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2,2,5,5-Tetramethylpyrrolidin-3-one-1-sulfinyl Group for 5′-Hydroxyl Protection of Deoxyribonucleoside Phosphoramidites in the Solid-Phase Preparation of DNA Oligonucleotides

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Abstract: Several nitrogen−sulfur reagents have been investigated as potential 5′-hydroxyl protecting groups for deoxyribonucleoside phosphoramidites to improve the synthesis of oligonucleotides on glass microarrays. Out of the nitrogen−sulfur-based protecting groups so far investigated, the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group exhibited near optimal properties for 5′-hydroxyl protection by virtue of the mildness of its deprotection conditions. Specifically, the iterative cleavage of a terminal 5′-sulfamidite group in the synthesis of 5′-d(ATCCGTAGCCAAGGTCATGT) on controlled-pore glass is efficiently accomplished by treatment with iodine in the presence of an acidic salt. Hydrolysis of the oligonucleotide to its 2′-deoxyribonucleosides upon exposure to snake venom phosphodiesterase and bacterial alkaline phosphatase did not reveal the formation of any nucleobase adducts or other modifications. These findings indicate that the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group for 5′-hydroxyl protection of phosphoramidites, such as **10a**−**d**, may lead to the production of oligonucleotide microarrays exhibiting enhanced specificity and sensitivity in the detection of nucleic acid targets.

Introduction

Rapid advances in DNA microarray technology has empowered researchers with an unprecedented parallelism in biological analysis. This technology has allowed one to (i) monitor differences in the expression levels of a large number of genes,[1,2] (ii) identify expression patterns associated with particular physiological and pathological states,[3,4] and (iii) determine gene functions.[5] Although these applications represent only a fraction of the many possible diagnostic applications of DNA microarrays, the technology is still evolving in the context of the methods used to immobilize DNA on glass surfaces.[6] In this regard, we have been particularly interested in the in situ synthesis of oligonucleotides on planar glass surfaces because of the versatility of this method in determining the experimental parameters necessary for optimal hybridization specificity and sensitivity with complementary nucleic acid targets.[7] Oligonucleotide microarrays have particularly been powerful in the detection of single nucleotide polymorphisms and discrimination of sequences between closely related gene families.[8]

One approach to the in situ synthesis of oligonucleotides on microarrays employs deoxyribonucleoside phosphoramidites functionalized with photolabile 5′-*O*-protecting groups and photolithographic masks to direct synthesis to specific areas of the planar glass surface.[9] This method has, however, not produced stepwise coupling efficiencies better than 92−94% presumably because of incomplete recovery of photodeprotected

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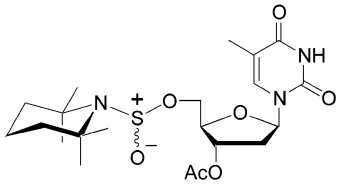
5′-hydroxyl groups.[9c] Improved coupling yields via 5′-/3′-*O*-[2-(2-nitrophenyl)propyloxycarbonyl]-2′-deoxyribonucleoside phosphoramidites[10] have recently been reported,[10d] presumably as a result of a more efficient 5′-/3′-*O*-photodeprotection and the use of a maskless array synthesis technology.[11] These improvements thus validate the photolithographic method as a viable approach to the preparation of oligonucleotide microarrays. An alternative approach to the synthesis of oligonucleotides on planar glass surfaces entails the use of conventional 5′-*O*-(4,4′-dimethoxytrityl) (DMTr)-2′-deoxyribonucleoside phosphoramidites and computer-controlled inkjet delivery systems.[7,12] Although the synthesis of oligonucleotides on microarrays has evolved from these recent technological advances, inherent problems associated with the iterative photochemical or acidic cleavage of 5′-/3′-*O*-protecting groups during oligonucleotide assembly along with those imparted by the nucleophilic conditions required for final oligonucleotide deprotection at pH > 10.0 may cause irreversible damages to the glass surface and to the oligonucleotides covalently bound to it. For example, photochemical and/or chemical DNA modifications effected by light-induced radicals and/or harsh chemicals may lead to poorer performance of the array in terms of hybridization specificity, whereas the loss of oligonucleotides from the glass surface would result in a decreased detection sensitivity and a reduced dynamic range.

To minimize or avoid these potential problems, we proposed a "heat-driven" approach to the synthesis of DNA oligonucleotides on microarrays.[13] The method would involve the use of heat-sensitive groups for 5′-/3′-hydroxyl and phosphate protection. Thus, instead of using UV irradiation or acids for iterative removal of the 5′-/3′-hydroxyl protecting group throughout oligonucleotide assembly, heating the glass surface would simultaneously generate a free terminal 5′-/3′-hydroxy group on the growing DNA chain and cleave phosphate protecting groups. To achieve these primary objectives, several heat-sensitive phosphate protecting groups have already been developed in our laboratories. These groups can be cleaved from DNA oligonucleotides upon heating at either 55[14] or 90 °C[15] in an aqueous buffer (pH 7.0). Moreover, a number of thermolabile carbonates have been investigated for 5′-hydroxyl protection of deoxyribonucleosides.[16] Although these protecting groups are promising, it is difficult to engineer a thermolabile 5′-hydroxyl protecting group for phosphoramidite monomers that both is completely stable at ambient temperature to prevent multiple incorporations of the incoming phosphoramidite during each coupling step and can undergo complete and rapid (<10 min) thermolytic 5′-*O*-deprotection to permit the next chain extension cycle. We are therefore searching for novel 5′-/3′-hydroxyl protecting groups for deoxyribonucleoside phosphoramidite monomers that would iteratively be deprotected under mild conditions after incorporation into oligonucleotides, preferably while performing the mandatory oxidation step of the automated solid-phase synthesis program.[17] In addition to these requirements, the protecting group should (i) easily mask the 5′-/3′-hydroxy group of conventional *N*-protected deoxyribonucleosides, (ii) be stable to the conditions used for the preparation, purification, and utilization of the purporting phosphoramidite monomers in solid-phase oligonucleotide synthesis, and (iii) be cleaved rapidly and efficiently, when needed, without damaging the oligonucleotide through nucleobase modification and/or chain cleavage. Our search for a 5′-/3′-hydroxyl protecting group that would comply with these stringent requirements led us to investigate a number of nitrogen−sulfur reagents considering their reported sensitivity to both iodine oxidation and acidic conditions.[18] We now wish to report our findings on the properties of selected aminosulfinyl groups as 5′-hydroxyl protecting groups for deoxyribonucleosides and the conversion of these protected nucleosides to phosphoramidite derivatives. The performance of the resulting phosphoramidite monomers in the synthesis of DNA oligonucleotides on controlled-pore glass (CPG) will also be evaluated.[19]

Results and Discussion

The preparation of *N*,*N*-dialkylaminosulfinyl chlorides from thionyl chloride and secondary amines or their trimethylsilyl derivatives was reported in the literature several decades ago;[20] noteworthy is the relative stability of 1-bromosulfinyl-2,2,6,6-tetramethylpiperidine, which was isolated as a solid.[21] Given that several ethyl *N*,*N*-dialkylaminosulfinates have been prepared and isolated by distillation,[18] we set out to prepare the nucleosidic 5′-*O*-piperidinosulfinate **1** in a one-pot reaction from 1-chlorosulfinyl-2,2,6,6-tetramethylpiperidine[22] and 3′-*O*-acetylthymidine.



1

The reaction product was purified by silica gel chromatography, and pure **1** was mixed with a solution of 0.45 M 1*H*-

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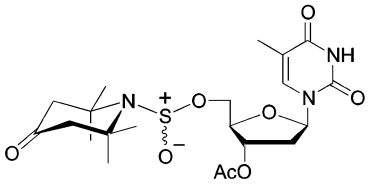
(19) It should however be understood that our findings are aimed at improving the current methods for the synthesis of oligonucleotides on microarrays. In this regard, replacement of the DMTr group with an aminosulfinyl group should not be viewed as a loss of a color-producing indicator for phosphoramidite coupling efficiency given that the DMTr assay is not sensitive enough to reliably assess phosphoramidite coupling efficiency on microarrays.

