The 4-[*N*-Methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl Group as an Alternative to the 2-Cyanoethyl Group for Phosphate Protection in the Synthesis of Oligodeoxyribonucleotides

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The 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl group for phosphate protection in the synthesis of oligodeoxyribonucleotides has been developed to completely prevent nucleobase alkylation by acrylonitrile that could potentially occur upon deprotection of the traditional 2-cyanoethyl phosphate protecting group. The properties of this new phosphate protecting group were evaluated using the model phosphotriester **9**. The mechanism of phosphate deprotection was studied by treating **9** with concentrated NH_4OH. NMR analysis of the deprotection reaction demonstrated that cleavage of the *N*-trifluoroacetyl group is rate-limiting. The resulting phosphotriester intermediate **13** was also shown to undergo rapid cyclodeesterification to produce *O*,*O*-diethyl phosphate **15** and *N*-methylpyrrolidine **16** (Scheme 2). Given the facile removal of the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting group under mild basic conditions, its utilization in oligonucleotide synthesis began with the preparation of the deoxyribonucleoside phosphoramidites **4a**−**d** (Scheme 3). The coupling efficiency of **4a**−**d** and conventional 2-cyanoethyl deoxyribonucleoside phosphoramidites **24a**−**d** was then compared in the solid-phase synthesis of the 20-mer d(ATCCGTAGCTAAGGTCATGC). As previously observed in the deprotection of **9**, the 4-[*N*-methyl,*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting groups were easily and completely removed from the oligonucleotide by using either concentrated NH_4OH or pressurized ammonia gas. Analysis of the deprotected oligomer by polyacrylamide gel electrophoresis (Figure 3) indicated that the phosphoramidites **4a**−**d** are as efficient as the 2-cyanoethyl phosphoramidites **24a**−**d** in the synthesis of the 20-mer. Furthermore, following digestion of the crude 20-mer by snake venom phosphodiesterase and bacterial alkaline phosphatase, HPLC analysis showed complete hydrolysis to individual nucleosides and no detectable nucleobase modification.

The facile and efficient production of synthetic oligonucleotides has spurred interest in the application of these biomolecules to a variety of diagnostics and therapeutic indications.[1] Oligonucleotides functionalized with reporter groups, intercalators, cross-linkers, affinity ligands, and DNA/RNA cleaving agents are examples of such applications.[2]

Since its inception in the early 1980s, the phosphoramidite method for oligonucleotide synthesis[3] has been extensively applied to the production of synthetic oligomers on solid supports.[4] The method is most convenient when using the 2-cyanoethyl group for phosphate protection.[5] This group is eventually removed along with nucleobase protecting groups by treatment with either concentrated NH_4OH or gaseous ammonia during oligonucleotide deprotection.[6] Under these conditions, 2-cyanoethyl groups undergo β-elimination with the concomitant formation of acrylonitrile.[7] This side product is a potent carcinogen,[8] and alkylation of the nucleobase of nucleosides and oligonucleotides by acrylonitrile is well-documented in the literature.[8−13]

While such production of acrylonitrile may appear inconsequential for small-scale oligonucleotide deprotec-

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(1) For a comprehensive review on the subject, see: Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 1925−1963. See also: Agrawal, S.; Iyer, R. P. *Curr. Opin. Biotech.* **1995**, *6*, 12−19.

(2) Beaucage, S. L. In *Comprehensive Natural Products Chemistry, Vol. 7*: *DNA and Aspects of Molecular Biology*; Kool, E. T., Ed.; Elsevier Science: London, 1999; pp 153−250.

(3) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859−1862. See also: (a) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223−2311. (b) Beaucage, S. L. In *Methods in Molecular Biology, Vol. 20: Protocols for Oligonucleotides and Analogues*; Agrawal, S., Ed.; Humana Press: Totowa, 1993; pp 33−61. (c) Beaucage, S. L.; Caruthers, M. H. In *Bioorganic Chemistry: Nucleic Acids*; Hecht, S. M., Ed.; Oxford University Press: New York, 1996; pp 36−74. (d) Beaucage, S. L.; Caruthers, M. H. In *Current Protocols in Nucleic Acid Chemistry*; Bergstrom, D. E., Glick, G. D., Jones, R. A., Beaucage, S. L., Eds.; John Wiley & Sons: New York, 1999; pp 3.3.1−3.3.20.

