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Solid-Phase Purification of Synthetic DNA Sequences

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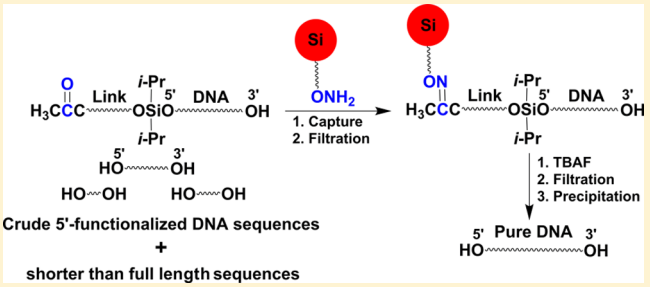
Supporting Information

ABSTRACT: Although high-throughput methods for solid-phase synthesis of DNA sequences are currently available for synthetic biology applications and technologies for large-scale production of nucleic acid-based drugs have been exploited for various therapeutic indications, little has been done to develop high-throughput procedures for the purification of synthetic nucleic acid sequences. An efficient process for purification of phosphorothioate and native DNA sequences is described herein. This process consists of functionalizing commercial aminopropylated silica gel with aminooxyalkyl functions to enable capture of DNA sequences carrying a 5′-siloxyl ether linker with a "keto" function through an oximation reaction. Deoxyribonucleoside phosphoramidites functionalized with the 5′-siloxyl ether linker were prepared in yields of 75−83% and incorporated last into the solid-phase assembly of DNA sequences. Capture of nucleobase- and phosphate-deprotected DNA sequences released from the synthesis support is demonstrated to proceed near quantitatively. After shorter than full-length DNA sequences were washed from the capture support, the purified DNA sequences were released from this support upon treatment with tetra-*n*-butylammonium fluoride in dry DMSO. The purity of released DNA sequences exceeds 98%. The scalability and high-throughput features of the purification process are demonstrated without sacrificing purity of the DNA sequences.



INTRODUCTION

The ability to design and synthesize DNA and RNA sequences has had a huge impact on biotechnology, particularly in the rapidly growing fields of synthetic biology and nucleic acid-based drug development. Indeed, the use of synthetic DNA/RNA sequences and their analogues for recognition and binding to mRNAs encoding disease-causing proteins has led to the production of nucleic acid-based drugs capable of inhibiting the expression of those proteins through either an antisense[1] or an RNA interference[2] pathway. Such applications require the production of synthetic nucleic acid sequences in large quantities (e.g., millimoles) and high purity for preclinical and clinical investigations. In contrast, total gene synthesis for synthetic biology applications requires small amounts (e.g., nanomoles) of numerous highly pure synthetic DNA sequences. Although the chemical synthesis of nucleic acid sequences using the phosphoramidite chemistry[3,4] is efficient and can be scaled up for pharmaceutical production, the purification of those sequences presents a formidable challenge. Despite the fact that the coupling efficiency of phosphoramidite monomers is near quantitative on a controlled-pore glass (CPG) support, the full-length nucleic acid sequences are mixed with shorter sequences, resulting from a near complete phosphoramidite coupling at each cycle of the nucleic acid sequence assembly. Other process-related impurities include deletion sequences due to failure to either quantitatively prevent the growth of shorter than full-length sequences or to completely remove the 5′-hydroxyl protecting group at each step of the nucleic acid sequence assembly. Furthermore, the formation of longer than full-length nucleic acid sequences occurs when the activation of phosphoramidite monomers by a weak acid prompts the premature cleavage of the acid-labile 5′-hydroxyl protecting group of the newly extended nucleic acid sequence. Although these impurities are produced in small amounts, their physicochemical similarity to the desired nucleic acid sequence makes them very difficult to remove. In the context of large-scale nucleic acid-based drug production, HPLC-based methods including reversed-phase (RP) HPLC and anion-exchange HPLC are currently the preferred techniques for purification of nucleic acid sequences. These methods require high capacity instruments and accessories (e.g., preparative columns) in addition to large volumes of buffered aqueous and organic elution solvents. This process is neither cost-effective nor amenable to parallel purification processes; only a single nucleic acid sequence can be purified per run unless numerous instruments are available for this purpose. HPLC-based purification processes are time-consuming given that, depending on the nature of individual nucleic acid sequence, more than one purification run may be required to achieve the level of sequence purity required for pharmaceutical applications. One important limitation of any large-scale HPLC purification process is the burdensome removal of large volumes of aqueous solvents produced during purification, which may also depend on the

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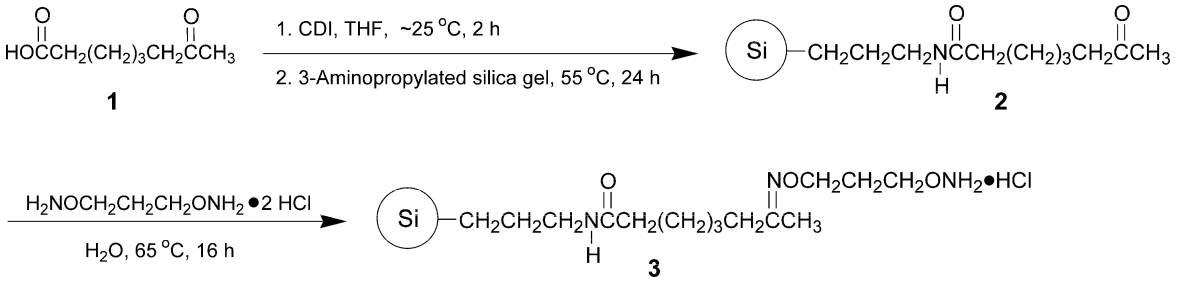
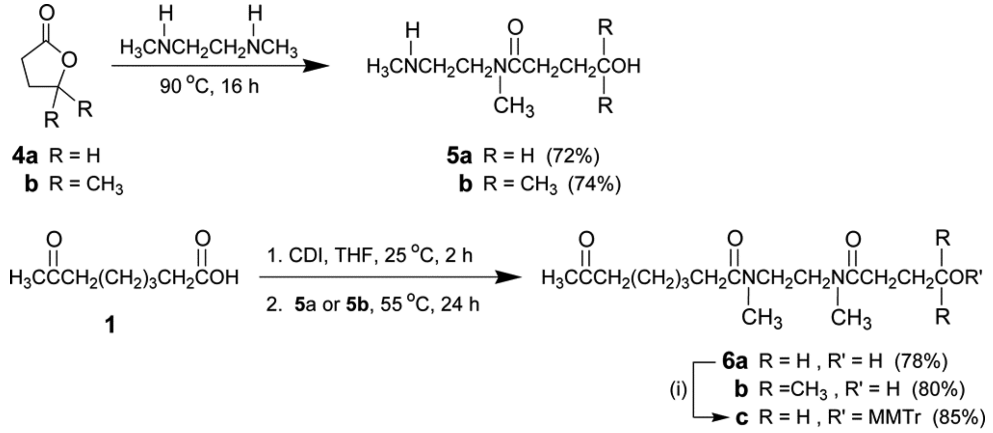
Scheme 1. Preparation of the Capture Support 3[a]



[a]Abbreviations: CDI, 1,1′-carbonyldiimidazole; Si, 3-aminopropylated silica gel.

Scheme 2. Synthesis of Linkers To Be Used for the Solid-Phase Capture of DNA Sequences or Measuring the Concentration of Aminooxy Functions Conjugated to Support 3[a]



[a]Conditions: (i) MMTr-Cl, dry pyridine, 25 °C, 4 h. Abbreviations: CDI, 1,1′-carbonyldiimidazole; MMTr, 4-monomethoxytrityl.

physicochemical properties of each nucleic acid sequence; this operation requires costly equipment, as well. With regard to the small-scale purification of DNA sequences for total gene construction in the realm of synthetic biology applications, HPLC-based methods can also be used for this purpose, but as discussed above, these methods are not amenable to cost-effective parallel purification of nucleic acid sequences. Furthermore, HPLC-based purification methods are time-consuming and often may not completely resolve shorter than full-length sequences from the desired DNA sequences. Although polyacrylamide gel electrophoresis (PAGE) can efficiently separate shorter nucleic acid sequences from full-length DNA sequences, recovery of the purified sequences from the gel matrix is cumbersome and laborious, with limited potential for parallel purification of nucleic acid sequences. A number of orthogonal methods have, however, been proposed for the purification of nucleic acid sequences. These methods are based on affinity,[5−11] hydrophobic[12] or ion-pair[13] chromatography, and solid-phase removal of shorter than full-length nucleic acid sequences by enzymatic hydrolysis[14] or by hydrophobic retention of the full-length sequences.[15] All of those techniques are either not amenable to highly parallel or large-scale purification of nucleic acid sequences or both. A conceptually different approach to the purification of synthetic DNA sequences has been proposed[16,17] and consists of catching shorter than full-length sequences through a polymerization process; the full-length sequence is then extracted from the polymer and isolated by precipitation in *n*-butanol. This process has been claimed to be potentially useful for the large-scale and high-throughput purification of DNA sequences.[17]

We report herein an innovative solid-phase-assisted purification method for the purification of synthetic unmodified and modified (e.g., phosphorothioate) DNA sequences. The objectives of this work are to (i) demonstrate the efficient solid-phase capture of synthetic nucleic acid sequences through an oximation reaction and subsequent fluoride-assisted release of the pure sequences from the capture support; (ii) apply the method to the purification of a relatively long (60-mer) synthetic DNA sequence; and (iii) demonstrate the parallel solid-phase purification of a phosphorothioate DNA sequence (20-mer) and the scalability of the process in terms of yield and purity of the sequence.