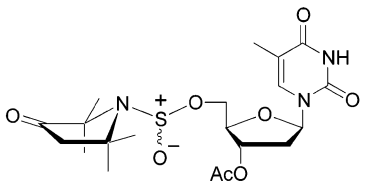
tetrazole in MeCN to assess whether the 5′-*O*-piperidinosulfinate group is completely stable to the conditions under which phosphoramidite coupling reactions are performed. Thin-layer chromatography (TLC) analysis of the solution shows that **1** is almost (>90%) completely converted to 3′-*O*-acetylthymidine within 5 min at ambient temperature and thus confirms that the 2,2,6,6-tetramethylpiperidine-1-sufinyl group would not be satisfactory for 5′-hydroxyl protection of deoxyribonucleoside phosphoramidites during solid-phase oligonucleotide synthesis. When solubilized in 0.02 M I_2 in THF/pyridine/H_2O, **1** is completely converted to 3-*O*-acetylthymidine within 5 min at 25 °C. These findings suggest that if a 5′-*O*-aminosulfinyl protecting group could be stable to phosphoramidite coupling conditions, it might then be possible to effect its removal during the mandatory iodine oxidation step. This approach would result in a significantly simpler and faster process for solid-phase oligonucleotide synthesis. Encouraged by these preliminary results, we decided to improve the stability of **1** to acidic conditions. We rationalized that the incorporation of an electron-withdrawing group into the piperidine ring of the sulfinyl protecting group might achieve our objective. To test this rationale, synthesis of the 5′-*O*-piperidinosulfinate **2** was carried out, much like that of **1**, from 1-chlorosulfinyl-2,2,6,6-tetramethylpiperidin-4-one[23] and 3′-*O*-acetylthymidine. As in the case of **1**, the nucleoside **2** was purified by silica gel chromatography and isolated as an amorphous solid.



2

As expected, **2** is significantly more stable than **1** to 0.45 M 1*H*-tetrazole in MeCN. After a contact time of 5 min, TLC reveals the presence of **2** (~60%) and 3′-*O*-acetylthymine (~40%). Interestingly, **2** is completely converted to 3′-*O*-acetylthymine upon mixing with 0.02 M I_2 in THF/pyridine/H_2O for 5 min at ambient temperature. These results prompted us to generate an inductive effect stronger than that produced by the 4-oxo group through the piperidine ring system. One way to achieve this goal is to bring the carbonyl group closer to the amine function to further decrease its basicity and thus increase the stability of the nitrogen−sulfur linkage to acidic conditions. Contraction of the six-membered piperidine ring to a five-membered pyrrolidine ring would effectively bring the carbonyl group one bond length *closer* to the amino group. Synthesis of the pyrrolidine **3** was achieved in one step from commercial 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide[24] via a Hofmann rearrangement.[25] Synthesis and purification of the 5′-*O*-pyrrolidinosulfinate **4** were performed in a manner similar to that of **1** and **2**.

3



4

Much to our expectation, the stability of **4** to 0.45 M 1*H*-tetrazole in MeCN is such that the formation of 3′-*O*-acetylthymidine becomes apparent (TLC) only after a ~15 min exposure to the weak acid. However, **4** is completely transformed into 3′-*O*-acetylthymidine when subjected to 0.02 M I_2 in THF/pyridine/H_2O for 15 min. These properties suggest that the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group may be stable as a 5′-hydroxyl protecting group for deoxyribonucleoside phosphoramidite monomers throughout each coupling step of the DNA oligonucleotide assembly. The protecting group may also be cleaved while performing the iodine oxidation step that is necessary for the conversion of the newly formed internucleosidic phosphite triester linkage to its phosphate triester counterpart. The next objective is to functionalize thymidine with a 5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl) group and phosphinylate its 3′-hydroxyl to a phosphoramidite derivative for further testing. Given that **4** is generally sensitive to protic or Lewis acids, a protection/deprotection strategy involving the use of a 3′-*O*-protecting group that can be removed under near neutral conditions had to be devised to enable the addition of a 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group to the 5′-hydroxyl of thymidine and that of deoxyribonucleosides functionalized with conventional base-labile nucleobase protecting groups. Out of the plethoric number of groups that have been developed over the years for hydroxyl protection of nucleosides,[26] the levulinyl group appears ideal considering its compatibility with standard base-labile nucleobase protecting groups.[27] The synthesis of **8a** from **5a** was performed to assess whether the 3′-*O*-levulinyl group can be chemoselectively cleaved in the presence of a 5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl) group. The preparation of **6a** and **7a** from **5a** was accomplished under conditions similar to those reported in the literature,[28] as specified in Scheme 1.

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(22) 1-Chlorosulfinyl-2,2,6,6-tetramethylpiperidine is prepared in situ from equimolar amounts of $SOCl_2$ and 2,2,6,6-tetramethylpiperidine and Et_3N (3 mol equiv) in MeCN under an inert atmosphere at 5 °C.

(23) 1-Chlorosulfinyl-2,2,6,6-tetramethylpiperidin-4-one is also prepared in situ at 5 °C from equimolar amounts of $SOCl_2$ and commercial 2,2,6,6-tetramethyl-4-piperidone and Et_3N (3.3 mol equiv) in MeCN under an atmosphere of argon gas.

(24) Alternatively, 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide is inexpensively prepared in two steps from 2,2,6,6-tetramethylpiperidin-4-one.[25a]

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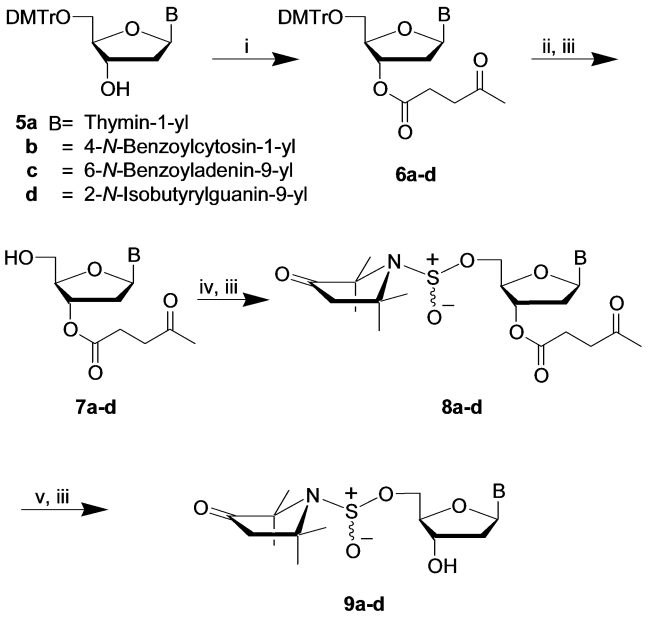
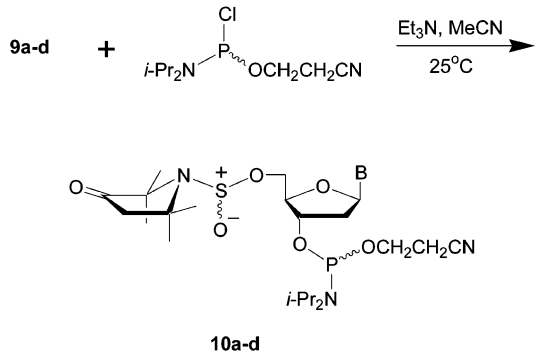
Scheme 1. Preparation of 5′-*O*-(2,2,5,5-Tetramethylpyrrolidin-3-one-1-sulfinyl) Deoxyribonucleosides (**9a**−**d**)[a]



[a] Conditions: (i) Levulinic acid (6 mol equiv), DCC (3 mol equiv), and DMAP (0.5 mol equiv) in dioxane, 25 °C, 3 h; (ii) 80% AcOH, 0.5−2 h, 25 °C; (iii) silica gel chromatography; (iv) 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl chloride (2.5 mol equiv), Et_3N (40 mol equiv) in MeCN, 10 min, 5 °C under Ar, then 1 h at 25 °C; (v) 0.4 M hydrazine hydrate in pyridine/AcOH (3:2 v/v), 25 °C, 3 min. Keys: DMTr, 4,4′-dimethoxytrityl; DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine.

Scheme 2. Synthesis of 5′-*O*-(2,2,5,5-Tetramethylpyrrolidin-3-one-1-sulfinyl) Deoxyribonucleoside Phosphoramidites (**10a**−**d**)



Treatment of **8a** with hydrazine hydrate (3 mol equiv) in pyridine/acetic acid (3:2 v/v) for 3 min at 25 °C was sufficient to remove most of the 3′-*O*-levulinyl group without detectable (TLC) loss of the 5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl) group, thereby confirming the orthogonality of these hydroxyl protecting groups.

The reaction conditions depicted in Scheme 1 are also applicable to the preparation of **8b**−**d**, which were isolated in yields ranging from 71% to 83% based on **7b**−**d** as the reference starting material. Hydrazinolysis of the 3′-*O*-levulinyl group from **8a**−**d** under the conditions described in Scheme 1 afforded **9a**−**d** in yields varying from 48% to 86% after silica gel chromatography. Phosphinylation of **9a**−**d** was performed as shown in Scheme 2 using 2-cyanoethyl diisopropylchlorophosphoramidite and triethylamine in dry MeCN.