(4) Pon, R. T.; Buck, G. A.; Niece, R. L.; Robertson, M.; Smith, A. J.; Spicer, E. *BioTechniques* **1994**, *17*, 526−534.

(5) Sinha, N. D.; Biernat, J.; McManus, J.; Köster, H. *Nucl. Acids Res.* **1984**, *12*, 4539−4557.

(6) Boal, J. H.; Wilk, A.; Harindranath, N.; Max, E. E.; Kempe, T.; Beaucage, S. L. *Nucl. Acids Res.* **1996**, *24*, 3115−3117.

(7) Tener, G. M. *J. Am. Chem. Soc.* **1961**, *83*, 159−168.

(8) Solomon, J. J.; Cote, I. L.; Wortman, M.; Decker, K.; Segal, A. *Chem.−Biol. Interact.* **1984**, *51*, 167−190.

(9) Prokopczyk, B.; Bertinato, P.; Hoffman, D. *Carcinogenesis* **1988**, *9*, 2125−2128.

(10) Crippa, S.; Di Gennaro, P.; Lucini, R.; Orlandi, M.; Rindone, B. *Gazz. Chim. Ital.* **1993**, *123*, 197−203.

(11) Chambers, R. W. *Biochemistry* **1965**, *4*, 219−226.

(12) Mag, M.; Engels, J. W. *Nucl. Acids Res.* **1988**, *16*, 3525−3543.

(13) Ogilvie, K. K.; Beaucage, S. L. *Nucl. Acids Res.* **1979**, *7*, 805−823.

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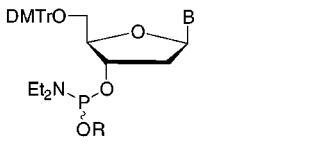
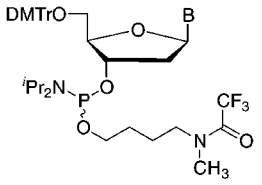
tion, it may not be the case for large-scale oligonucleotide deprotection. On the basis of economical and practical considerations, large-scale oligonucleotide syntheses intended for therapeutic applications necessitate minimization of reagent and solvent consumption. Reducing solvent volume could result in an undesirably high concentration of acrylonitrile, which could irreversibly modify nucleobases during the oligonucleotide deprotection process. In an effort to completely suppress nucleobase modification (and loss of oligonucleotide therapeutic efficacy), and to facilitate the purification of large quantities of therapeutic oligonucleotides, an alternative to the 2-cyanoethyl group for phosphate protection is indicated.

Recently, we reported the use of the 4-[*N*-(2,2,2-trifluoroacetyl)amino]butyl group for phosphodiester protection in the synthesis of oligodeoxyribonucleotides.[14] Deprotection of these oligomers is easily accomplished by treatment with concentrated ammonium hydroxide. A rate-limiting cleavage of the 2,2,2-trifluoroacetyl groups is followed by rapid cyclodeesterification of the resulting 4-aminobutyl phosphotriesters to give the corresponding phosphodiesters with the simultaneous formation of pyrrolidine.[14] Under these conditions, no internucleotidic phosphodiester cleavage or nucleobase modification was detected during deprotection of octadecathymidylic (dT_{18}) and octadecadeoxycytidylic (dC_{18}) acids. Recognizing the significance of the cyclodeesterification step, we additionally investigated the 5-[*N*-(2,2,2-trifluoroacetyl)amino]-pentyl[14] and 2-[2-[*N*-(2,2,2-trifluoroacetyl)amino]ethyloxy]-ethyl[15] groups for phosphate protection in DNA oligonucleotide synthesis. Interestingly, in these cases we found cyclodeesterification, not cleavage of the 2,2,2-trifluoroacetyl group, to be rate-limiting. Thus, the 5-[*N*-(2,2,2-trifluoroacetyl)amino]pentyl and 2-[2-[*N*-(2,2,2-trifluoroacetyl)amino]ethyloxy]ethyl phosphotriesters gave the desired phosphodiesters with concurrent generation of piperidine and morpholine, respectively.[16]

The high coupling efficiency of the deoxyribonucleoside phosphoramidites **1**−**3** and their stability in acetonitrile solutions together with the relative ease of phosphate deprotection encouraged us to evaluate the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl group as a phosphodiester protecting group in the synthesis of oligodeoxyribonucleotides.