RESULTS AND DISCUSSION

Selection and Design of the Capture Solid Support. An appropriate selection of the solid support is critical for optimal capture and release of nucleic acid sequences. The support should be totally insoluble when immersed in either aqueous or polar and nonpolar organic solvents to prevent any loss of the support-anchored nucleic acid sequence when washing off the shorter than full-length sequences. The solid support should not swell or shrink in any solvents in order to provide optimal capture and release kinetics of the full-length DNA sequence. Such requirements exclude the use of most organic polymeric supports. Another critically important criterion in the selection of a solid support is its loading capacity, which should be of the highest for optimal capture of nucleic acid sequences and scale-

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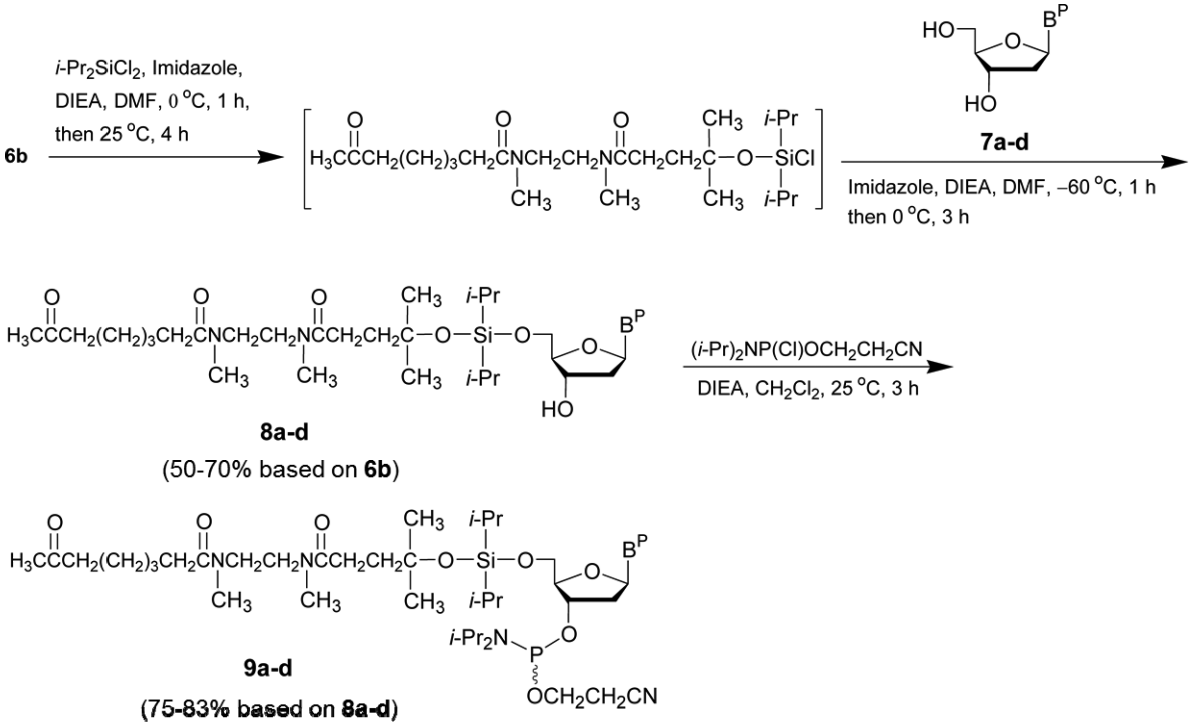
Scheme 3. Synthesis of 5′-Functionalized Deoxyribonucleosides (8a−d) and Deoxyribonucleoside Phosphoramidites (9a−d) for Solid-Phase Capture of DNA Sequences[a]



[a]Abbreviations: DIEA, *N*,*N*-diisopropylethylamine; B^P, (a) N^6-benzoyladenin-9-yl, (b) N^4-benzoylcytosin-1-yl, (c) N^2-isobutyrylguanin-9-yl, or (d) thymin-1-yl; *i*-Pr, isopropyl; DMF, *N*,*N*-dimethylformamide.

up potential for the large-scale purification of nucleic acid-based drugs. We identified 3-aminopropyl-functionalized silica gel as a suitable support for the intended purpose; the support is commercially available and loaded with ∼1 mmol of primary aminopropyl functions per gram of support. As shown in Scheme 1, functionalization of the aminopropylated support begins with the reaction of 7-oxooctanoic acid (**1**) with 1,1′-carbonyldiimidazole in THF to provide the ketoalkyl amidoalkylated support **2**; unreacted amino functions are inactivated upon reaction with excess acetic anhydride in dry pyridine.

The support **2** was then suspended in a solution of *O*,*O*′-1,3-propanediylbishydroxylamine dihydrochloride in H_2O to provide the aminooxy-functionalized support **3**. The selection of the aminooxy group for the functionalization of support **3** is based on the high reactivity of this function by virtue of the well-known α-effect[18] with carbonyl groups. The concentration of aminooxy functions covalently bound to support **3** was determined upon reaction of the 4-monomethoxytritylated ketone **6c** with **3** and subsequent release of the 4-monomethoxytrityl cation under acidic conditions; spectrophotometric measurement of the yellow-colored cation at 478 nm reveals an aminooxy concentration of 146 ± 7 μmol per gram of support **3**. As shown in Scheme 2, **6c** is prepared from the 4-monomethoxytritylation of **6a**, which was obtained from the reaction sequence **4a** → **5a**. Although the reactions of aminooxy functions with carbonyl groups were shown to produce stable oxime ethers with a variety of nucleosides and nucleic acid sequences,[19−29] oximation reactions have not been used, to the best of our knowledge, for solid-phase purification of nucleic acid sequences.

Functionalization of Deoxyribonucleoside Phosphoramidites with a Linker To Enable the Capture of Nucleic Acid Sequences. Our objectives are to functionalize nucleoside phosphoramidites with a linker that will efficiently react with the aminooxy function of the capture support **3**. Such a linker is prepared from the reaction of an aqueous solution of *N*,*N*′-dimethylethylenediamine with 4,4-dimethyl-γ-butyrolactone (**4b**) to provide the aminated amido alcohol **5b** in a postpurification yield of 74% (Scheme 2). The reaction of CDI-activated 7-oxooctanoic acid **1** with **5b** gives the keto diamidated alcohol **6b** in a yield of 80% after purification on silica gel. The conjugation of **6b** to the 5′-hydroxy function of 2′-deoxythymidine and N-protected 2′-deoxyribonucleosides (**7a−d**) was performed through the formation of a diisopropylsiloxyl linkage[7,30−32] upon reaction with equimolar amounts of dichlorodiisopropylsilane in the presence of imidazole to provide the 5′-*O*-diisopropylsiloxyl ether derivatives[5−7] **8a−d** (Scheme 3) with yields in the range of 50−70% after silica gel purification. The alcohols **5a**,**b** and **6a**,**b**, the 4-methoxytrityl ether **6c**, and the silica-gel-purified 5′-functionalized deoxyribonucleosides **8a−d** were fully characterized by ^{1}H and ^{13}C NMR spectroscopies and by high-resolution mass spectrometry (HRMS); the characterization data are presented in the Experimental Section and in the Supporting Information (see Figures S8−S22, S24−S26, S28−S30, S32−S34, and S36−S38). The purity of **8a−d** was assessed by RP-HPLC, the chromatograms of which are shown in the Supporting Information (see Figures S23, S27, S31, and S35). It should be noted that the two rotameric tertiary amides[33] in each of **8a−d** have contributed to the high complexity of their ^{1}H and ^{13}C NMR spectra. Although the use of secondary amides could have been an option for reducing the complexity of NMR data, the selection of tertiary instead of secondary amides was motivated by the inherent imidic acid tautomerism of secondary amides, which could potentially lead to the formation of

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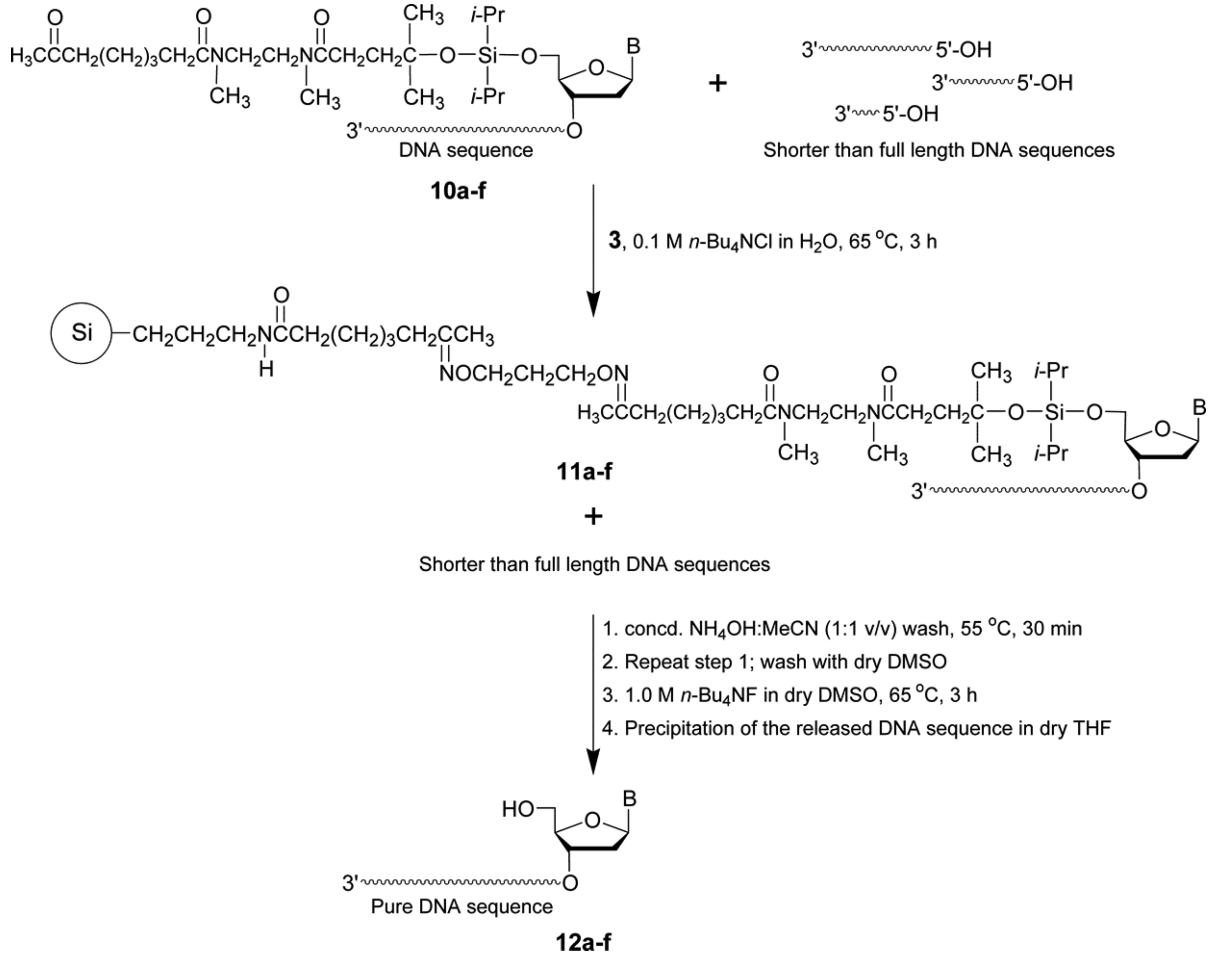
Scheme 4. Capture of Crude DNA Sequences 10a–f by the Solid Support 3 and Release of These Sequences from the Solid Supports 11a–f as Purified Sequences 12a–f[a]