Similar to standard phosphoramidites, **10a**−**d** were purified by silica gel chromatography and isolated as pure amorphous solids in yields varying from 54% to 83%. These phosphoramidites are stable for months when stored at −20 °C under a dry and inert atmosphere. Moreover, TLC analysis of 1 week old 0.1 M solutions of **10a**−**d** in dry MeCN shows that these phosphoramidites are as stable as commercial 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites when stored under identical conditions at ambient temperature.

The phosphoramidites **10a**−**d** were then employed in the solid-phase preparation of 5′-d(CTCTCTACGT) to determine optimal synthesis parameters.[29] Furthermore, the standard capping step was intentionally omitted throughout oligonucleotide assembly considering that this iterative step would not be a critical one in the context of oligonucleotide synthesis on glass surfaces. Since oligonucleotide purification is not performed on microarrays, a stepwise capping reaction would essentially be useless and wasteful in terms of reagents and time.

The decamer produced from each optimization experiment was fully deprotected and analyzed by RP-HPLC. The results of such an analysis were compared with those obtained from an identical decamer synthesized via commercial 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites under conditions defined in the Experimental Section. This comparative approach reveals that 1*H*-tetrazole is not rigorously adequate for the activation of **10a**−**d** given the presence of peaks corresponding to oligonucleotides larger than full-length in the RP-HPLC chromatogram. The 5′-*O*-sulfinyl protecting group in **10a**−**d** is presumably cleaved to some extent during activation with 1*H*-tetrazole, and as minimal this cleavage may be, it does lead to incorporation of the activated phosphoramidite more than once in any given coupling step. Replacement of the standard 1*H*-tetrazole activator solution with 0.25 M 4,5-dicyanoimidazole[30] in MeCN almost completely eliminated this problem as a consequence of its relatively lower acidity.[31] The optimized coupling reaction time for **10a**−**d** under these conditions is 30 s. Optimization of the parameters controlling the oxidation/5′-*O*-deprotection reaction is more complex considering the lack of detailed information on the mechanism whereby a terminal 5′-*O*-sulfinyl group is cleaved by iodine in the presence of acidic salts, which accelerate synergistically the deprotection kinetics of such groups.[32] Optimally, the iterative removal of a 5′-*O*-sulfinyl group incorporated into an oligonucleotide via phosphoramidites **10a**−**d** involves a 1 min treatment with a commercial iodine solution (0.02 M I_2 in THF:pyridine:H_2O) to safely convert the internucleoside phosphite triester linkage to its parent phosphate triester, followed by exposure to a solution of 0.1 M I_2, 0.25 M 3-acetylpyridine ($pK_{BH^+} = 3.26$), 0.125 M trichloroacetic acid in THF:H_2O (9:1 v/v) for 8 min.[33,34] Incidentally, replacement of the commercial iodine solution with

(29) The most important parameters investigated were (i) phosphoramidite coupling time and coupling efficiency, and (ii) time required for oxidative 5′-*O*-deprotection relative to the concentration of the acidic salt being used in the reaction.

(30) Vargeese, C.; Carter, J.; Yegge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. *Nucl. Acids. Res.* **1998**, *26*, 1046−1050.

(31) The pK_a of 4,5-dicyanoimidazole is 5.2, whereas that of 1*H*-tetrazole is 4.8, see: Jin, Y.; Just, G. *J. Org. Chem.* **1998**, *63*, 3647−3654.

(32) The details of this complex deprotection mechanism are still being investigated.

(33) Skipping the 1 min treatment with a commercial iodine solution leads to premature cleavage of the internucleoside phosphite triester linkage caused by the acidity of the 0.1 M iodine solution.

(34) Replacement of the 0.1 M iodine solution with a solution of 0.25 M 3-acetylpyridine and 0.125 M trichloroacetic acid in THF:H_2O (9:1 v/v) results in a noticeably slower deprotection of the 5′-*O*-sulfinyl group and thus underscores the synergistic participation of iodine in the cleavage of the protecting group.

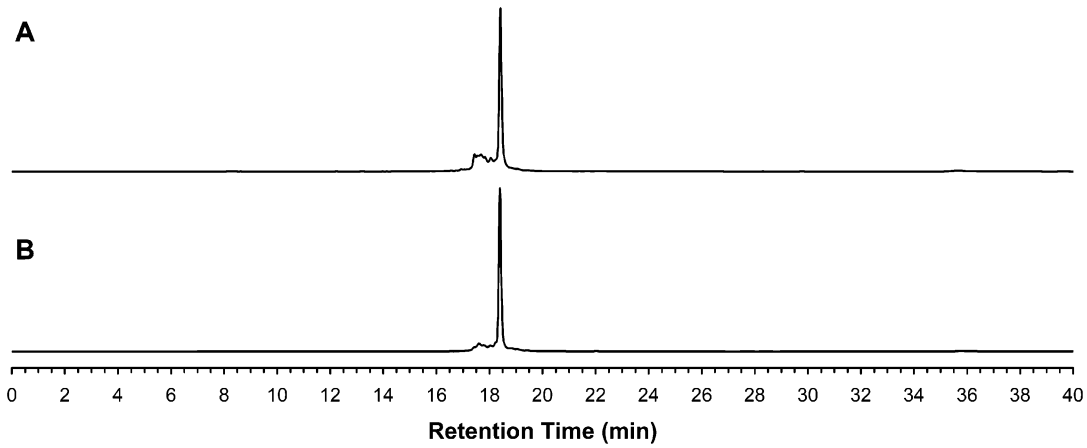


Figure 1. RP−HPLC chromatograms of crude, deprotected, and desalted 5′-d(ATCCGTAGCCAAGGTCATGT). (A) Chromatogram of the 20-mer synthesized via **10a**−**d** and deprotected upon heating in concentrated NH_4OH for 10 h at 55 °C. (B) Chromatogram of the 20-mer synthesized from commercial 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites and deprotected under conditions identical to those described in A. RP-HPLC analyses were performed using a 5 μm Supelcosil LC-18S column (25 cm × 4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min and then held isocratically for 10 min. Peak heights are normalized to the highest peak, which is set to 1 arbitrary unit.

a 0.05 M solution of 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in MeCN[35] for a 1 min exposure followed by treatment with the mildly acidic iodine solution for 8 min allows the preparation of phosphorothioated oligodeoxyribonucleotides.[36]

Under optimized conditions for phosphoramidite activation and oxidative 5′-*O*-deprotection, the purity of crude 5′-d(CTCTCTACGT) synthesized from **10a**−**d** is very similar to that of the same oligomer synthesized from commercial 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites on the basis of RP-HPLC analysis of the fully deprotected decamers (data shown in Supporting Information). Encouraged by these promising results, the synthesis of a representative 20-mer was undertaken employing **10a**−**d** as 0.1 M solutions in dry MeCN under the optimized solid-phase synthesis cycle conditions described above for the preparation of 5′-d(CTCTCTACGT). RP-HPLC analysis of the crude and deprotected 20-mer is presented in Figure 1. It is apparent that the concentration of "failure" sequences (t_R = 17−18 min) is slightly higher when phosphoramidites **10a**−**d** are used to synthesize the 20-mer (Figure 1A) than when standard phosphoramidites are employed for the synthesis (Figure 1B). Polyacrylamide gel electrophoresis (PAGE) analysis of the crude oligonucleotide under denaturing conditions is consistent with RP-HPLC data and shows a small band exhibiting a lower electrophoretic mobility than that of the 20-mer (Figure 2, right lane). The sensitivity of the 5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl) group to 4,5-dicyanoimidazole is likely responsible for producing a population of larger than full-length oligonucleotides. Interestingly, the retention time of these oligonucleotides on a C_{18} RP-HPLC column is shorter (t_R ≈17.5 min) than that of the 20-mer (t_R = 18.5 min, see Figure 1A). Consequently, the concentration of

Figure 2. Polyacrylamide gel electrophoresis analysis of crude, deprotected, and desalted 5′-d(ATCCGTAGCCAAGGTCATGT) under denaturing conditions (7 M urea, 1X TBE buffer, pH 8.3). Left lane: 20-mer synthesized from commercial 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites and deprotected by treatment with concentrated NH_4OH for 10 h at 55 °C. Right lane: 20-mer synthesized from **10a**−**d** and deprotected under conditions identical to those used for the oligomer shown in the left lane. Oligonucleotides are visualized as blue bands upon staining the gel with Stains-all. Bromophenol blue is used as a marker and shows as a large band, in each lane, at the bottom of the gel.

larger than full-length oligonucleotides partially accounts for what is usually viewed as shorter than full-length oligonucleotides or failure sequences and does not commensurately reflect a lower coupling efficiency of phosphoramidites **10a**−**d** relative to that of conventional 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites.