1 R = $(CH_2)_4NHCOCF_3$
2 R = $(CH_2)_5NHCOCF_3$
3 R = $(CH_2)_2O(CH_2)_2NHCOCF_3$
DMTr = 4,4′-dimethoxytrityl
B = Thy or BzCyt

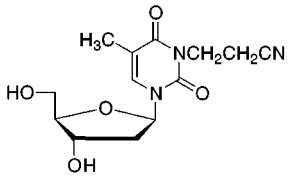


4a B = Thy
b B = BzCyt
c B = BzAde
d B = IbuGua

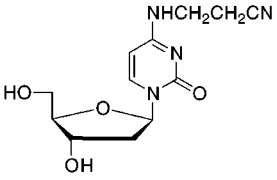
We now wish to report the preparation and characterization of the deoxyribonucleoside phosphoramidites **4a**−**d** and their application to the solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC). The deprotection kinetics of the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting group is also presented.

Results and Discussion

Nucleobase Alkylation of Deoxyribonucleosides by Acrylonitrile under Conditions Similar to Those Used for Large-Scale Oligonucleotide Deprotection. Alkylation of deoxyribo- and ribonucleosides by prolonged exposure to acrylonitrile at pH 5.0−11.5 has been extensively studied.[8−11] However, little is known about the alkylation of these nucleosides and N-protected deoxyribonucleosides under conditions that are used for oligonucleotide deprotection. Thus, thymidine, N^4-benzoyl-2′-deoxycytidine, 2′-deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, 2′-deoxyadenosine, N^2-isobutyryl-2′-deoxyguanosine, and 2′-deoxyguanosine were separately reacted with acrylonitrile and 25% NH_4OH in acetonitrile at 55 °C for 10 h to simulate large-scale oligonucleotide deprotection conditions. Each reaction was then analyzed by reversed-phase (RP) high-performance liquid chromatography (HPLC) to assess the extent of nucleobase alkylation. 2′-Deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, 2′-deoxyadenosine, N^2-isobutyryl-2′-deoxyguanosine, and 2′-deoxyguanosine did not produce any significant quantity of alkylation products under the conditions studied. Conversely, thymidine reacted with acrylonitrile to produce N^3-(2-cyanoethyl) thymidine **5** in 11% yield. The same reaction at 25 °C generated **5** in 2% yield within 2 h. The modified thymidine derivative **5** has been characterized by ^1H and ^{13}C NMR spectroscopy and accurate mass by fast atom bombardment (FAB) mass spectrometry. The characterization data are identical to those of an analytical sample of **5** prepared according to the method of Mag and Engels.[12]



5



6

The conversion of N^4-benzoyl-2′-deoxycytidine to N^4-(2-cyanoethyl)-2′-deoxycytidine **6** under the conditions described for the alkylation of thymidine is particularly interesting. The N^4-alkylated deoxycytidine derivative **6** is produced in 7% yield as a major reaction product. Following isolation, the characterization of **6** was accomplished as reported above for **5** by ^1H and ^{13}C NMR spectroscopy and accurate mass determination. The structure of **6** was further confirmed by its synthesis via another route. Specifically, when N^4-benzoyl-2′-deoxycytidine was treated with excess 3-aminopropionitrile fumarate in the presence of triethylamine in aqueous acetonitrile, the only nucleoside product other than 2′-deoxycytidine had an RP-HPLC retention time identical to that of **6**. Since alkylation of 2′-deoxycytidine by acrylonitrile at pH 3.0−7.0 is known to produce an N^3-adduct,[8] our findings suggest that **6** is generated by nucleophilic displacement of benzamide at C-4. This is presumably initiated by 3-aminopropionitrile that is produced in situ from the reaction of concentrated am-

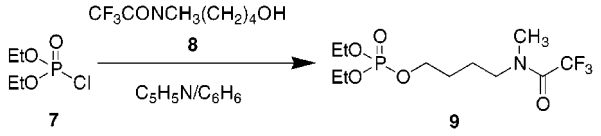
(14) Wilk, A.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem.* **1997**, *62*, 6712−6713.
(15) Wilk, A.; Beaucage, S. L. Unpublished results.
(16) Cyclodeesterification of 5-aminopentyl and 2-(2-aminoethyloxy)-ethyl phosphotriesters in concentrated NH_4OH at 55 °C was complete within 2 and 10 h, respectively.