[a]Abbreviations: Si, 3-aminopropylated silica gel; B, adenin-9-yl, cytosin-1-yl, guanin-9-yl, or thymin-1-yl; **12a**, 5′-d($A_{PS}C_{PS}A_{PS}C_{PS}T_{PS}G_{PS}T_{PS}G_{PS}A_{PS}A_{PS}T_{PS}C_{PS}G_{PS}A_{PS}T_{PS}G_{PS}C_{PS}C_{PS}A_{PS}T$); **12b**, 5′-d($C_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}C_{PS}C_{PS}T_{PS}T_{PS}A_{PS}C_{PS}G_{PS}T_{PS}C_{PS}T_{PS}T_{PS}G_{PS}T$); **12c**, 5′-d($G_{PS}T_{PS}G_{PS}A_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}G_{PS}A_{PS}A_{PS}C_{PS}G_{PS}T_{PS}G_{PS}A_{PS}A_{PS}G_{PS}T$); **12d**, 5′-d($T_{PS}A_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}T_{PS}A_{PS}A_{PS}C_{PS}C_{PS}G_{PS}T_{PS}C_{PS}A_{PS}G_{PS}T$); **12e**, 5′-d($A_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PC_PA_PT$); **12f**, 5′-d($T_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PA_PA_PT_PT_PG_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PA_PA_PT_PC_PG_PA_PT_PG_PC_PC_PA_PT_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PC_PA_PT$); PS, phosphorothioate diester; P, phosphate diester.

"branched sites" amenable to multiple self-condensations with activated phosphoramidites **9a–d**. This would ultimately result in the exponential formation of numerous adducts and poorer coupling efficiency of **9a–d** as a consequence of its rapidly decreasing concentration caused by numerous self-condensations. On the basis of this rationale, the selection of tertiary amides in the preparation of **8a–d** was made in order to ensure optimum coupling efficiency of **9a–d** for its intended purpose. This action is justified despite the increased NMR complexity of rotameric tertiary amide-containing products. In this context, comparing side-by-side the (i) chemical shifts and multiplicity of the ^{1}H NMR signals, (ii) chemical shifts of the ^{1}H-decoupled ^{13}C and ^{31}P NMR signals, where applicable, and (iii) HRMS data is a reasonable approach to support reproducibility in the preparation of **8a–d**.

Phosphitylation of **8a–d** (Scheme 3) was performed in anhydrous CH_2Cl_2 using commercial 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite in the presence of *N*,*N*-diisopropylethylamine.[34−36] It is worth mentioning that the benzoyl and isobutyryl groups protecting the exocyclic amino function of cytosine, adenine, and guanine were selected to provide a worst-case scenario in the solid-phase purification of nucleic acid sequences in terms of their postsynthesis nucleobase deprotection. Given that silica gel purification of the nucleoside phosphosphoramites **9a–d** was conducted in the presence of triethylamine in order to prevent premature activation of the phosphoramidites when exposed to the inherent acidity of silica gel, it is critically important that residual triethylamine be removed from **9a–d**.[35] Neutralization of 1*H*-tetrazole or any other acidic activator by triethylamine will result in decreased coupling efficiency of the phosphoramidites during the course of solid-phase DNA synthesis.[35] Lyophilization of frozen benzene solutions of **9a–d** under high vacuum was found to be effective at removing residual triethylamine from phosphoramidite monomers.[35] Triethylamine-free deoxyribonucleoside phosphoramidites (**9a–d**) were isolated as viscous oils, the yields of which were in the range of 75–83%. These phosphoramidites were satisfactorily characterized by ^{31}P NMR spectroscopy and HRMS; the characterization data are presented in the Experimental Section, whereas the NMR and HRMS spectra are included in the Supporting Information (see Figures S39–S46). As discussed above, the two rotameric tertiary amides

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within **8a−d** have not only contributed to the complexity of their ^{1}H and ^{13}C NMR spectra but also added to the complexity of the diastereomeric ^{31}P NMR signals recorded for **9a−d**. It should also be emphasized that the presence of adventitious moisture in **9a−d** and commercial phosphoramidites will result in lower phosphoramidite coupling efficiencies. It is therefore recommended that all deoxyribonucleoside phosphoramidites needed for solid-phase synthesis of nucleic acid sequences be dried overnight under high vacuum in a desiccator containing an efficient drying agent (e.g., phosphorus pentoxide) prior to use.

Solid-Phase Synthesis of 5′-Functionalized Phosphorothioate and Native DNA Sequences. The solid-phase synthesis of phosphorothioate and native DNA sequences (**10a−d** and **10e,f**, respectively) was conducted using a commercial long-chain alkylamine controlled-pore glass support (LCAA-CPG) according to standard protocols[37] with the following exception: the capping step was performed after the oxidation reaction. The phosphite triester function was oxidized using 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN[37] or 0.02 M iodine solution in THF/pyridine/water for phosphorothioate or native DNA sequences, respectively. It is critically important that the coupling efficiency of phosphoramidites **9a−d** and the capping of unreacted 5′-hydroxy functions be optimal for solid-phase purification of DNA sequences; less than optimal coupling and capping reactions will result in poorer recovery of solid-phase-purified DNA sequences. In this regard, the coupling time of DNA phosphoramidites **9a−d** was extended to 180 s to ensure the highest coupling efficiency of these 5′ sterically demanding phosphoramidite monomers. Postsynthesis deprotection and release of the DNA sequences **10a−f** from LCAA-CPG were performed under basic conditions according to standard protocols.[36]

Solid-Phase Capture and Release of Phosphorothioate and Native DNA Sequences. Upon release from the LCAA-CPG support, the aqueous ammonia solution containing the crude DNA sequence **10a−f** and shorter than full-length sequences (Scheme 4) was evaporated to one-half of its original volume. Solid tetra-*n*-butylammonium chloride (TBACl) and the capture support **3** were then sequentially added to the aqueous solution of DNA sequences; the suspension was kept at 65 °C over a period of 3 h. The use of TBACl was intended to exchange the ammonium counterions of thiophosphate or phosphate diester functions with tetraalkylammonium ions with the objective of enhancing the solubility of mostly phosphorothioate DNA sequences in aqueous solvents.

As shown in Figure 1, RP-HPLC analysis of the pre- and postcapture solutions of each DNA sequence (Figures 1A,B and S1A,B, S2A,B, S3A,B, and S4A,B) indicates that the oxidation reaction resulting in the capture of each 5′-functionalized DNA sequence is in all cases near complete. The solid supports **11a−f** were then treated twice with a warm (55 °C) solution of aqueous ammonia in acetonitrile (Scheme 4, step 1) to wash off unbound shorter than full-length DNA sequences by filtration; a final wash with anhydrous DMSO (Scheme 4, step 2) was necessary to ensure efficient release of the DNA sequences **12a−f** from their respective supports (**11a−f**) upon treatment with 1.0 M tetra-*n*-butylammonium fluoride (TBAF) in dry DMSO at 65 °C over a period of 3 h. The DNA sequences were isolated by precipitation in dry THF and characterized by ESI-TOF-MS. The purity of these sequences was evaluated by RP-HPLC and by PAGE under denaturing conditions (Figures 1C and S1C, S2C, S3C, and S4C).

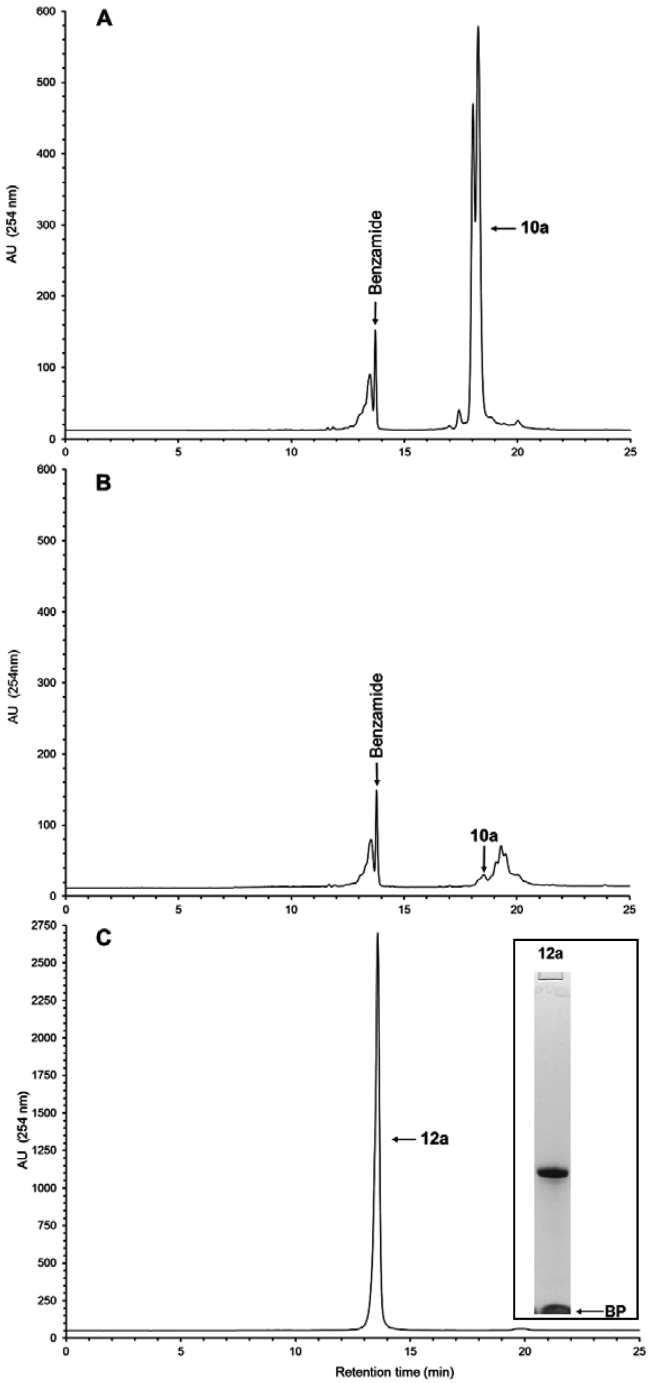

Figure 1. Solid-phase purification of the phosphorothioate DNA sequence **12a**. (A) RP-HPLC profile of unpurified **10a** (5′-functionalized **12a**). (B) RP-HPLC profile of unpurified **10a** after capture by the support **3**. (C) RP-HPLC analysis of solid-phase-purified **12a** that was released from the support **11a**. Inset: Purity analysis of the solid-phase-purified **12a** by PAGE. Chromatographic and electrophoretic conditions are described in the Materials and Methods of the Experimental Section. Abbreviation: BP, bromophenol blue dye.