The crude, deprotected, and desalted 20-mer is further analyzed by enzymatic hydrolysis catalyzed by snake venom phosphodiesterase and bacterial alkaline phosphatase to assess whether the DNA nucleobases have been modified during solid-phase oligonucleotide synthesis when employing phosphoramidites **10a**−**d** under the optimized conditions defined for the

(35) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693−4699. See also: Regan, J. B.; Phillips, L. R.; Beaucage, S. L. *Org. Prep. Proc. Int.* **1992**, *24*, 488−492.

(36) Although the scope of this work did not specifically address the solid-phase synthesis of phosphorothioated DNA oligonucleotides using **10a**−**d**, the manual synthesis of d($T_{PS}C_{PS}G_{PS}A_{PS}T$) has nonetheless been performed to demonstrate the stability of the 5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl) group to sulfurization conditions. A RP-HPLC chromatogram of crude and deprotected d($T_{PS}C_{PS}G_{PS}A_{PS}T$) is provided in the Supporting Information and compared with a chromatogram of the same oligonucleotide synthesized from standard 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites under similar conditions.

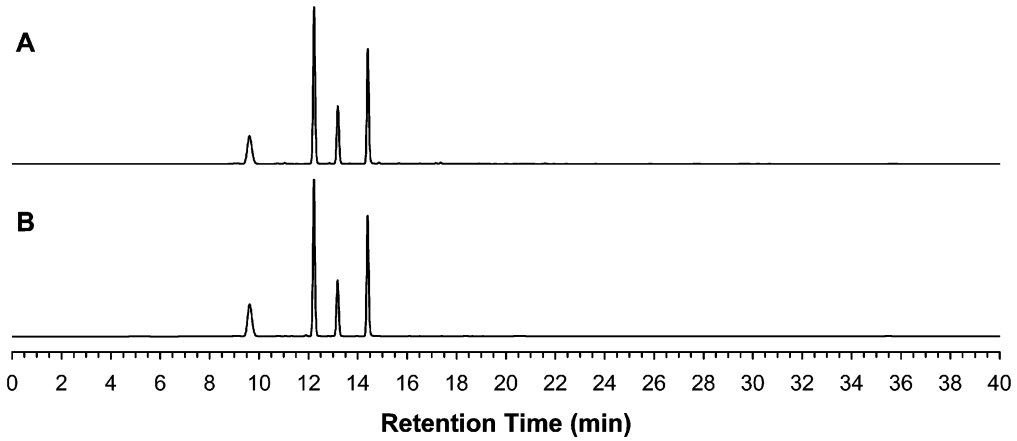


Figure 3. RP−HPLC analysis of crude, deprotected, and desalted 5′-d(ATCCGTAGCCAAGGTCATGT) after digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase (37 °C, 16 h). (A) Hydrolysates of the 20-mer synthesized via **10a**−**d** and deprotected upon heating to 55 °C in concentrated NH_4OH for 10 h. (B) Hydrolysates of the 20-mer synthesized from commercial 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites and deprotected under conditions identical to those described in A. RP-HPLC analyses were performed using a 5 μm Supelcosil LC-18S column (25 cm × 4.6 mm) under the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min. Peak heights are normalized to the highest peak, which is set to 1 arbitrary unit. Identities of the RP-HPLC peaks from left to right are as follows: dC, dG, dT, and dA when compared to authentic commercial samples.

coupling and oxidation/5′-*O*-deprotection steps. The enzymatic conversion of the 20-mer to its 2′-deoxyribonucleosides is analyzed by RP-HPLC. A chromatogram of the digest is shown in Figure 3A and does not reveal significant nucleobase modifications. The chromatogram is very similar to that of the same 20-mer synthesized from commercial 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites when subjected to enzymatic hydrolysis under identical conditions (see Figure 3B). These results indicate that phosphoramidites **10a**−**d** produce DNA oligonucleotides of genetic integrity comparable to those prepared from standard phosphoramidites.

Conclusion

Our investigations of nitrogen−sulfur reagents as potential 5′-hydroxyl protecting groups for deoxyribonucleoside phosphoramidites have enabled us to identify the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group as a protecting group that can be cleaved, when needed, under mild conditions. The protecting group is efficiently prepared in situ via thionyl chloride and 2,2,5,5-tetramethylpyrrolidin-3-one. Condensation of the resulting pyrrolidinosulfinyl chloride derivative with the 5′-hydroxyl of 3′-*O*-levulinyl-2′-deoxyribonucleosides (**7a**−**d**) proceeded efficiently. Orthogonal deprotection of the 3′-*O*-levulinyl group from **8a**−**d** allowed the preparation of phosphoramidites **10a**−**d** according to standard methods. Because of the inherent sensitivity of the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group to acidic conditions, 4,5-dicyanoimidazole had to be employed instead of 1*H*-tetrazole for activation of **10a**−**d** in the solid-phase synthesis of a 20-mer DNA oligonucleotide. As shown in Figure 1, the coupling efficiency of **10a**−**d** is nearly as good as that of commercial 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites. The presence of a small population of oligomers larger than the full-length 20-mer, shown in Figure 2, accounts in part for the apparent lower coupling efficiency of **10a**−**d**. One way to possibly prevent the formation of larger than full-length oligonucleotides is to use the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group for 3′-hydroxyl rather than 5′-hydroxyl protection; it is generally accepted that 3′-hydroxyl protecting groups are relatively more stable to deprotection under basic or acidic conditions than 5′-hydroxyl protecting groups, presumably because of subtle electronic and steric factors. Such a strategy would be particularly beneficial for the synthesis of oligonucleotide on microarrays, as it will permit the synthesis to proceed in the 5′→3′ direction[10d,37] via 3′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl) deoxyribonucleoside phosphoramidites. This approach would produce oligonucleotides with free 3′-hydroxyl groups for subsequent enzymatic reactions typically catalyzed by polymerases and/or ligases.[10d,37] Another way to potentially avoid the formation of larger than full-length oligonucleotides when using **10a**−**d** is to design an activator that is slightly more basic than 4,5-dicyanoimidazole without significantly impairing its ability to activate **10a**−**d**.

To the best of our knowledge, there are no related aminosulfinyl groups that have so far been reported as hydroxyl protecting groups for nucleosides. The uniqueness of the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group described herein remains in its application to 5′-hydroxyl protection of deoxyribonucleoside phosphoramidites in the solid-phase synthesis of oligonucleotides and its facile removal under oxidative and mildly acidic conditions. In addition, use of the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group for 5′-hydroxyl protection of deoxyribonucleoside phosphoramidites is perfectly compatible with conventional base-labile nucleobase protecting groups and, consequently, does not require elaborating a different nucleobase protection strategy. The phosphoramidites **10a**−**d** can be employed in the solid-phase synthesis of DNA oligonucleotides without the need for a capping step, which is normally used to avoid subsequent chain extension of oligonucleotides left unreacted after any given coupling step and to destroy most nucleobase adducts that might have been generated during each of these iterative coupling steps.[38] Thus, the use of **10a**−**d** in the absence of a capping step in the solid-phase synthesis of a 20-mer DNA oligonucleotide did not produce nucleobase adducts or any other nucleobase modifications. Such

(37) (a) Pirrung, M. C.; Wang, L.; Montague-Smith, M. P. *Org. Lett.* **2001**, *3*, 1105−1108. (b) Beier, M.; Hoheisel, J. D. *J. Biotechnol.* **2002**, *94*, 15−22.

nucleobase alterations would have been detected by RP-HPLC analysis of the 2′-deoxyribonucleosides obtained after enzymatic hydrolysis of the 20-mer catalyzed by snake venom phosphodiesterase and bacterial alkaline phosphatase. Figure 3 shows that each nucleoside is free of any detectable nucleobase modifications and thus validates the use of **10a**−**d** in the solid-phase synthesis of oligodeoxyribonucleotides without a capping step integrated into the chain assembly program.

It seems reasonable to speculate that the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group may find application as a hydroxyl protecting group in the synthesis of ribonucleotides, carbohydrates, and other biomedically important products. Attempts at imparting fluorescent properties to the 2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl group through its 3-oxo function, as a means to monitor the coupling efficiency of phosphoramidite monomers carrying this protecting group, is currently under way in the laboratory. Results of our findings in regard to their potential application to the synthesis of oligonucleotides on microarrays will be communicated as they become available.