monium hydroxide with acrylonitrile.[17] Primary amines have been previously reported to attack N^4-benzoyl-2′-deoxycytidine at C-4.[18]

It should be noted that alkylation of N^4-benzoylcytosine by acrylonitrile during large-scale oligodeoxyribonucleotide deprotection might be minimized, if not eliminated altogether, by appropriate selection of a more base-labile protecting group for the exocyclic amino function of cytosine. In this context, alkylation of thymine may be significantly suppressed by protection at either N-3 or O-4. A variety of protecting groups have been developed over the years specifically for this purpose.[3a] However, this strategy would adversely affect the economics of large scale oligonucleotide syntheses. It would therefore appear that the only practical solution to *completely* inhibit nucleobase alkylation by acrylonitrile during large-scale oligonucleotide deprotection is to eliminate the presence of this alkylating agent.

The 4-[*N*-Methyl-*N*-(2,2,2-trifluoroacetyl)amino]-butyl Group as an Alternative to the 2-Cyanoethyl Group for Phosphate Protection. In an effort to develop an approach to the synthesis of oligonucleotides that would block formation of modified nucleobases, we searched for phosphate protecting groups that would produce only inert side products during oligonucleotide deprotection. During the course of our investigations, we discovered that deprotection of oligodeoxyribonucleotides bearing 4-[*N*-(2,2,2-trifluoroacetyl)aminobutyl phosphate protecting groups produced 2,2,2-trifluoroacetamide and pyrrolidine.[14] Although pyrrolidine is inert relative to acrylonitrile in terms of nucleobase modification, production of, for example, a poorly nucleophilic tertiary amine during oligonucleotide deprotection would be ideal. In this regard, we rationalized that the use of the 4-[*N*-*methyl*-*N*-(2,2,2-trifluoroacetyl)amino]butyl group as a phosphodiester protecting group for oligodeoxyribonucleotides (Figure 1) would innocuously generate 2,2,2-trifluoroacetamide and *N*-methylpyrrolidine upon deprotection with concentrated NH_4OH. In contrast to the alkylating properties of acrylonitrile, which is generated during the deprotection of the popular 2-cyanoethyl phosphate protecting group,[7] these side products are essentially inert toward DNA nucleobases.

To determine whether base-assisted deprotection of oligonucleotides carrying 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting groups would proceed as shown in Figure 1, the simpler phosphotriester model **9** was first selected for mechanistic NMR studies. This phosphotriester is prepared by condensing *O*,*O*-diethyl phosphorochloridate **7** with 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol **8** and pyridine.



Pure **9** is isolated from this reaction in a near-quantitative yield (99%); further processing is unnecessary. The amido alcohol **8** is in turn prepared by the reaction of γ-butyrolactone **10** with methylamine (ca. 2.5 bar) to generate the *N*-methylamido alcohol **11** in essentially quantitative yields (Scheme 1). Reduction of **11** with LAH in Et_2O affords the *N*-methylamino alcohol **12** in an isolated yield of 67%. Conversion of **12** to **8** is effected by treatment of **12** with methyl 2,2,2-trifluoroacetate.The