Although Figures 1C and S1C, S2C, S3C, and S4C show that the purity of solid-phase-purified DNA sequences (**12a−f**) is essentially 100% based on RP-HPLC analyses, PAGE analyses of the same sequences show small amounts of shorter than full-length DNA sequences, indicating that the coupling efficiency of commercial phosphoramidites and that of **9a−d** are less than 100%. The presence of shorter than full-length sequences in trace

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amounts also underscores the affinity of those sequences for silica gel, which makes it difficult to quantitatively wash them off supports **11a–f** before release. The efficiency of the solid-phase purification of DNA sequences was further demonstrated by spiking the 5′-functionalized phosphorothioate DNA sequence **10d** with three synthetic phosphorothioate DNA sequences (14-, 16-, and 18-mers) according to the following ratios: **10d**/14-mer/16-mer/18-mer (5:1:1:1) based on OD_{260} measurements. As shown in Figure 2, the solid-phase purification process was efficient at eliminating shorter than full-length DNA sequences.

Efficiency of DNA Sequence Recovery from the Solid-Phase Purification Process. The most meaningful and reliable approach to measure efficiency of the solid-phase purification of nucleic acid sequences is to use a spectrophotometrically measured amount of a RP-HPLC-purified and desalted 5′-functionalized DNA sequence (**10a**) and subject it to capture by the solid support **3** under the conditions described above. Upon exposure of the solid support **11a** to TBAF and subsequent precipitation of the released DNA sequence under the conditions reported in the Experimental Section, the total amount of **12a**, as measured by UV spectroscopy at 260 nm, is 90% the amount of **10a** used for capture.

In order to assess whether the solid supports **3** and/or **11** did or did not detrimentally affect the quality of the DNA sequence during the capture and release steps of the solid-phase purification process, the purity of **12a** was evaluated by PAGE and compared to that of unpurified **12a** and of **12a** obtained directly from treatment of RP-HPLC-purified **10a** with TBAF under conditions identical to those used for the release of **12a** from **11a**. Figure 3 clearly shows that the purity of **12a** that had been subjected to the solid-phase purification process was highly comparable to the purity of **12a** that had not been in contact with the capture solid support **3**.

Solid-Phase Purification Process Is Highly Parallel and Scalable. With the objective of demonstrating that the solid-phase purification process can be qualified as highly parallel and scalable, the solid-phase synthesis of 10 identical phosphorothioate DNA sequences (**10a**) was carried out, each on a scale of 1 μmol using the conditions described in the Experimental Section. Upon completion of the syntheses, deprotection, and release of each sequence from the CPG support, the ammoniacal solution of each sequence was pooled together and rotoevaporated to dryness under low pressure. The amounts of the capture support **3**, reagents, and solvent required for the capture of **10a** were increased by 10-fold while keeping the final concentration of the reagents the same as that reported for individual syntheses. The capture reaction is performed under conditions identical to those described above (Scheme 3) for individual syntheses in terms of reaction time and temperature. An aliquot of the capture reaction was subjected to RP-HPLC analysis showing, as anticipated, near complete (>98%) disappearance of the DNA sequence **10a** within 3 h (Figure S5A,B). Release of the DNA sequence **12a** from the support **11a** (Figure S5C) was carried out using a 10-fold increase of 1.0 M TBAF/DMSO solution that had been required for a 1 μmol scale reaction while keeping reaction time and temperature conditions the same. A 10-fold increase in the volume of THF is necessary to precipitate **12a**. The yield of the solid-phase-purified DNA sequence was determined by UV spectroscopy at 260 nm and found to be 995 ODs; this yield is nearly proportional to that of solid-phase-purified **12a** (105 ODs) isolated from a 1 μmol process scale. These results conclusively demonstrate that the solid-phase purification of nucleic acid sequences can be achieved in a highly parallel

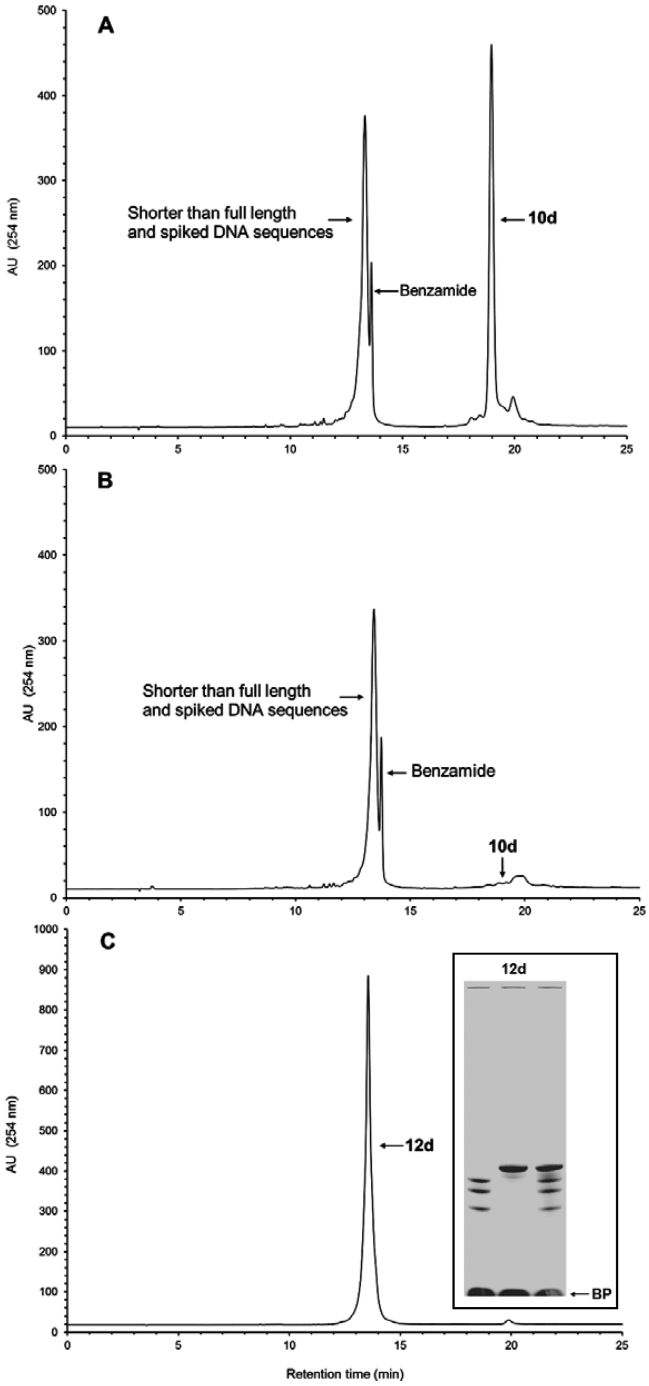

Figure 2. Solid-phase purification of the phosphorothioate DNA sequence **12d**. (A) RP-HPLC profile of unpurified **10d** (5′-functionalized **12d**) spiked with a 14-, 16-, and 18-mer phosphorothioate DNA sequence in a ratio of a 5:1:1:1, respectively. (B) RP-HPLC profile of the phosphorothioate DNA sequence **10d** after capture by the support **3**. (C) RP-HPLC analysis of solid-phase-purified **12d** that was released from the support **11d**. Inset: Purity analysis of the solid-phase-purified **12d** by PAGE. Left lane: solid-phase capture of **10d** spiked with unpurified 14-, 16-, and 18-mer phosphorothioate DNA sequences. Middle lane: solid-phase-purified **12d**. Right lane: unpurified DNA sequence **10d** spiked with unpurified 14-, 16-, and 18-mer phosphorothioate DNA sequences. Chromatographic and electrophoretic conditions are described in the Materials and Methods of the Experimental Section. Abbreviation: BP, bromophenol blue dye.

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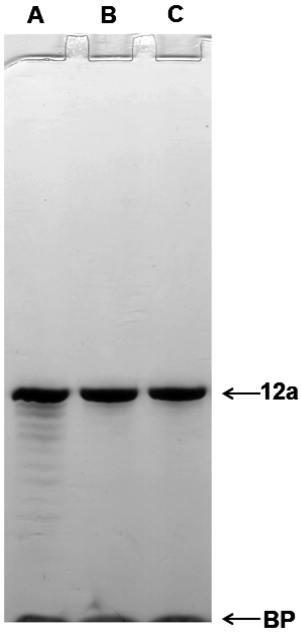

Figure 3. PAGE analysis of the purity of phosphorothioate DNA sequence **12a** recovered from the solid-phase purification process and from the RP-HPLC purification process. (A) Unpurified **12a**. (B) Solid-phase recovery of **12a** obtained from RP-HPLC-purified **10a**. (C) Recovery of **12a** from RP-HPLC-purified **10a** that had been exposed to TBAF. Electrophoretic conditions are described in the Materials and Methods of the Experimental Section. Abbreviation: BP, bromophenol blue dye.

manner and in yields comparable to those obtained from the 1 μmol scale. ESI-TOF-MS analysis of the DNA precipitate revealed a mass consistent with the theoretical molecular weight of the DNA sequence **12a** (see Figure S47).

CONCLUSION

We have functionalized commercial 3-aminopropyl silica gel with aminooxy groups to serve as a capture support for synthetic native and phosphorothioate DNA sequences, each carrying at the 5′-terminus a siloxyl ether linker with a ketone function. The ketone allows the 5′-modified DNA sequences to be captured by the support **3** through an oximation reaction when released from the LCAA-CPG support. The capture process is efficient. Indeed, when a RP-HPLC-purified 5′-modified phosphorothioate DNA sequence was subjected to capture by and released from the support **3**, 90% of the DNA sequence was recovered based on OD_{260} UV measurements. Although the purity of six solid-phase-purified DNA sequences appeared to be 100% by C18-RP-HPLC, the purity of the same sequences by PAGE under denaturing conditions was estimated to be 98%. Such a purity level was adequate for most biological and pharmaceutical applications.