Experimental Section

2,2,5,5-Tetramethylpyrrolidin-3-one (3). This compound is prepared from an adaptation of published methods.[25] Typically bromine (5.0 mL, 97 mmol) is slowly added to a cold (5 °C) and vigorously stirred solution of NaOH (19.4 g, 495 mmol) in H_2O (75 mL). Once the addition of bromine is complete, the solution is stirred for 10 min longer, whereupon a solution of 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide (13.6 g, 80.8 mmol) in H_2O (125 mL) is added. The solution is then heated for 1 h at 100 °C. To the intensely red solution that has been cooled to ambient temperature is added solid NaOH (75 g). A red oil separates from the aqueous phase and is immediately steam-distilled. The distillate is collected in an ice-cold Erlenmeyer flask and saturated with the addition of solid NaOH and NaCl. An orange oil separates and is extracted with petroleum ether (3 × 100 mL). The solution is dried over anhydrous $MgSO_4$, and the solvent is evaporated to an oil. The orange oil is distilled under reduced pressure, affording a colorless oil (4.34 g, 40%). Bp 32−33 °C @ 2 mmHg (lit.[25a] bp 169 @ 747 mmHg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.38 (s, 2H), 1.29 (s, 6H), 1.25 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.8, 30.9, 50.7, 53.7, 62.5, 221.8. FAB−HRMS: calcd for $C_8H_{16}NO$ $(M + H)^+$ 142.1232; found 142.1235.

General Method for the Preparation of 3′-*O*-Levulinyl-2′-Deoxyribonucleosides 7a−d from 5a−d. This method is an adaptation of the procedure reported by Kumar and Poonian.[28] Thus, **5a**−**d** (16.4 mmol) is dissolved in anhydrous dioxane (100 mL), and levulinic acid (10.1 mL, 98.6 mmol) is added to the magnetically stirred solution. Then 1,3-dicyclohexylcarbodiimide (49.3 mmol) and (*N*,*N*-dimethylamino)pyridine (8.2 mmol) in anhydrous dioxane (50 mL) are slowly added to the solution, which is left stirring for 3 h at ambient temperature under an inert atmosphere. TLC analysis of the reaction mixture indicated complete conversion of **5a**−**d** to **6a**−**d**. Insoluble 1,3-dicyclohexylurea is removed by filtration, and the filtrate is partitioned between CH_2Cl_2 (150 mL) and H_2O (150 mL). The organic layer is washed with 2% $NaHCO_3$ (150 mL) and H_2O (150 mL), dried over anhydrous $MgSO_4$, and evaporated to dryness affording **6a**−**d** as an oil. The crude oil is mixed with aqueous 80% AcOH (60 mL) and stirred for 2 h at 25 °C. Excess AcOH is then removed under reduced pressure, and the material left is dissolved in CH_2Cl_2 (200 mL) and washed with 10% $NaHCO_3$ (200 mL). The aqueous phase is extracted with CH_2Cl_2 (3 × 50 mL) and then with CH_2Cl_2:MeOH (85:15 v/v) (7 × 50 mL)[39] until no more product can be recovered. The combined organic extracts are dried over anhydrous $MgSO_4$ and evaporated to dryness. The crude material is purified by silica gel chromatography using a gradient of CH_2Cl_2 in hexane (0−100%), followed by a gradient of CH_3OH in CH_2Cl_2 (0−6%). The appropriate fractions are pooled together, and the solvent is removed under reduced pressure affording **7b**−**d** as an amorphous solid. Unlike **7b**−**d**, **7a** was isolated as an oil, which was then dissolved in 50 mL of EtOH:H_2O (1:5 v/v), frozen, and lyophilized to give a powder.

***3′*-O-Levulinyl-2′-deoxythymidine (7a).** Yield: 96% based on **5a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.54 (q, J = 1.1 Hz, 1H), 6.23 (dd, J = 6.0, 8.1 Hz, 1H), 5.36 (ddd, J = 3.1, 5.6, 7.9 Hz, 1H), 4.10 (m, 1H), 3.90 (ddd, J = 2.6, 2.8, 11.9 Hz, 2H), 2.78 (m, 2H), 2.59 (m, 2H), 2.44 (ddd, J = 5.6, 8.1, 14.1 Hz, 1H), 2.37 (m, 1H), 2.20 (s, 3H), 1.92 (d, J = 1.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5, 27.9, 29.7, 37.1, 37.7, 62.4, 74.9, 85.0, 85.9, 111.2, 136.5, 150.6, 164.0, 172.5, 206.7.

***N*^{4}-Benzoyl-3′-*O*-levulinyl-2′-deoxycytidine (7b).** Yield: 61% based on **5b**. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.36 (d, J = 7.6 Hz, 1H), 8.00 (dd, J = 1.3, 7.1 Hz, 2H), 7.62 (dd, J = 7.3, 7.5 Hz, 1H), 7.50 (dd, J = 7.1, 7.5 Hz, 2H), 7.35 (bd, J = 7.6 Hz, 1H), 6.17 (dd, J = 5.9, 8.0 Hz, 1H), 5.22 (ddd, J = 2.0, 4.1, 6.2 Hz, 1H), 4.19 (m, 1H), 3.64 (m, 2H), 2.75 (m, 2H), 2.52 (m, 2H), 2.48 (ddd, J = 2.0, 5.9, 14.2 Hz, 1H), 2.23 (ddd, J = 6.2, 8.0, 14.2 Hz, 1H), 2.13 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.9, 25.5, 27.9, 29.8, 33.8, 37.8, 38.4, 49.2, 62.3, 74.6, 85.8, 88.2, 127.6, 129.0, 133.2, 145.4, 162.4, 172.6, 206.4.

***N*^{6}-Benzoyl-3′-O-levulinyl-2′-deoxyadenosine (7c).** This compound is prepared as described above, but exposure to aqueous 80% AcOH is shortened to 30 min. Yield: 82% based on **5c**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.13 (s, 1H), 8.03 (dd, J = 1.6, 7.1 Hz, 1H), 7.60 (t, J = 7.3 Hz, 1H), 7.52 (dd, J = 7.1, 7.6 Hz, 2H), 6.37 (dd, J = 5.3, 9.7 Hz, 1H), 5.57 (bd, J = 5.5 Hz, 1H), 4.30 (m, 1H), 3.98 (dd, J = 1.8, 12.7 Hz, 1H), 3.89 (bd, J = 12.7 Hz, 1H), 3.16 (ddd, J = 5.7, 9.7, 14.0 Hz, 1H), 2.81 (m, 2H), 2.61 (m, 2H), 2.50 (bdd, J = 5.7, 14.0 Hz, 1H), 2.21 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.9, 29.7, 37.7, 37.8, 63.1, 76.4, 87.2, 87.5, 124.5, 127.9, 128.8, 132.9, 133.4, 142.4, 150.3, 150.7, 152.1, 164.6, 172.2, 206.5.

***N*^{2}-Isobutyryl-3′-*O*-levulinyl-2′-deoxyguanosine (7d).** Yield: 91% based on **5d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.62 (bs, 1H), 7.79 (s, 1H), 6.18 (dd, J = 5.5, 9.4 Hz, 1H), 5.46 (ddd, J = 1.3, 6.2, 7.5 Hz, 1H), 4.22 (m, 1H), 3.94 (dd, J = 2.3, 12.3 Hz, 1H), 3.85 (m, 1H), 2.96 (ddd, J = 6.2, 9.4, 14.1 Hz, 1H), 2.80 (m, 2H), 2.69 (sept, J = 6.8 Hz, 1H), 2.60 (m, 2H), 2.44 (ddd, J = 1.3, 5.5, 14.1 Hz, 1H), 2.21 (s, 3H), 1.28 (d, J = 6.8 Hz, 3H), 1.26 (d, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.8, 18.9, 27.9, 29.8, 36.2, 37.4, 37.8, 49.1, 62.7, 75.8, 86.1, 122.2, 138.5, 147.3, 147.8, 155.2, 172.4, 179.3, 206.8.