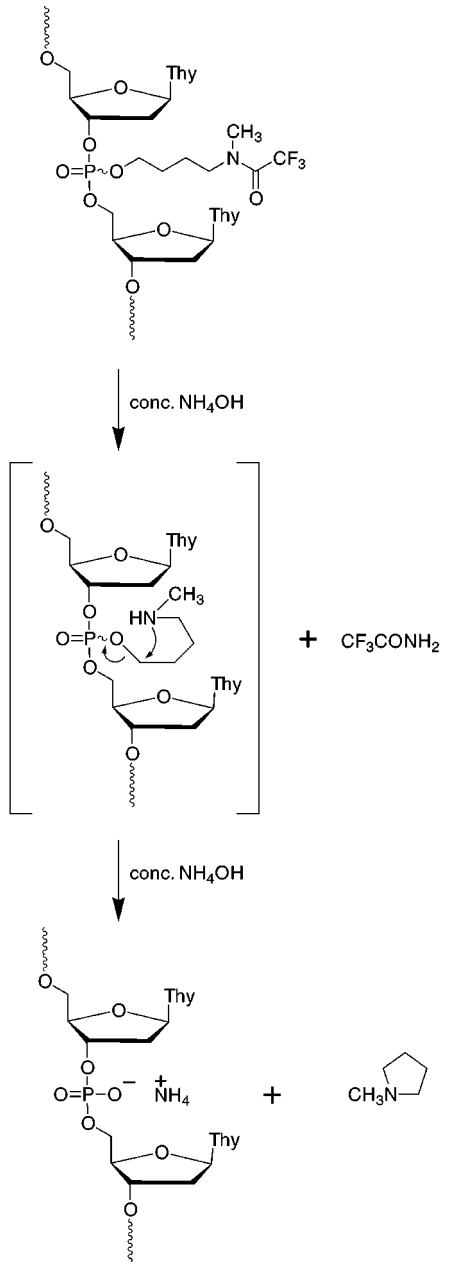
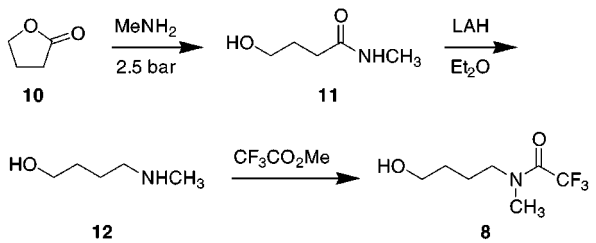


Figure 1. Proposed mechanism for base-assisted deprotection of oligodeoxyribonucleoside 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)-amino]butyl phosphotriesters.

Scheme 1



(17) The preparation of 3-aminopropionitrile from ammonia and acrylonitrile is well documented in the patent literature, see: *Chem. Abstr.* **1995**, *122*, P264930k and P105276k. See also: Jenner, G. *Tetrahedron* **1996**, *52*, 13557−13568.

(18) Weber, H.; Khorana, H. G. *J. Mol. Biol.* **1972**, *72*, 219−249. See also: Reddy, M. P.; Hanna, N. B.; Farooqui, F. *Tetrahedron Lett.* **1994**, *35*, 4311−4314 and references therein.

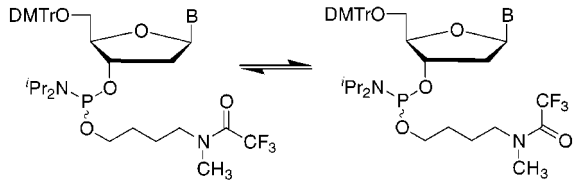
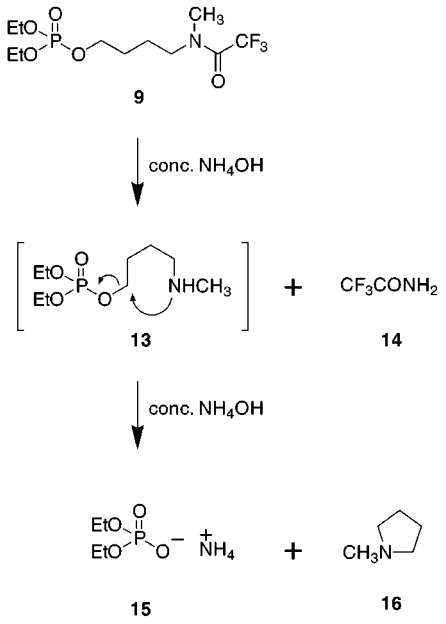


Figure 2. Tentative structure of the stable amide rotamers observed for the deoxyribonucleoside phosphoramidites **4a**−**d** by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy.

Scheme 2

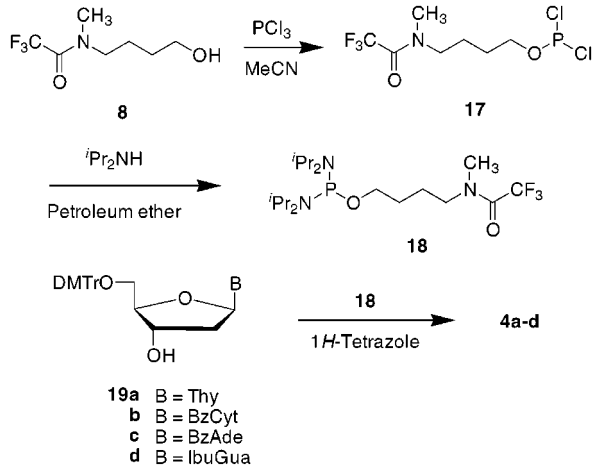


amido alcohol **8** is isolated by high-vacuum distillation in yields exceeding 90%.

