 15. The photoreaction was carried out using a Spectroline germicidal UV sterilizing lamp (Dual-tube, 15 w, intensity: 1550 μw cm^{-2}) with the samples ∼9 cm to the lamp using the protocol described in our previous publication.[31] Product analyses by LC−MS found no formation of the corresponding SP products.

Oligonucleotide Synthesis. The oligonucleotides was synthesized using standard solid-phase synthesis conditions, in a column type reactor fitted with a sintered glass frit which could be maintained airtight with a serum cap.[64−68] All of the synthetic cycles were performed on a 2 μmol scale. The average coupling yield for SP incorporation was >90%, as estimated by HPLC analysis.

After the solid-phase synthesis, the SEM- and DMTr-protecting groups were removed by stirring with 1 M $SnCl_4$ in CH_2Cl_2 for 0.5 h at room temperature under anhydrous conditions. The oligonucleotides were cleaved from resin with concentrated aq. $NH_3{\cdot}H_2O$ at 55 °C for 18 h in a sealed tube. The methyl group from the SP phosphotriester moiety was also removed by this ammonium hydroxide treatment.[42−44] The resins were then washed with H_2O for 3 times and the washing solutions combined. For the dinucleoside SP TT reaction, an additional step is needed to remove the TBDPS protecting group, which was achieved by the following procedure: the resulting cleavage solution was dried by lyophilization, heated at 65 °C in a mixture of anhydrous DMSO and triethylamine trihydrofluoride ($TEA{\cdot}(HF)_3$) for 1 h, and precipitated by addition of butanol. After HPLC purification, the yields for the 10-mer and 20-mer sequence were found to be 63% and 30%, respectively, in the dinucleotide SP TpT incorporation and 60% and 23%, respectively, in the dinucleoside SP TT incorporation. The yields were calculated based on the amount of resin used.

HPLC Assay for Product Purification. HPLC chromatography was performed at room temperature with a Waters (Milford, MA) breeze HPLC system with a 2489 UV/Visible detector at 260 nm. An Waters XBridge OST C18 column (2.5 μm, 4.6 × 50 mm) was equilibrated with 5% CH_3CN in 0.1 M TEAA buffer at pH 7.0 (buffer A), and compounds were eluted with an ascending gradient (0−35%) of buffer B in 15 min which is composed of 70% buffer A and 30% acetonitrile at a flow rate of 1 mL/min. The SP containing oligonucleotides were collected using 1.5 mL Eppendorf tubes that were lyophilized and redissolved in 10 μL of dd H_2O. The resulting solution (0.5 μL) was then injected into the Agilent 6520 Accurate-Mass Q-TOF LC/MS spectrometer, and the data was acquired and analyzed via Agilent MassHunter software.

Measurement of Oligonucleotide Melting Point (T_m). UV melting curves of oligonucleotide duplexes were obtained with a Perkin-Elmer UV/vis/NIR spectrometer (Lambda 19) equipped with a PTP-1 peltier temperature programmer. Quarts cuvettes with 1 cm optical path length were employed. The variation of UV absorbance with temperature was monitored at λ = 260 nm. The temperature was scanned between 4 and 94 °C from both directions, and the rate of temperature change was 0.4 °C/min. The experiments were carried out in 10 mM sodium phosphate buffer at pH 7.0, which contains 150 mM NaCl, and duplex concentrations of 0.4, 0.8, 1.6, and 3.2 μM for 20-mer oligonucleotide and 0.6, 1.2, 2.4, and 4.8 μM for 10-mer oligonucleotide. Melting points were obtained from the inflection points of the baseline corrected averaged melting curves. Thermodynamic parameters were calculated using the published protocol.[58−61]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectroscopic characterization of the new compounds. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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