As demonstrated above, the solid-phase purification of multiple DNA sequences can be carried out simultaneously and scaled up without adversely affecting the purity of the sequences. Although RP-HPLC and ion-exchange HPLC procedures were developed for large-scale purification of nucleic acid sequences, those purification methods are associated with prohibitive costs in terms of instrumentation and accessories (e.g., columns and detection systems) in addition to daily operational costs (i.e., elution solvents, buffers, solvent/buffer removal, etc.). Moreover, chromatographic methods are not amenable to a high-throughput mode. Thus, the proposed solid-phase purification process provides a cost-effective high-throughput alternative to conventional chromatographic methods for the purification and production of phosphorothioate and native DNA sequences with a comparable level of purity. The solid-phase purification process is currently being adapted to the purification of (i) nucleobase- and/or sugar-modified DNA sequences, (ii) native and/or phosphorothioate RNA sequences, and (iii) uncharged nucleic acid sequences in our laboratory. Progress in any of these projects will be reported in due course.

EXPERIMENTAL SECTION

Materials and Methods. All reactions sensitive to air and/or moisture were carried out under an atmosphere of argon in dry solvents using oven-dried glassware. Common solvents (acetonitrile, benzene, dichloromethane, chloroform, methanol, 2-propanol, hexane, acetone, ethyl acetate, THF, formamide, DMF, and DMSO), anhydrous solvents (acetonitrile, dichloromethane, THF, DMF, pyridine, and DMSO), deuterated solvents (benzene-d_6 and DMSO-d_6), and chemicals including 7-oxooctanoic acid, 1,1′-carbonyldiimidazole, 3-aminopropyl silica gel, acetic anhydride, 1-methyl imidazole, *O*,*O*′-1,3-propanediylbishydroxylamine dihydrochloride, triethylamine, *N*,*N*-diisopropylethylamine, *N*,*N*′-dimethylethylenediamine, concentrated (28%) aqueous ammonia, 5,5-dimethyldihydrofuran-2-one, imidazole, dichlorodiisopropylsilane, methoxytrimethylsilane, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, tetra-*n*-butylammonium chloride, tetra-*n*-butylammonium fluoride, and anhydrous sodium sulfate were all purchased from commercial sources and used without further purification. Ancillary reagents commonly used in solid-phase DNA synthesis including 5′-*O*- and nucleobase-protected deoxyribonucleosides phosphoramidites, 1*H*-tetrazole, 3*H*-1,2-benzodithiol-3-one, 1,1-dioxide, and a succinylated LCAA-CPG support functionalized with 2′-deoxythymidine as the leader nucleoside were obtained from reputable commercial sources and were dried over fresh P_2O_5 in a desiccator under high vacuum prior to use. Reagents for enzymatic hydrolysis of native DNA sequences including magnesium chloride, Tris-Cl buffer, snake venom phosphodiesterase (*Crotallus adamanteus*), and bacterial alkaline phosphatase (*Escherichia coli*) were all purchased from commercial sources and used as received. Flash chromatography purifications were performed on glass columns (6.0 or 2.5 cm i.d.) packed with silica gel 60 (EMD, 230−400 mesh), whereas analytical thin-layer chromatography analyses were conducted on 2.5 cm × 7.5 cm glass plates coated with a 0.25 mm thick layer of silica gel 60 F_{254} (EMD). Analytical RP-HPLC analyses of silica-gel-purified 5′-functionalized, nucleobase-protected, 2′-deoxyribonucleosides 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 5.0% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min. All other 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 2.5% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min. Triethylammonium acetate buffer (2 M) was purchased from Applied Biosystem and diluted to 0.1 M with HPLC-grade water prior to use. RP-HPLC-purified DNA sequences were desalted using commercial PD-10 (Sephadex G-25M) columns. All NMR experiments were performed using a spectrometer operating at 300.13, 75.47, and 121.5 MHz for one-dimensional ^{1}H, ^{1}H-decoupled ^{13}C, and ^{1}H-decoupled ^{31}P, respectively. Samples were maintained at a temperature of 298 K. All spectra were recorded in deuterated solvents, and chemical shifts δ are reported in parts per million (ppm) relative to appropriate internal references. High-resolution and low-resolution mass spectrometry analyses of new compounds and DNA sequences were performed under contract at a reputable mass spectrometry facility.

Preparation of Solid Support 2. To a solution of 7-oxooctanoic acid (**1**, 0.58 g, 3.0 mmol) in dry THF (4 mL) was added 1,1′-carbonyldiimidazole (CDI, 0.49 g, 3.0 mmol); the solution was stirred for 2 h at 25 °C. Commercial 3-aminopropyl silica gel (1.0 g, ∼1 mmol

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NH_2) was added to the solution after being washed with 20% triethylamine in MeCN (20 mL), filtered, and dried under argon. The suspension was mechanically shaken at 65 °C for 24 h. After filtration, the solid support was washed successively with THF (20 mL) and MeCN (20 mL). The solid support was then suspended over a period of 30 min in a commercial solution (20 mL) of acetic anhydride, 1-methylimidazole, and pyridine in THF to inactivate unreacted amine functions. After filtration, the support was washed with MeCN (2 × 20 mL) and then dried under high vacuum to give the functionalized solid support **2**.

Preparation Solid Support 3. Solid support **2** (1.0 g) was placed in a 10 mL glass vial to which was added a solution of *O*,*O*′-1,3-propanediylbishydroxylamine dihydrochloride (537 mg, 3.00 mmol) in H_2O (4 mL). The glass vial was sealed, and the suspension was mechanically shaken for 16 h at 65 °C. The suspension was filtered, washed with DMF (20 mL) and MeCN (20 mL), and dried under high vacuum to give the solid support **3**, which was stored at −20 °C until needed. The concentration of aminooxy functions covalently attached to **3** was measured according to the following protocol: The support **3** (200 mg) was washed with a solution (2 mL) of Et_3N in MeCN (1:2 v/v) followed by MeCN (10 mL). The air-dried support (20 mg) was then added to a solution (300 μL) of **6c** (60 mg, 0.1 mmol) in DMSO/H_2O (5:1 v/v). The suspension was mechanically agitated at 25 °C over a period of 24 h and filtered. The 4-monomethoxytritylated support was washed with MeCN (3 × 5 mL) and air-dried. Treatment of an accurately measured amount of support with an accurate volume of 3% trichloroacetic acid in CH_2Cl_2 released the yellow-colored 4-methoxytrityl cation, the absorbance of which was spectrophotometrically measured at 478 nm to provide a surface density of 146 ± 7 μmol of aminooxy functions per gram of support **3**.

4-Hydroxy-*N*-methyl-*N*-(2-(methylamino)ethyl)butanamide (5a). *N*,*N*′-Dimethylethylenediamine (3.25 g, 40.0 mmol) and commercial γ-butyrolactone (**4a**, 1.72 g, 20.0 mmol) were placed in a 25 mL glass vial, which was sealed and heated at 90 °C for 16 h. The reaction mixture was then rotoevaporated to an oil under reduced pressure. The oily material was loaded on the top of a glass column packed with silica gel (~40 g) pre-equilibrated in $CHCl_3$/MeOH (9:1 v/v). The product was eluted from the column using a gradient of MeOH (10 → 20%) in $CHCl_3$ to afford **5a** (2.32 g, 14.4 mmol) as an oil in a yield of 72%: ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.39 (dt, *J* = 6.6, 2.0 Hz, 2H), 3.32 (dt, *J* = 6.6, 2.0 Hz, 2H), 2.95 (s, 1.5H), 2.79 (s, 1.5H), 2.59 (t, *J* = 6.6 Hz, 1H), 2.53 (t, *J* = 6.6 Hz, 1H), 2.34 (t, *J* = 7.4 Hz, 1H), 2.29 (t, *J* = 7.4 Hz, 1H), 2.28 (s, 1.5H), 2.25 (s, 1.5H), 1.63 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 171.92, 171.91, 60.3, 49.7, 49.0, 48.9, 46.6, 36.2, 36.0, 35.4, 33.1, 29.3, 28.6, 28.3, 28.0; +ESI-TOF-HRMS calcd for $C_8H_{18}N_2O_2$ $[M + H]^+$ 175.1400, found 175.1405.

4-Hydroxy-*N*,4-dimethyl-*N*-(2-(methylamino)ethyl)-pentanamide (5b). The preparation of **5b** was performed at the same scale and conditions used for the preparation of **5a** with the exception of replacing **4a** with commercial 5,5-dimethyldihydrofuran-2-one (**4b**, 2.28 g, 20.0 mmol). The purification of **5b** was carried out as described above for **5a** and isolated as an oil (2.99 g, 14.8 mmol) in a yield of 74%: ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.23 (br s, 1H), 3.33 (q, *J* = 6.6 Hz, 2H), 2.97 (s, 1.5H), 2.79 (s, 1.5H), 2.61 (t, *J* = 6.6 Hz, 1H), 2.53 (t, *J* = 6.6 Hz, 1.5H), 2.37−2.27 (m, 3H), 2.28 (s, 1.5H), 2.25 (s, 1.5H), 1.57 (m, 2H), 1.08 (s, 3H), 1.07 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.5, 172.4, 79.1, 49.7, 49.0, 48.9, 46.6, 38.7, 38.3, 36.2, 35.9, 33.1, 28.9, 28.1, 27.4; +ESI-TOF-HRMS calcd for $C_{10}H_{22}N_2O_2$ $[M + H]^+$ 203.1754, found 203.1763.