After **9** was treated with concentrated NH_4OH in CD_3CN (1:1 v/v), the reaction mixture was analyzed by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy.[19] ^{31}P NMR data show that **9** (δ −3.5 ppm) is first converted to the corresponding *O*,*O*-diethyl-*O*-[4-(*N*-methyl)amino]butyl phosphotriester intermediate **13** (δ −3.3 ppm). This intermediate rapidly underwent cyclodeesterification to *O*,*O*-diethyl phosphate **15** (δ −2.5 ppm) with concurrent formation of *N*-methylpyrrolidine **16** (Scheme 2). The generation of **16** was confirmed by ^{1}H NMR spectroscopy, which showed two multiplets at 1.78 (4H) and 2.53 ppm (4H) and a singlet at 2.33 ppm (3H).[19] These chemical shifts are identical to those recorded from a commercial sample of *N*-methylpyrrolidine in the same solvent. Further, ^{13}C NMR showed signals at 56.2, 41.9, and 24.4 ppm, perfectly matching those of commercial *N*-methylpyrrolidine obtained under the same conditions.[19] Thus, this NMR mechanistic study established the base-assisted deprotection of **9** to proceed in a manner similar to that of *O*,*O*-diethyl-*O*-[4-[*N*-(2,2,2-trifluoroacetyl)amino]butyl] phosphate investigated previously.[14]

Having successfully completed the study of phosphate protection/deprotection of our model system, we then applied this strategy to the preparation of the deoxyribonucleoside phosphoramidites **4a**−**d** (Scheme 3). The reaction of an equimolar ratio of amido alcohol **8** and phosphorus trichloride in dry acetonitrile gave the corresponding phosphorodichloridite **17** in 92% yield. Addition of *N*,*N*-diisopropylamine (4 equiv) to **18** in anhydrous petroleum ether afforded the phosphordiamidite **18** in yields exceeding 95%. This diamidite can be purified by silica gel chromatography to a viscous oil, or alternatively, it can be used without further purification in the phosphinylation of deoxyribonucleosides.[20] The phosphoramidites **4a**−**d** were purified by silica gel chromatography, and isolated yields were greater than 92%. These phosphoramidites were characterized by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, and by accurate mass determination. It should be noted that the ^{1}H and ^{13}C NMR data for **4a**−**d** are quite complex due to a number of factors. One factor relates to the generation of stable rotamers caused by partial double-bond character of the C−N bond in the 4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)-amino] group. As shown in Figure 2, the phosphoramidites **4a**−**d** exist as a mixture of rotamers in ratio of about 2:1. Multiplicity of ^{1}H and ^{13}C NMR signals associated with tertiary amide rotamers is well-documented.[21] Other factors contributing to the complexity of the NMR data include the chirality of **4a**−**d** at phosphorus, ^{1}H- and ^{13}C-spin−spin couplings to phosphorus and fluorine, and closely related methylenes.[22] In this regard, proton-decoupled ^{31}P NMR spectra of **4a**−**d** show two sets of doublets demonstrating phosphorus chirality and a rotameric population of the 4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino] function.[19]

Scheme 3



The deprotection kinetics of the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting group was investigated using the model dinucleoside phosphotriester **20** and its phosphorothioate analogue **21**.[23] HPLC-purified **20** (or **21**) was treated as before with

(19) Data shown as Supporting Information.

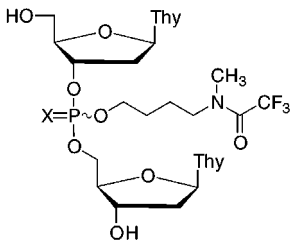
(20) Lee, H.-J.; Moon, S.-H. *Chem. Lett.* **1984**, 1229−1232. See also: Barone, A. D.; Tang, J.-Y.; Caruthers, M. H. *Nucl. Acids Res.* **1984**, *12*, 4051−4061.