General Procedure for the Preparation of 5′-*O*-(2,2,5,5-Tetramethylpyrrolidin-3-one-1-sufinyl)-3′-*O*-levulinyl-2′-deoxyribonucleosides (8a−d). 2,2,5,5-Tetramethylpyrrolidin-3-one (3, 2.03 g, 14.3 mmol) is dissolved in anhydrous MeCN (20 mL) under an inert atmosphere, and anhydrous Et_3N (32 mL, 230 mmol) is added to the solution. To the cold (5 °C) and vigorously stirred solution is added thionyl chloride (10.5 mL, 14.3 mmol), dropwise, under an inert atmosphere. Once the addition is complete, the reaction mixture is magnetically stirred for 10 min longer at 5 °C. Then a solution of **7a**−**d** (5.74 mmol) in anhydrous MeCN (40 mL) is added, dropwise, over a period of ∼10 min through the use of a cannula. Once the addition is complete, the reaction mixture is stirred for an additional 2 h at 25 °C. TLC analysis of the suspension indicated complete transformation of **7a**−**d** to a faster-moving product. The reaction mixture is partitioned between CH_2Cl_2 (200 mL) and H_2O (100 mL). The aqueous phase is

(38) (a) Eadie, J. S.; Davidson, D. S. *Nucl. Acids Res.* **1987**, *15*, 8333−8349. (b) Farrance, I. K.; Eadie, J. S.; Ivarie, R. *Nucl. Acids Res.* **1989**, *17*, 1231−1245.

(39) These additional extractions are required only to optimize the recovery of **7b**.

further extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts are dried over anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. The desired product is isolated by silica gel flash chromatography using a gradient of CH_2Cl_2 (0−80%) in hexane containing 5% Et_3N. The appropriate fractions are pooled together, and the solvent is removed under reduced pressure. The product **8a−d** is obtained as an orange-colored foam in yields ranging from 71% to 87%.

5′-*O*-(2,2,5,5-Tetramethylpyrrolidin-3-one-1-sufinyl)-3′-*O*-levulinyl-2′-deoxythymidine (8a). Yield: 87% based on **7a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.65 (q, J = 1.3 Hz, 0.5H), 7.41 (q, J = 1.3 Hz, 0.5H), 6.35 (2×dd, J = 5.5, 9.4 Hz, 1H), 5.27 (2×ddd, J = 1.7, 3.6, 6.6 Hz, 1H), 4.17 (m, 3H), 2.79 (m, 2H), 2.62 (2×d, J = 17.4 Hz, 1H), 2.60 (m, 2H), 2.47 (2×d, J = 17.4 Hz, 1H), 2.43 (2×ddd, J = 1.7, 5.5, 14.0 Hz, 1H), 2.21 (s, 3H), 2.16 (2×ddd, J = 6.6, 9.4, 14.0 Hz, 1H), 1.97 (d, J = 1.3 Hz, 1.5H), 1.94 (d, J = 1.3 Hz, 1.5H), 1.68 (s, 3H), 1.57 (2×s, 3H), 1.54 (s, 3H), 1.51 (2×s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.1, 12.2, 12.4, 26.5, 26.7, 27.8, 28.0, 28.1, 29.7, 30.0, 30.2, 37.0, 37.2, 37.7, 46.0, 51.2, 60.5, 63.0, 64.1, 66.60, 66.65, 74.5, 74.8, 82.98, 83.05, 84.1, 84.8, 111.4, 111.8, 134.8, 135.2, 150.3, 150.5, 163.56, 163.65, 172.3, 172.4, 206.2, 212.8. FAB−HRMS: calcd for $C_{23}H_{33}N_3O_9S$ (M + Cs)$^+$ 660.0992; found 660.0997.

***N*^{4}-Benzoyl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-3′-*O*-levulinyl-2′-deoxycytidine (8b).** In the synthesis of this compound, **7b** is dissolved in anhydrous CH_2Cl_2:MeCN (1:1 v/v) and the reaction time is extended to 5 h. Yield: 71% based on **7b**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.32 (d, J = 7.5 Hz, 0.5H), 8.18 (d, J = 7.5 Hz, 0.5H), 7.91 (dd, J = 1.6, 7.4 Hz, 2H), 7.62 (m, 2H), 7.52 (dd, J = 7.4, 7.9 Hz, 2H), 6.31 (2×dd, J = 5.3, 8.4 Hz, 1H), 5.28 (2×ddd, J = 1.8, 6.5, 8.4 Hz, 1H), 4.38 (m, 0.5H), 4.32 (m, 0.5H), 4.21 (ddd, J = 2.6, 3.4, 11.4 Hz, 1.4H), 4.11 (ddd, J = 2.6, 3.4, 11.4 Hz, 0.6H), 2.79 (2×ddd, J = 1.8, 5.3, 14.3 Hz, 1H), 2.77 (m, 2H), 2.62 (2×d, J = 17.4 Hz, 1H), 2.59 (m, 2H), 2.21 (s, 3H), 2.47 (2×d, J = 17.4 Hz, 1H), 2.11 (2×ddd, J = 6.5, 8.4, 14.3 Hz, 1H), 1.67 (s, 3H), 1.55 (s, 3H), 1.53 (s, 3H), 1.50 (2×s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.6, 26.7, 27.8, 28.0, 28.1, 29.7, 30.1, 30.2, 37.7, 38.6, 38.9, 51.2, 60.50, 60.53, 63.0, 63.7, 66.68, 66.71, 74.5, 74.9, 83.9, 84.1, 86.7, 87.3, 96.6, 96.8, 127.5, 129.0, 133.2, 143.9, 144.5, 162.3, 172.30, 172.34, 206.2, 212.76, 212.83. FAB−HRMS: calcd for $C_{29}H_{37}N_4O_9S$ (M + H)$^+$ 617.2281; found 617.2290.

***N*^{6}-Benzoyl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-3′-*O*-levulinyl-2′-deoxyadenosine (8c).** In the synthesis of this compound, **7c** is dissolved in anhydrous CH_2Cl_2. Yield: 78% based on **7c**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.54 (s, 1H), 8.28 (s, 1H), 8.03 (dd, J = 1.6, 7.1 Hz, 2H), 7.61 (2×t, J = 7.3 Hz, 1H), 7.53 (2×dd, J = 7.1, 7.3 Hz, 2H), 6.53 (2×dd, J = 5.7, 8.8 Hz, 1H), 5.48 (2×ddd, J = 2.2, 4.0, 6.3 Hz, 1H), 4.37 (2×ddd, J = 2.2, 4.0, 6.0 Hz, 1H), 4.19 (2×ddd, J = 4.0, 6.0, 11.5 Hz, 2H), 2.94 (2×ddd, J = 6.3, 8.8, 14.0 Hz, 1H), 2.81 (m, 2H), 2.70 (2×ddd, J = 1.9, 5.7, 14.0 Hz, 1H), 2.63 (m, 2H), 2.60 (2×d, J = 17.4 Hz, 1H), 2.45 (2×d, J = 17.4 Hz, 1H), 2.22 (s, 3H), 1.64 (s, 1.5H), 1.63 (s, 1.5H), 1.55 (s, 1.5H), 1.54 (s, 1.5H), 1.50 (s, 1.5H), 1.49 (s, 1.5 H), 1.48 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.5, 26.6, 27.9, 28.0, 29.7, 30.1, 37.5, 37.8, 51.2, 60.4, 60.5, 63.1, 63.8, 66.6, 66.7, 74.7, 75.0, 83.6, 83.8, 84.0, 84.7, 123.2, 123.4, 127.8, 128.8, 132.7, 133.6, 141.2, 141.7, 149.5, 151.5, 151.6, 152.7, 164.5, 172.2, 172.3, 206.2, 213.0, 213.1. FAB−HRMS: calcd for $C_{30}H_{37}N_6O_8S$ (M + H)$^+$ 641.2393; found 641.2397.

***N*^{2}-Isobutyryl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sufinyl)-3′-*O*-levulinyl-2′-deoxyguanosine (8d).** Yield: 83% based on **7d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74 (s, 0.5H), 7.71 (s, 0.5H), 6.20 (dd, J = 6.0, 8.5 Hz, 0.5H), 6.11 (dd, J = 5.2, 9.6 Hz, 0.5H), 5.48 (2×ddd, J = 1.6, 3.9, 6.0 Hz, 1H), 4.50 (dd, J = 5.8, 11.2 Hz, 0.5H), 4.38 (dt, J = 1.6, 5.5 Hz, 0.5H), 4.32 (dd, J = 4.0, 12.0 Hz, 0.5H), 4.31 (dt, J = 1.5, 5.5 Hz, 0.5H), 4.13 (2×dd, J = 5.3, 11.5 Hz, 1H), 3.32 (ddd, J = 6.1, 9.6, 13.8 Hz, 0.5H), 3.16 (ddd, J = 5.8, 8.4, 14.4 Hz, 0.5H), 2.67 (qt, J = 6.8 Hz, 1H), 2.81 (m, 2H), 2.62 (d, J = 17.4 Hz, 0.5H), 2.60 (m, 2H), 2.59 (d, J = 17.4 Hz, 0.5H), 2.47 (2×d, J = 17.4 Hz, 1H), 2.36 (m, 1H), 2.21 (s, 3H), 1.68 (s, 1.5H), 1.62 (s, 1.5H), 1.57 (s, 1.5H), 1.56 (s, 1.5H), 1.52 (2×s, 4.5H), 1.48 (s, 1.5H), 1.25 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 10.4, 18.8, 19.1, 26.6, 27.8, 28.2, 29.7, 29.9, 36.1, 36.2, 37.8, 46.1, 51.1, 51.2, 60.8, 61.0, 62.8, 63.8, 66.6, 66.7, 74.9, 75.1, 85.8, 86.1, 122.5, 122.8, 128.2, 138.1, 138.9, 147.5, 147.7, 148.0, 155.6, 172.2, 172.4, 179.1, 179.2, 206.4, 212.6, 212.7. FAB−HRMS: calcd for $C_{27}H_{39}N_6O_9S$ (M + H)$^+$ 623.2499; found 623.2510.