***N*-(2-(4-Hydroxy-*N*-methylbutanamido)ethyl)-*N*-methyl-7-oxooctanamide (6a).** To a solution of 7-oxooctanoic acid (**1**, 2.45 g, 15.5 mmol) in dry THF (15 mL) was added 1,1′-carbonyldiimidazole (2.52 g, 15.5 mmol). The solution was stirred for 2 h at 25 °C, and upon addition of **5a** (2.32 g, 14.4 mmol), the reaction mixture was allowed to stir at 65 °C for 24 h. The solution was rotoevaporated under reduced pressure; the material left was dissolved in $CHCl_3$ (40 mL) and vigorously mixed with water (20 mL). The organic phase was collected and rotoevaporated to dryness under low pressure. The crude product was then dissolved in a minimal volume of $CHCl_3$ (4 mL) and loaded on the top of a glass column packed with silica gel (~40 g) pre-equilibrated in $CHCl_3$. The product **6a** was eluted from the column using a gradient of MeOH (0 → 4%) in $CHCl_3$. Pure **6a** was isolated as an oil (3.57g, 11.3 mmol) in a yield of 78%: ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.44 (t, *J* = 5 Hz, 1H), 3.46 (s, 0.7H), 3.39 (s, 3.5H), 2.97 (s, 0.7H), 2.96 (s, 0.8H), 2.92 (s, 1.1H), 2.91 (s, 1.1H), 2.81 (m, 2.1H), 2.40 (t, *J* = 7.3 Hz, 2H), 2.32−2.15 (m, 4H), 2.07 (s, 3H), 1.62 (m, 2H), 1.45 (m, 4H), 1.22 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.5, 172.4, 172.3, 172.2, 172.1, 172.0, 171.9, 171.86, 171.81, 60.3, 60.24, 60.20, 47.3, 46.5, 46.4, 45.8, 45.6, 44.2, 42.6, 35.9, 35.8, 35.1, 33.5, 33.4, 33.12, 33.08, 32.4, 32.3, 31.4, 29.7, 29.3, 29.2, 28.4, 28.3, 28.28, 28.20, 28.0, 27.9, 24.7, 24.6, 24.31, 24.26, 23.1; +ESI-TOF-HRMS calcd for $C_{16}H_{30}N_2O_4Cs$ $[M + Cs]^+$ 447.1255, found 447.1258.

***N*-(2-(4-Hydroxy-*N*,4-dimethylpentanamido)ethyl)-*N*-methyl-7-oxooctanamide (6b).** The preparation of **6b** was performed at the same scale and conditions used for the preparation of **6a** with the exception of replacing **5a** with **5b** (2.91 g, 14.4 mmol). The purification of **6b** was carried out as described above for **6a** and isolated as an oil (3.97 g, 11.6 mmol) in a yield of 80%: ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.22 (m, 1H), 3.47 (s, 0.4H), 3.39 (m, 3H), 3.33 (s, 0.4H), 2.98 (s, 0.6H), 2.96 (s, 0.6H), 2.94 (s, 1H), 2.91 (s, 1H), 2.82 (d, *J* = 2.1 Hz, 0.6H), 2.80 (s, 1H), 2.40 (t, *J* = 7.3 Hz, 2H), 2.33−2.21 (m, 3H), 2.17 (t, *J* = 7.3 Hz, 0.8H), 2.06 (s, 3H), 1.56−1.52 (m, 1.8H), 1.50−1.38 (m, 4.2H), 1.22 (m, 2H), 1.08 (s, 1.4H), 1.06 (s, 3.8H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.4, 172.9, 172.7, 172.5, 172.4, 172.2, 172.0, 171.8, 171.7, 79.1, 68.25, 68.21, 47.3, 47.2, 46.6, 46.4, 45.8, 45.6, 44.1, 44.0, 42.6, 38.7, 38.2, 36.0, 35.8, 35.1, 35.0, 33.40, 33.37, 33.05, 33.01, 32.4, 32.3, 31.4, 29.6, 29.2, 28.4, 28.3, 28.2, 28.14, 28.06, 27.2, 24.7, 24.6, 24.3, 24.2, 23.1; +ESI-TOF-HRMS calcd for $C_{18}H_{34}N_2O_4$ $[M + H]^+$ 343.2591, found 343.2623.

***N*-(2-(4-((4-Methoxyphenyl)diphenylmethoxy)-*N*-methylbutanamido)ethyl)-*N*-methyl-7-oxooctanamide (6c).** To a solution of **6a** (628 mg, 2.00 mmol) in dry pyridine was added 4-methoxytrityl chloride (927 mg, 3.00 mmol). The reaction mixture was stirred at 25 °C over a period of 4 h. The reaction was then quenched by adding water (5 mL) and subjected to extraction using $CHCl_3$. Upon phase separation, the organic phase was collected, dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated under reduced pressure to an oil. The oily material was loaded on the top of a glass column packed with silica gel (~40 g) pre-equilibrated in a solution of $CHCl_3/C_5H_5N$ (99.5:0.5 v/v). The reaction product was eluted from the column using a gradient of 0 → 2% MeOH in $CHCl_3/C_5H_5N$ (99.5:0.5 v/v). Fractions containing the product were collected and rotoevaporated to dryness under low pressure. The material left was coevaporated with toluene (3 × 5 mL) to remove residual C_5H_5N. Pure **6c** was isolated as an oil (1.01 g, 1.7 mmol) in a yield of 85%: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.39−2.29 (m, 8H), 7.25−7.20 (m, 4H), 6.89 (d, *J* = 8.8 Hz, 2H), 3.74 (s, 3H), 3.42 (s, 0.5H), 3.36 (s, 2H), 2.98 (m, 2H), 2.93 (s, 0.7H), 2.90 (s, 0.8H), 2.88 (s, 1H), 2.87 (s, 1H), 2.79 (m, 2H), 2.35 (m, 3H), 2.26−2.12 (m, 3H), 2.04 (s, 1H), 2.03 (s, 1H), 2.02 (s, 1H), 1.77 (m, 2H), 1.42 (m, 4H), 1.19 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.3, 172.1, 172.0, 171.9, 171.7, 171.6, 171.5, 171.4, 158.1, 144.59, 144.57, 135.4, 129.8, 128.8, 128.1, 127.9, 127.8, 126.7, 125.2, 113.1, 85.5, 62.6, 62.5, 62.4, 55.0, 47.3, 47.2, 46.4, 46.3, 45.8, 45.6, 44.13, 44.10, 42.6, 35.9, 35.8, 35.1, 35.0, 33.4, 33.3, 33.0, 32.34, 32.28, 31.4, 29.58, 29.56, 29.3, 29.2, 28.38, 28.35, 28.3, 28.2, 25.4, 25.1, 24.7, 24.5, 24.3, 24.2, 23.1, 21.0; +ESI-TOF-HRMS calcd for $C_{36}H_{46}N_2O_5Cs$ $[M + Cs]^+$ 719.2456, found 719.2458.

General Procedure for the Preparation of 5′-Functionalized Deoxyribonucleosides (8a−d). To **6b** (780 mg, 2.28 mmol) and imidazole (184 mg, 2.70 mmol) in a flame-dried 25 mL flask were added under argon dry DMF (5 mL) and *N*,*N*-diisopropylethylamine (2.35 mL,13.5 mmol); the solution was then cooled to 0 °C. Dichlorodiisopropylsilane (730 μL, 4.50 mmol) was added to the cold solution, which was left stirring for 1 h at 0 °C. The reaction mixture was allowed to warm to room temperature over a period of 4 h and then cooled to −60 °C. A solution of deoxyribonucleoside **7a** (1.31 g, 5.40 mmol) and imidazole (368 mg, 5.40 mmol) in dry DMF (5 mL) was added dropwise to the reaction mixture, which was kept stirring at −60 °C for 1 h. The reaction was then allowed to warm to 0 °C and was left stirring for 3 h at the same temperature. The reaction mixture was quenched by the

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J. Org. Chem. 2016, 81, 6165−6175

addition of cold (0 °C) 5% aq $NaHCO_3$ (40 mL) and EtOAc (40 mL); after vigorous shaking, the organic layer was collected and rotoevaporated to dryness under low pressure. The crude product was dissolved in a minimal volume of $CHCl_3$ (4 mL) and loaded on the top of a glass column packed with silica gel (~40 g) pre-equilibrated in $CHCl_3$. The product **8a** was eluted from the column using a gradient of MeOH (0 → 6%) in $CHCl_3$. Pure **8a** (1.32 g, 1.59 mmol) was isolated as a solid in a yield of 70%. The 5′-functionalized deoxyribonucleosides **8b–d** were similarly prepared, purified, and isolated as oily materials in yields in the range of 50–68%.