(21) Abraham, R. J.; Loftus, P. In *Proton and Carbon-13 NMR spectroscopy—An integrated Approach*; Heyden: London, 1980; pp 171−174.

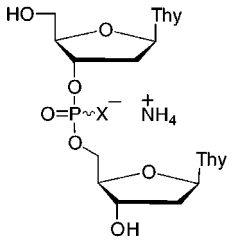
(22) An example of such complexity is provided by the 300 MHz ^{1}H NMR spectrum of the simpler phosphotriester **9** in DMSO-d_6 (30 °C), which shows two quartets at 2.97 ppm ($^{5}J_{HF}$ = 0.8 Hz) and 3.10 ppm ($^{5}J_{HF}$ = 1.8 Hz) corresponding to *N*-CH_3.[19] These signals were generated from long-range coupling with the CF_3 group of each rotamer. The coalescence of these quartets was observed at ca. 80 °C, and only a relatively sharp singlet was observed (3.08 ppm) at 120 °C indicating free rotation around the amide bond. The same rationale applies to the complex ^{1}H NMR signal corresponding to the methylene adjacent to *N*-CH_3 (3.44 ppm) and its coalescence to the expected triplet (3.47 ppm) at ca. 80 °C.[19]

concentrated NH_4OH, and the rate of phosphate (or thiophosphate) deprotection was monitored by HPLC. Complete conversion of **20** (or **21**) to **22** (or **23**) was observed within 2 h at 25 °C.

The 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting group can also be deprotected by pressurized ammonia gas. For example, while still covalently linked to controlled-pore glass (CPG), dinucleotide **20** (or **21**) can be exposed to ammonia gas (ca. 10 bar) for 10 h at 25 °C to produce exclusively **22** (or **23**) as assessed by HPLC analysis of the deprotected dimers. Since the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate (or thiophosphate) protecting group can be rapidly deprotected at room temperature by concentrated NH_4OH, this protecting group is thus as convenient to use as the 2-cyanoethyl group in the synthesis of oligonucleotides that carry base-sensitive nucleobases.

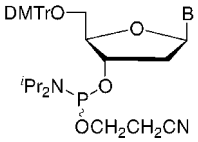


20 X = O
21 X = S



22 X = O
23 X = S

The coupling efficiency of **4a**−**d** was then studied and compared with that of the commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites **24a**−**d** by at-



24a B = Thy
b B = BzCyt
c B = BzAde
d B = IbuGua

tempting the solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) under identical conditions. After standard deprotection, the crude oligomer was analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (Figure 3). Comparison of the relative density of all PAGE bands in the left lane of the stained gel with those of the middle lane demonstrates the phosphoramidites **4a**−**d** to be as efficient as the 2-cyanoethyl phosphoramidites **24a**−**d** in the synthesis of the 20-mer. Furthermore, the phosphoramidites **4a**−**d** are quite stable and retain excellent coupling efficiency even after being in an acetonitrile solution for 1 week.

(23) The dinucleoside phosphotriester **20** and its phosphorothioate derivative **21** were pepared according to standard solid-phase techniques by the use of the phosphoramidite **4a**. The sulfurization step required for the preparation of **21** was effected by 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-doxide in MeCN according to a literature procedure.[28] Following cleavage of the 5′-*O*-DMTr group, the dinucleotides were released from the CPG support by a brief exposure (2 min) to methylamine gas under pressure (ca. 2.5 bar). The crude dinucleotides **20** and **21** were eluted off CPG with 50% aqueous acetonitrile (300 μL) and then purified by RP-HPLC under the conditions described for the analysis of deoxyribonucleoside alkylation by acrylonitrile (see the Experimental Section). Purified **20** displayed a retention time (t_R) of 33.3 and 34.0 min for its diastereoisomers, whereas diastereomeric **21** exhibited a t_R of 35.0 and 35.9 min.