General Procedure for the Conversion of 8a−d to 9a−d. Fully protected deoxyribonucleoside **8a−d** (4.87 mmol) is dissolved in anhydrous pyridine (20 mL) under an inert atmosphere, and 1 M hydrazine hydrate in pyridine/acetic acid (3:2 v/v, 14.6 mL) is added to the solution, which is stirred for 3 min at 25 °C. The reaction mixture is then chilled in an ice−water bath, and pentane-2,4-dione (1.25 mL) is added. After 2 min, the solution is partitioned between CH_2Cl_2 (150 mL) and H_2O (100 mL). The organic layer is separated and sequentially washed with 10% $NaHCO_3$ (50 mL) and H_2O (50 mL), dried over anhydrous $MgSO_4$, and evaporated to dryness under reduced pressure. TLC analysis of the material showed complete removal of the levulinyl group. The crude product is purified by silica gel flash chromatography using the following gradients: CH_2Cl_2 (0−100%) in hexane containing 5% Et_3N and acetone (0−80%) in CH_2Cl_2 containing 5% Et_3N. The appropriate fractions are combined together, and the solvent is removed under reduced pressure, affording **9a−d** as a pale yellow solid in yield ranging from 48% to 86%.

5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-2′-deoxythymidine (9a). Yield: 86% based on **8a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.46 (q, J = 1.3 Hz, 0.5H), 7.31 (q, J = 1.3 Hz, 0.5H), 6.30 (2dd, J = 6.1, 7.4 Hz, 1H), 4.49 (ddd, J = 3.7, 6.8, 10.2 Hz, 1H), 4.11 (m, 3H), 2.62 (d, J = 17.4 Hz, 1H), 2.47 (2×d, J = 17.4 Hz, 1H), 2.38 (ddd, J = 3.7, 6.1, 13.8 Hz, 1H), 2.17 (m, 1H), 1.95 (d, J = 1.3 Hz, 1.5H), 1.92 (d, J = 1.3 Hz, 1.5H), 1.67 (2×s, 3H), 1.57 (2×s, 3H), 1.53 (bs, 3H), 1.51 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 10.8, 12.2, 12.4, 26.5, 26.6, 27.9, 29.9, 30.0, 40.1, 45.9, 51.0, 60.26, 60.33, 63.2, 63.6, 66.5, 70.8, 71.0, 84.5, 84.8, 84.9, 110.8, 111.0, 135.2, 135.4, 150.4, 150.5, 163.9, 164.0, 212.9, 213.0. FAB−HRMS: calcd for $C_{18}H_{27}N_3O_7S$ (M + Na)$^+$ 452.1467; found 452.1446.

***N*^{4}-Benzoyl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-2′-deoxycytidine (9b).** Yield: 79% based on **8b**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.27 (d, J = 7.5 Hz, 0.5H), 8.21 (d, J = 7.5 Hz, 0.5H), 7.95 (dd, J = 1.6, 7.1 Hz, 2H), 7.60 (dd, J = 7.3, 7.5 Hz, 1H), 7.57 (d, J = 7.5 Hz, 1H), 7.50 (dd, J = 7.1, 7.9 Hz, 2H), 6.31 (dd, J = 6.2, 8.5 Hz, 1H), 4.46 (2×ddd, J = 2.0, 4.2, 6.3 Hz, 1H), 4.25 (m, 2H), 4.12 (m, 1H), 2.76 (2×ddd, J = 4.2, 6.2, 13.9 Hz, 1H), 2.63 (2×d, J = 17.6 Hz, 1H), 2.48 (2×d, J = 17.6 Hz, 1H), 2.15 (2×ddd, J = 2.0, 8.5, 13.9 Hz, 1H), 1.68 (2×s, 3H), 1.57 (2×s, 3H), 1.54 (2×s, 3H), 1.51 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.6, 28.0, 30.0, 30.1, 41.4, 51.1, 60.4, 60.6, 63.1, 63.3, 66.7, 70.5, 70.8, 85.7, 85.8, 87.1, 87.3, 96.8, 97.0, 127.8, 128.8, 133.0, 133.1, 144.1, 144.4, 162.6, 212.9, 213.1. FAB−HRMS: calcd for $C_{24}H_{31}N_4O_7S$ (M + H)$^+$ 519.1914; found 519.1924.

***N*^{6}-Benzoyl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-2′-deoxyadenosine (9c).** Yield: 62% based on **8c**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.75 (2×s, 1H), 8.26 (2×s, 1H), 8.02 (dd, J = 1.6, 7.1 Hz, 2H), 7.62 (dd, J = 7.3, 7.4 Hz, 1H), 7.53 (dd, J = 7.1, 7.3 Hz, 2H), 6.49 (2×t, J = 6.5 Hz, 1H), 4.22 (m, 2H), 4.79 (2×m, 1H), 2.96 (ddd, J = 6.5, 7.3, 13.5 Hz, 1H), 4.11 (m, 1H), 2.62 (2×ddd, J = 3.7, 6.5, 13.5 Hz, 1H), 2.46 (2×d, J = 17.5 Hz, 1H), 2.61 (2×d, J = 17.5 Hz, 1H), 1.63 (2×s, 3H), 1.55(s, 3H), 1.49 (2×s, 3H), 1.48 (2×s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.6, 28.0, 30.0, 39.7, 39.9, 51.2, 60.4, 60.5, 63.3, 63.7, 66.8, 71.4, 72.0, 84.4, 84.7, 85.3, 85.4, 123.4, 127.8, 128.8, 132.8, 133.6, 141.6, 141.8, 149.5, 151.3, 151.4, 152.5, 164.7, 212.9, 213.0. FAB−HRMS: calcd for $C_{25}H_{31}N_6O_6S$ (M + H)$^+$ 543.2026; found 543.2026.

***N*²-Isobutyryl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-2′-deoxyguanosine (9d).** Yield: 48% based on **8d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.87 (s, 0.5H), 7.86 (s, 0.5H), 6.26 (m, 1H), 4.82 (m, 1H), 4.29 (ddd, J = 4.3, 5.2, 14.9 Hz, 2H), 4.12 (ddd, J = 4.3, 5.2, 6.9 Hz, 1H), 2.96 (ddd, J = 5.9, 7.6, 13.6 Hz, 1H), 2.85 (qt, J = 6.8 Hz, 1H), 2.77 (qt, J = 6.8 Hz, 1H), 2.62 (d, J = 17.3 Hz, 0.5H), 2.58 (d, J = 17.3 Hz, 0.5H), 2.47 (d, J = 17.3 Hz, 0.5H), 2.46 (d, J = 17.3 Hz, 0.5H), 2.40 (m, 1H), 1.65 (s, 1.5H), 1.61 (s, 1.5H), 1.55 (s, 1.5H), 1.53 (s, 1.5H), 1.50 (2×s, 3H), 1.49 (s, 1.5H), 1.46 (s, 1.5H), 1.27 (d, J = 6.8 Hz, 3H), 1.25 (d, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 10.8, 18.9, 19.1, 26.7, 27.9, 28.0, 30.0, 36.0, 36.1, 39.5, 39.8, 46.2, 51.1, 51.2, 60.6, 60.7, 63.9, 64.3, 66.6, 71.4, 71.7, 85.1, 85.7, 121.5, 121.8, 128.3, 138.3, 139.0, 147.9, 148.2, 148.3, 155.9, 180.0, 180.2, 213.0, 213.1. FAB−HRMS: calcd for $C_{22}H_{33}N_6O_7S$ $(M + H)^+$ 525.2131; found 525.2136.