8a: ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.23 (s, 1H), 8.74 (s, 1H), 8.62 (s, 0.7H), 8.59 (s, 0.3H), 8.32 (s, 1H), 8.07 (d, J = 7.8 Hz, 2H), 7.65 (dd, J = 7.6, 7.4 Hz, 1H), 7.56 (dd, J = 7.6, 7.4 Hz, 2H), 6.51 (t, J = 6.8 Hz, 1H), 5.45 (m, 1H), 4.54 (br s, 1H), 3.98 (m, 2H), 3.85 (m, 1H), 3.38 (m, 4H), 2.92 (m, 4H), 2.79 (m, 2H), 2.38 (t, J = 7.3 Hz, 2H), 2.31–2.13 (m, 4H), 2.05 (s, 3H), 1.66 (m, 2H), 1.43 (m, 4H), 1.21 (m, 7H), 0.97 (m, 16H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.3, 172.4, 172.2, 172.1, 172.0, 171.9, 171.8, 171.71, 171.66, 165.6, 151.9, 151.4, 150.3, 143.0, 142.8, 133.4, 132.4, 128.5, 128.4, 125.9, 125.8, 87.2, 83.74, 83.66, 79.2, 73.2, 73.1, 70.1, 70.4, 68.3, 68.2, 62.9, 62.7, 47.2, 46.5, 46.4, 45.8, 45.7, 44.1, 44.0, 42.6, 38.5, 35.9, 35.8, 35.0, 33.5, 33.3, 33.2, 33.0, 32.4, 32.3, 31.5, 29.6, 29.3, 29.2, 28.4, 28.3, 28.2, 28.1, 28.0, 27.3, 27.1, 24.7, 24.6, 24.3, 24.2, 23.1, 17.60, 17.52, 17.47, 17.43, 17.3, 17.21, 17.18, 17.11, 17.0, 12.9, 12.7, 12.6, 12.32, 12.29; +ESI-TOF-HRMS calcd for $C_{41}H_{63}N_7O_8SiNa$ $[M + Na]^+$ 832.4399, found 832.4411. **8b:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.25 (s, 1H), 8.27 (d, J = 7.3 Hz, 1H), 8.01 (d, J = 7.3 Hz, 2H), 7.63 (m, 1H), 7.52 (m, 2H), 7.37 (d, J = 7.3 Hz, 1H), 6.16 (t, J = 6.2 Hz, 1H), 5.37 (d, J = 4.4 Hz, 1H), 4.28 (m, 1H), 4.01–3.89 (m, 3H), 3.47 (s, 0.4H), 3.38 (m, 3H), 2.98 (s, 0.7H), 2.95 (s, 0.7H), 2.94 (s, 1.1H), 2.90 (s, 1.1H), 2.81 (m, 2H), 2.38 (m, 5H), 2.23 (t, J = 7.3 Hz, 2H), 2.15 (m, 2H), 2.05 (s, 3H), 1.70 (m, 2H), 1.43 (m, 4H), 1.27 (s, 6H), 1.20 (m, 2H), 1.02 (m, 14H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.29, 208.27, 172.4, 172.2, 172.0, 171.9, 171.8, 171.70, 171.66, 167.3, 163.0, 154.3, 144.3, 133.1, 132.7, 128.41, 128.38, 95.8, 87.1, 86.0, 73.34, 73.30, 69.3, 62.3, 47.3, 47.2, 46.5, 46.4, 45.8, 45.7, 44.1, 44.0, 42.6, 40.8, 35.9, 35.8, 35.1, 35.0, 33.5, 33.3, 33.2, 33.0, 32.4, 32.3, 31.5, 29.6, 29.39, 29.37, 28.4, 28.3, 28.2, 28.13, 28.07, 27.3, 27.1, 24.7, 24.6, 24.31, 24.25, 23.1, 17.61, 17.59, 17.51, 17.48, 12.8, 12.6; +ESI-TOF-HRMS calcd for $C_{40}H_{63}N_5O_9SiNa$ $[M + Na]^+$ 808.4287, found 808.4298. **8c:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.07 (s, 1H), 11.68 (s, 1H), 8.16 (s, 1H), 6.22 (t, J = 6.6 Hz, 1H), 5.37 (d, J = 4.2 Hz, 1H), 4.42 (m, 1H), 3.88 (d, J = 7.8 Hz, 1H), 3.83 (m, 2H), 3.44–3.34 (m, 4H), 2.95 (s, 0.8H), 2.94 (s, 0.8H), 2.91 (s, 1.2H), 2.89 (s, 1.2H), 2.77 (m, 3H), 2.63 (m, 2H), 2.38 (t, J = 7.3 Hz, 2H), 2.33–2.13 (m, 5H), 2.05 (s, 3H), 1.66 (m, 2H), 1.43 (m, 4H), 1.21 (m, 7H), 1.12 (d, J = 6.8 Hz, 6H), 0.95 (m, 14H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.3, 180.1, 172.4, 172.2, 172.1, 172.0, 171.9, 171.8, 171.69, 171.66, 154.8, 148.3, 148.0, 137.2, 128.3, 120.3, 87.1, 82.8, 73.2, 73.1, 70.0, 62.9, 47.3, 47.2, 46.5, 46.3, 45.7, 45.6, 44.1, 44.0, 42.6, 35.9, 35.8, 35.05, 35.02, 34.7, 33.5, 33.3, 33.2, 33.0, 32.4, 32.3, 31.4, 29.6, 29.3, 28.4, 28.3, 28.2, 28.1, 28.0, 27.2, 27.1, 24.7, 24.5, 24.3, 24.2, 23.1, 18.83, 18.78, 17.55, 17.52, 17.48, 17.43, 17.1, 12.7, 12.6; +ESI-TOF-HRMS calcd for $C_{38}H_{65}N_7O_9SiNa$ $[M + Na]^+$ 814.4505, found 814.4518. **8d:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.33 (s, 1H), 7.42 (s, 1H), 6.17 (t, J = 6.8 Hz, 1H), 5.31 (d, J = 4.5 Hz, 1H), 4.25 (m, 1H), 3.85 (m, 3H), 3.46 (s, 0.4H), 3.39 (s, 2 H), 3.36 (m, 1.1H), 2.97 (s, 0.8H), 2.95 (s, 0.7H), 2.92 (s, 1H), 2.90 (s, 1H), 2.80 (m, 2H), 2.39 (t, J = 7.3 Hz, 2H), 2.13 (m, 1H), 2.22 (m, 2H), 2.12 (m, 3H), 2.06 (s, 3H), 1.76 (s, 3H), 1.67 (m, 2H), 1.44 (m, 4H), 1.21 (m, 8H), 1.00 (m, 13H), 0.95 (s, 1H), 0.93 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.3, 172.4, 172.2, 172.0, 171.9, 171.8, 171.71, 171.67, 163.1, 150.3, 135.6, 109.4, 86.5, 83.6, 73.3, 73.2, 70.0, 62.8, 47.3, 47.2, 46.5, 46.4, 45.8, 45.7, 44.1, 44.0, 42.6, 35.9, 35.8, 35.1, 35.0, 33.5, 33.3, 33.1, 33.0, 32.4, 32.3, 31.5, 29.6, 29.4, 28.4, 28.3, 28.2, 28.1, 28.0, 27.3, 27.1, 24.7, 24.6, 24.3, 24.2, 23.1, 17.60, 17.56, 17.50, 17.48, 17.3, 12.8, 12.63, 12.59, 12.7; +ESI-TOF-HRMS calcd for $C_{34}H_{60}N_4O_9SiNa$ $[M + Na]^+$ 719.4022, found 719.4031.

General Procedure for the Preparation of Nucleoside Phosphoramidites (9a–d). A solution of **8a** (897 mg, 1.00 mmol) in CH_2Cl_2 (10 mL) was placed in a flame-dried 100 mL round-bottom flask. *N*,*N*-Diisopropylethylamine (536 μL, 3.00 mmol) was added to the solution and followed by 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (335 μL,1.50 mmol). The reaction mixture was stirred at 25 °C for 3 h, quenched by the addition of H_2O (10 mL), and diluted with CH_2Cl_2 (25 mL). After vigorous shaking, the organic layer was collected, dried over anhydrous Na_2SO_4, and rotoevaporated under reduced pressure to afford an oil. The oily material was evenly spread on the top of a column packed with silica gel (~25 g) pre-equilibrated in benzene/Et_3N (9:1 v/v). The product was eluted from the column using benzene/Et_3N (9:1 v/v) as the eluent. Fractions containing the product were pooled together, concentrated under reduced pressure, and dissolved in dry benzene (7 mL). The solution was frozen and then lyophilized under high vacuum to afford triethylamine-free **9a** (750 mg, 0.83 mmol) as a colorless oil in 83% yield. The 5′-functionalized deoxyribonucleoside phosphoramidites **9b–d** were similarly prepared and purified. Triethylamine-free **9b–d** were isolated as colorless oils, the yields of which were in the range of 75–80%.

9a: ^{31}P NMR (121 MHz, C_6D_6) δ 149.0, 148.8, 148.76, 148.72; +ESI-TOF-HRMS calcd for $C_{50}H_{80}N_9O_9PSi$ $[M + H]^+$ 1010.5659, found 1010.5675. **9b:** ^{31}P NMR (121 MHz, C_6D_6) δ 148.3, 148.25, 148.22, 148.1, 147.91, 147.89; +ESI-TOF-HRMS calcd for $C_{49}H_{80}N_7O_{10}PSi$ $[M + H]^+$ 986.5546, found 986.5548. **9c:** ^{31}P NMR (121 MHz, C_6D_6) δ 148.77, 148.73, 148.0, 147.92, 147.88; +ESI-TOF-HRMS calcd for $C_{47}H_{82}N_9O_{10}PSi$ $[M + H]^+$ 992.5764, found 992.5773. **9d:** ^{31}P NMR (121 MHz, C_6D_6) δ 148.7, 148.6, 148.49, 148.46, 148.37; +ESI-TOF-HRMS calcd for $C_{43}H_{77}N_6O_{10}PSiCs$ $[M + Cs]^+$ 1029.4257, found 1029.4266.

Solid-Phase Synthesis and Deprotection of the DNA Sequences 10a–f. Solid-phase synthesis of the phosphorothioate DNAsequences **10a** [5′-d($A_{PS}C_{PS}A_{PS}C_{PS}T_{PS}G_{PS}T_{PS}G_{PS}A_{PS}A_{PS}T_{PS}C_{PS}G_{PS}A_{PS}T_{PS}G_{PS}C_{PS}C_{PS}A_{PS}T$)], **10b** [5′-d($C_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}C_{PS}C_{PS}T_{PS}T_{PS}A_{PS}C_{PS}G_{PS}T_{PS}C_{PS}T_{PS}T_{PS}G_{PS}T$)], **10c** [5′-d($G_{PS}T_{PS}G_{PS}A_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}G_{PS}A_{PS}A_{PS}C_{PS}G_{PS}T_{PS}G_{PS}A_{PS}A_{PS}G_{PS}T$)], **10d** [5′-d($T_{PS}A_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}T_{PS}A_{PS}A_{PS}C_{PS}G_{PS}T_{PS}C_{PS}A_{PS}G_{PS}T$)], **10e** [5′-d($A_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PC_PA_PT$)], and **10f** [5′-d($T_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PA_PA_PT_PT_PG_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PC_PA_PT_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PC_PA_PT$)] was conducted on a scale of 1 μmol using a succinyl long-chain alkylamine controlled-pore glass (500 Å LCAA-CPG or 2000 Å LCAA-CPG for **10f**) support functionalized with 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxythymidine as the leader nucleoside. The syntheses were carried out using a DNA/RNA synthesizer and commercial 5′-*O*-(4,4′-dimethoxytrityl)-dA^{Bz}, -dG^{iBu}, -dC^{Bz}, -dT phosphoramidite monomers, which were each dissolved in dry MeCN to give a 0.1 M solution. Each modified deoxyribonucleoside phosphoramidite **9a–d** was dissolved in dry MeCN to provide a 0.15 M solution. Each solution was placed in a vial connected to the DNA/RNA synthesizer through an additional delivery port. Commercial 1*H*-tetrazole solution was used for phosphoramidite activation in the solid-phase synthesis of **10a–f**. The reaction times for the coupling, capping, and oxidation steps in the synthesis of native and phosphorothioate DNA sequences were 120, 60, and 60 s, respectively. It should, however, be noted that the capping step in the synthesis of phosphorothioate DNA sequences was performed after the oxidative sulfuration step, which was effected using 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN; the standard 0.02 M iodine solution in THF/pyridine/water was employed in the oxidation step of native DNA sequences. The last coupling reaction of each synthesis was performed using any of the activated deoxyribonucleoside phosphoramidites **9a–d** over a period of 180 s. The LCAA-CPG-linked DNA sequence was then transferred to a 4 mL glass vial, to which was added concentrated aq ammonia (1 mL). The tightly capped glass vial was placed in a heat block and kept at 65 °C for 16 h. The ammoniacal solution was transferred to another 4 mL glass vial and evaporated to one-half of its original volume using a stream of air.