General Method for the Preparation of the Deoxyribonucleoside Phosphoramidites 10a−d. To a stirred solution of **9a−d** (2.33 mmol) in anhydrous MeCN is added anhydrous Et_3N (1.6 mL, 11.7 mmol) followed by 2-cyanoethyl diisopropylchlorophosphoramidite (1.6 mL, 3.03 mmol) under an inert atmosphere. Progress of the reaction is monitored by TLC, which indicates complete disappearance of **9a−d** within 1 h at 25 °C. The reaction mixture is then diluted upon addition of CH_2Cl_2 (150 mL) and aqueous saturated $NaHCO_3$ (100 mL). The organic phase is collected and washed further with a saturated solution of $NaHCO_3$ (3 × 50 mL). The organic extracts are dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The material left is then purified by silica gel chromatography. Typically phosphoramidites **10a** and **10b** are eluted from the column using a solution of 5% Et_3N in EtOAc:hexane (1:1 v/v), whereas a solution of 5% Et_3N in EtOAc:hexane (1:3 v/v) is required for the elution of **10c**. The more polar **10d** is eluted with a gradient of acetone (0−50%) in EtOAc containing 5% Et_3N. Fractions containing the product are pooled together and evaporated to dryness to give **10a−d** as a pale yellow foam in yields varying from 54% to 83%.[40]

5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethyloxy)]phosphinyl-2′-deoxythymidine (10a). Yield: 83% based on **9a**. ^{31}P NMR (121 MHz, $CDCl_3$): δ 150.4, 150.3, 150.2, 149.9. FAB−HRMS: calcd for $C_{27}H_{44}N_5O_8PS$ $(M + Cs)^+$ 762.1703; found 762.1691.

***N*⁴-Benzoyl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethyloxy)]phosphinyl-2′-deoxycytidine (10b).** Yield: 76% based on **9b**. ^{31}P NMR (121 MHz, $CDCl_3$): δ 149.8, 149.7, 149.2. FAB−HRMS: calcd for $C_{33}H_{47}N_6O_8PS$ $(M + Cs)^+$ 851.1968; found 851.1970.

***N*⁶-Benzoyl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethyloxy)]phosphinyl-2′-deoxyadenosine (10c).** Yield: 69% based on **9c**. ^{31}P NMR (121 MHz, $CDCl_3$): δ 150.54, 150.46, 150.41, 150.36. FAB−HRMS: calcd for $C_{34}H_{47}N_8O_7PS$ $(M + Cs)^+$ 875.2080; found 875.2050.

***N*²-Isobutyryl-5′-*O*-(2,2,5,5-tetramethylpyrrolidin-3-one-1-sulfinyl)-3′-*O*-[(*N*,*N*-diisopropylamino)(2-cyanoethyloxy)]phosphinyl-2′-deoxyguanosine (10d).** Yield: 54% based on **9d**. ^{31}P NMR (121 MHz, $CDCl_3$): δ 149.7, 149.2, 149.1, 148.8. FAB−HRMS: calcd for $C_{31}H_{49}N_8O_8PS$ $(M + Cs)^+$ 857.2186; found 857.2160.

Preparation and Characterization of Oligonucleotides. It is critically important to free **10a−d** from the triethylamine and adventitious moisture carried through the purification process. This is accomplished by (i) repeatedly coevaporating each phosphoramidite with anhydrous MeCN under reduced pressure, (ii) dissolving each phosphoramidite in dry benzene and freezing the resulting solution, and (iii) lyophilizing each frozen solution under high vacuum overnight. Failure to perform these precautionary steps may result in less efficient coupling reactions during oligonucleotide assembly given that the activator, 4,5-dicyanoimidazole, is neutralized by Et_3N. The phosphoramidites **10a−d** are further exposed to P_2O_5, overnight, under high vacuum prior to use. The triethylamine-free and dry phosphoramidites are then dissolved in anhydrous MeCN under an inert atmosphere and employed as 0.1 M solutions in the automated solid-phase synthesis of 5′-d(CTCTCTACGT) and 5′-d(ATCCGTAGCCAAGGTCATGT) on a 0.2 μmol scale. Ancillary reagents for the preparation of oligonucleotides are purchased and utilized as recommended by the manufacturer. The computerized synthesis cycle for oligonucleotide assembly is modified to accommodate the use of **10a−d**. Specifically, the dedimethoxytritylation and capping steps are eliminated from the synthesis cycle. The leader nucleoside attached to long-chain alkylamine controlled-pore glass (LCAA-CPG) through a 3′-*O*-succinyl linker is manually dedimethoxytritylated under standard conditions (3% TCA in CH_2Cl_2) prior to use. 4,5-Dicyanoimidazole is used as a 0.25 M solution in MeCN for the activation of **10a−d,** and the phosphoramidite coupling time is set at 30 s. The oxidation/5′-*O*-deprotection step consists of a 1 min treatment with 0.02 M I_2 in THF/pyridine/H_2O followed by a 8 min exposure to a solution of 0.1 M I_2, 0.25 M 3-acetylpyridine, and 0.125 M TCA in THF:H_2O (9:1 v/v). Low-water content MeCN is used to perform the washes after the coupling step and the oxidation/5′-*O*-deprotection step.

The solid-phase synthesis of 5′-d(CTCTCTACGT) and 5′-d(ATCCGTAGCCAAGGTCATGT) via standard 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites is carried out on a 0.2 μmol scale following a modified synthesis cycle designed to closely match the one developed for the use of **10a−d** to comparatively evaluate synthesis performance. Typically the didemethoxytritylation step remains unchanged from the standard synthesis cycle. The coupling step is performed using 0.25 M 4,5-dicyanoimidazole in MeCN for the activation of 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites, and the coupling time is set at 30 s. The capping step is deleted from the cycle, whereas the oxidation step consists of a 1 min treatment with 0.02 M I_2 in THF/pyridine/H_2O. Washes between each step of the synthesis cycle are also effected with low-water content MeCN.

Upon completion of the synthesis, the crude oligomers are released from the CPG support by treatment with concentrated NH_4OH for 30 min at 25 °C. Each oligonucleotide solution is then heated for 10 h at 55 °C to ensure complete nucleobase deprotection. The ammoniacal solution of each 20-mer is evaporated to near dryness under reduced pressure and, ddH_2O (250 μL) is added. Each oligonucleotide solution is applied to the top of a prepacked PD-10 Sephadex G-25M column equilibrated in ddH_2O to eliminate benzamide. The crude oligonucleotides are eluted with ddH_2O, and 1 mL fractions are collected. Fractions containing each oligonucleotide are identified by UV at 260 nm and pooled together. The crude oligonucleotides are analyzed by RP-HPLC to assess purity and compare synthesis yields. RP-HPLC chromatograms are shown in Figure 1 along with a description of the chromatographic conditions used for the analysis.

The crude, fully deprotected, and desalted oligomers (0.25 OD_{260} unit, each) are further characterized by PAGE using 20% polyacrylamide−7 M urea gels (40 cm × 20 cm × 0.75 mm), which were prepared using electrophoresis purity reagents. Gels are stained by soaking in a solution of Stains-all as described elsewhere.[41] A photograph of a comparative gel is shown in Figure 2.

Enzymatic digestion of crude, deprotected, and desalted 5′-d(ATCCGTAGCCAAGGTCATGT) synthesized via either **10a−d** or standard 5′-*O*-DMTr-deoxyribonucleoside phosphoramidites is effected by snake venom phosphodiesterase (*Crotalus adamanteus*) and bacterial alkaline phosphatase according to a published procedure.[42] RP-HPLC profiles of the enzymatic digests are presented in Figure 3, along with a narrative of the chromatographic conditions used for the analysis to assess

(40) Proton-decoupled ^{31}P NMR analysis of **10a**,**c**,**d** reveals four signals, whereas only three signals are resolved for **10b** in $CDCl_3$. These signals are consistent with the four diastereomers emerging from the asymmetry of the phosphorus and sulfur atoms.

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nucleobase modifications that might have occurred during oligonucleotide synthesis and deprotection.

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Supporting Information Available: Materials and Methods; ^{1}H NMR spectra of **7a**−**d**; ^{1}H and ^{13}C NMR spectra of **3**, **8a**−**d**, and **9a**−**d**; ^{31}P NMR spectra of **10a**−**d**; RP-HPLC retention times (t_R) of **9a**−**d**; RP-HPLC chromatograms of 5′-d(CTCTCTACGT) and that of 5′-d($T_{PS}C_{PS}G_{PS}A_{PS}T$). This material is available free of charge via the Internet at http://pubs.acs.org.

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