Solid-Phase Capture of the DNA Sequences. The amino-oximated solid support **3** (150 mg) was washed with 20% triethylamine in MeCN (1 mL), filtered, blow-dried under argon, and placed into a 1 mL glass vial. The above solution of unpurified 5′-functionalized phosphorothioate or native DNA sequence **10a** or **10e** (~500 μL) was

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J. Org. Chem. 2016, 81, 6165–6175

added to the glass vial along with tetra-*n*-butylammonium chloride (14 mg, 50 μmol); the solid-phase capture of the DNA sequence **10a** or **10e** was carried out over a period of 3 h at 65 °C. Near complete capture was achieved upon oximation of the DNA sequence mediated by support **3** to produce the silica gel support **11a** or **11e**. RP-HPLC analysis of the capture reaction mixture confirmed the near absence of the DNA sequence **10a** or **10e** (see Figure 1B or Figure S3B). Solid-phase capture of the DNA sequences **10b−d** and **10f** was performed as described for **10a**. RP-HPLC analysis of the capture reaction mixtures also confirmed the near absence of the DNA sequences **10b−d** and **10f** (see Figures 2B and S1B, S2B, and S4B), thereby indicating essentially complete oximation of those sequences by the support **3**.

Release of the DNA Sequences from the Supports 11a−f. The supports **11a−f** were placed in a 4 mL screw-capped glass vial to which was added a solution (1 mL) of concentrated aq NH_3/MeCN (1:1 v/v); the glass vial was heated at 65 °C for 30 min and then subjected to filtration. This wash step was repeated once more under identical conditions and was followed by five DMSO washes (1 mL each). Release of the purified DNA sequence from **11a−f** was effected by treatment with 1.0 M TBAF in dry DMSO (0.5 mL) in a sealed glass vial kept at 65 °C over a period of 3 h. Methoxytrimethylsilane (200 μL) and MeCN (200 μL) were added to the suspension, which was kept at 25 °C for 30 min and was subsequently filtered through a scintered glass funnel; the filtrate was collected. The solid support was suspended in a solution (1 mL) of concentrated aq NH_3/MeCN (1:1 v/v), heated at 65 °C for 30 min, and filtered again through a glass-scintered funnel; the filtrate was collected and combined with the previous filtrate. The process was repeated once more under identical conditions. All collected filtrates were pooled together and concentrated under vacuum to approximately 100 μL. THF (1 mL) was added to the concentrated solution in order to precipitate the DNA sequences **12a−f**. The precipitate was centrifuged at 14 000*g* for 15 min at 25 °C; the supernatant was then carefully removed by suction. The DNA pellet was washed with THF (3 × 1 mL); the pure DNA sequences **12a−f** were dried under reduced pressure and stored at −20 °C until further use. The DNA sequences **12a−f** were characterized by mass spectrometry, and their purity was analyzed by both RP-HPLC and PAGE.

12a: 5′-d($A_{PS}C_{PS}A_{PS}C_{PS}T_{PS}G_{PS}T_{PS}G_{PS}A_{PS}A_{PS}T_{PS}C_{PS}G_{PS}A_{PS}T_{PS}G_{PS}C_{PS}C_{PS}A_{PS}$T); −ESI-TOF-MS calcd 6406, found, 6407. **12b:** 5′-d($C_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}C_{PS}C_{PS}T_{PS}T_{PS}A_{PS}C_{PS}G_{PS}T_{PS}C_{PS}T_{PS}T_{PS}G_{PS}$T); −ESI-TOF-MS calcd 6315, found, 6315. **12c:** 5′-d($G_{PS}T_{PS}G_{PS}A_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}G_{PS}A_{PS}A_{PS}C_{PS}G_{PS}T_{PS}G_{PS}A_{PS}A_{PS}G_{PS}$T); −ESI-TOF-MS calcd 6551, found, 6551. **12d:** 5′-d($T_{PS}A_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}T_{PS}A_{PS}A_{PS}C_{PS}G_{PS}T_{PS}C_{PS}A_{PS}G_{PS}$T); −ESI-TOF-MS calcd 6397, found, 6397. **12e:** 5′-d($A_PC_PA_PC_PT_PG_PT_PG_PA_PA_PT_PC_PG_PA_PT_PG_PC_PC_PA_P$T); −ESI-TOF-MS calcd 6101, found, 6101.

PAGE Analysis of the Solid-Phase-Purified DNA Sequences 12a−f. An aqueous solution (0.25 OD_{260}) of each solid-phase-purified phosphorothioate and native DNA sequence, in a 1.5 mL microcentrifuge tube, was evaporated to dryness under reduced pressure. To each tube was added 10 μL of loading buffer [10× Tris borate EDTA buffer (TBE), pH 8.3, in formamide 1:4 (v/v) containing 2 mg/mL of bromphenol blue]. The solution was then vigorously vortexed, centrifuged, and loaded into a 2 cm wide well of a 20 × 40 cm, 7 M urea/20% polyacrylamide gel (**12a−e**) or 7 M urea/18% polyacrylamide gel (**12f**). Electrophoresis was performed at 375 V using 1× TBE buffer, pH 8.3, as an electrolyte until the bromphenol blue dye traveled ∼80% the length of the gel. The electrophoresis apparatus was dismantled and the gel immersed in a solution (250 mL) composed of *i*-PrOH/H_2O/formamide (2:8:0.4 v/v/v) to which was added a 1 mg/mL Stains-All solution (10 mL) in formamide. The gel was agitated for 3−4 h in the dark, whereupon the staining solution was discarded and the gel washed with distilled water (3 × 250 mL). The gel was then exposed to natural light until the disappearance of the purple background and scanned. The DNA sequences appeared as blue (**12e,f**) or purple bands (**12a−d**) against a white background. The results are shown in Figures 1, 2, and S1−S4.

Enzymatic Hydrolysis of the Native DNA Sequences 12e−f. One OD_{260} unit of an aqueous solution of each of the solid-phase-purified and desalted native DNA sequences **12e** and **12f** was pipetted into separate microcentrifuge tubes. Each DNA solution was evaporated to dryness under reduced pressure, whereupon 1.0 M Tris-Cl buffer, pH 9.0 (6 μL), 1.0 M $MgCl_2$ (8 μL), and water (75 μL) were added followed by, after mixing, snake venom phosphodiesterase (*Crotallus adamanteus*, 0.015 U, 5 μL) and bacterial alkaline phosphatase (*E. coli*, 0.7 U, 6 μL). The enzymatic reactions were allowed to proceed at 37 °C for 16 h. Deactivation of the enzymes was carried out by heating the digests at 90 °C for 3 min; the digests were then centrifuged at 14 000*g* for 5 min at 25 °C. Immediately after centrifugation, an aliquot (50 μL) of each digest was analyzed by RP-HPLC 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 2.5% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min. RP-HPLC chromatograms of the digests are shown in Figures S6 and S7.

Determination of the Efficiency of the Solid-Phase Purification Process. To a RP-HPLC-purified and desalted DNA sequence (**10a**, 70 OD_{260}) in a 1 mL glass vial was added a 0.1 M solution of tetra-*n*-butylammonium chloride in H_2O (150 μL) and support **3** (70 mg). The suspension was processed as described above for the solid-phase purification of unpurified **10a** to give **12a** after release from **11a** and precipitation in THF. The efficiency of the solid-phase purification process was determined from the following equation: (OD_{260} of the DNA sequence **12a** recovered after the solid-phase purification process/OD_{260} of the DNA sequence **10a** before solid-phase purification) × 100, that is, (63 OD_{260}/70 OD_{260})100 = 90%.

Ten-fold Scale up of the Solid-Phase Purification of the Phosphorothioate DNA Sequence 12a. Ten individual solid-phase syntheses of the phosphorothioate DNA sequence **10a** were each conducted on a scale of 1 μmol under conditions identical to those described above for the DNA sequences **10a−d**. Upon completion of the syntheses, each individual DNA sequence was subjected to a standard deprotection and release from the CPG support protocol under basic conditions. Upon complete deprotection and release of each DNA sequence from the CPG support, the individual ammoniacal solutions of the 10 DNA sequences were pooled together and rotoevaporated to one-half their original volume (∼5 mL) under reduced pressure. A 0.1 M solution of tetra-*n*-butylammonium chloride in DMSO/H_2O (1:1 v/v) (5.0 mL) was added to the crude DNA solution. This solution was then added to a 20 mL glass vial containing the aminooximated solid support **3** (1.50 g), which had previously been washed with 20% triethylamine in MeCN (10 mL), filtered, and blow-dried under argon; the glass vial was tightly sealed and mechanically agitated over a period of 3 h at 65 °C. An aliquot (0.5 μL) of the capture reaction mixture was analyzed by RP-HPLC, which has only revealed the presence of benzamide and shorter than full-length DNA sequences relative to a similar analysis performed prior to the capture of the crude DNA sequence **10a** (Figure S5A,B). The solid support **11a** was suspended in a solution of 10% Et_3N in MeCN/H_2O (1:1 v/v) (10 mL) and heated at 65 °C for 30 min, whereupon the suspension was filtered through a glass-scintered funnel. This process was repeated twice under identical conditions and was followed by multiple anhydrous DMSO (5 × 10 mL) washes. Release of the purified DNA sequence from **11a** was effected by treatment with 1.0 M TBAF in dry DMSO (5 mL) in a sealed 20 mL glass vial kept at 65 °C over a period of 3 h. Methoxytrimethylsilane (2 mL) and MeCN (2 mL) were added to the reaction mixture, which was allowed to stand at 25 °C for 30 min in order to consume unreacted fluoride ions. The suspension was filtered; the solid support was suspended in a solution of 10% Et_3N in MeCN/H_2O (1:1 v/v) (10 mL), heated at 65 °C for 30 min, and then filtered through a glass-scintered funnel. This process was repeated once more under identical conditions. All the postrelease filtrates were pooled together and rotoevaporated under vacuum until approximately 1 mL of the original volume was left. THF (20 mL) was then added to the filtrate; the DNA precipitate was centrifuged, and the supernatant was carefully removed by suction. The DNA was washed with THF (3 × 10 mL) and subjected to identical centrifugation and supernatant removal conditions. The pure nucleic acid sequence **12a** was dried under reduced pressure and stored at −20 °C. The solid-phase scale-up purification of **12a** is presented in Figure S5C,D.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01020.

RP-HPLC chromatograms and PAGE electropherograms of solid-phase-purified DNA sequences; ^{1}H, ^{13}C, and ^{31}P NMR spectra of all new compounds; ESI-HRMS and ESI-MS spectra of all new compounds; and photograph of the precipitated phosphorothioate DNA sequence **12a** (PDF)

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Notes

The authors declare no competing financial interest.

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