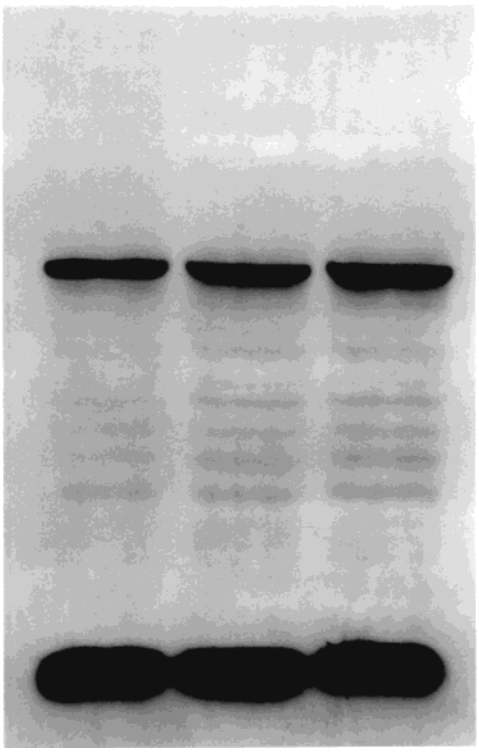


Figure 3. Electrophoretic analysis of d(ATCCGTAGCTAAGGTCATGC) prepared from phosphoramidites **4a**−**d** or **24a**−**d** using a 20% polyacrylamide−7 M urea gel at pH 8.3 (1× TBE buffer). Left lane: crude oligomer synthesized from freshly dissolved **24a**−**d** and then treated with concentrated NH_4OH for 10 h at 55 °C. Middle lane: crude oligomer prepared from freshly dissolved **4a**−**d** and then treated with concentrated NH_4OH for 10 h at 55 °C. Right lane: crude oligomer synthesized from one week old solutions of **4a**−**d** and then treated with ammonia gas under pressure (ca. 10 bar) for 10 h at 25 °C. Bromophenol blue is used as a marker, and shows as the large band, in each lane, at the bottom of the gel.

The PAGE bands that appear below the major product band in the right lane of the gel (Figure 3) are of comparable density to those generated by using freshly prepared phosphoramidite solutions (middle lane).

The above crude 20-mer was digested completely by snake venom phosphodiesterase (svPDE) and bacterial alkaline phosphatase (bAP) to individual nucleosides, as shown by HPLC analyses of the digests.[19] These results indicate the absence of nucleobase modification and lack of 4-(*N*-methylamino)butyl phosphate acylation by transamidation with neighboring N-protected nucleobases upon removal of the 2,2,2-trifluoroacetyl groups. As with nucleobase modifications, transamidation products would have shown resistance to hydrolysis by svPDE and bAP and would have been easily detected in HPLC analysis of the hydrolysates as peaks having longer retention times than those of the individual nucleosides.

Conclusion

The findings reported herein strongly support phosphoramidites **4a**−**d** as excellent alternatives to the conventional 2-cyanoethyl phosphoramidites **24a**−**d** in the rapid and efficient synthesis of oligodeoxyribonucleotides on any scale. The phosphoramidites **4a**−**d** are very soluble and stable in acetonitrile. For example, **4d**, unlike the 2-cyanoethyl phosphoramidite **24d**, does not precipitate upon prolonged storage in acetonitrile solutions. In

this regard, the stability of the phosphoramidites **4a**−**d** in acetonitrile is such that the coupling efficiency of these monomers is essentially unaffected even after being in solution for 1 week. Most importantly, deprotection of oligodeoxyribonucleotides prepared from phosphoramidites **4a**−**d**, unlike those prepared with the conventional phosphoramidites **24a**−**d**, proceeds without generating potent mutagens. For this reason, using the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)]aminobutyl group for phosphate protection is most certainly indicated over the 2-cyanoethyl group (from which nucleobase alkylation by acrylonitrile may result during deprotection) for any large-scale production of therapeutic oligonucleotides.

Furthermore, should the use of the (9-fluorenylmethoxy)carbonyl (Fmoc)[24] or the (2-dansylethoxy)carbonyl (Dnseoc)[25] group for 5′-hydroxyl protection in conjunction with the 2-cyanoethyl phosphate protecting group in DNA or RNA oligonucleotide synthesis be required for specific applications, the removal of the Fmoc or Dnseoc effected by DBU in acetonitrile[24,25] will dramatically increase the probability of nucleobase alkylation by acrylonitrile released from the concomitant DBU-assisted phosphate deprotection.[26] In such cases, the use of the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting group is definitely recommended over the 2-cyanoethyl group for small-scale oligonucleotide syntheses.

Finally, it is safe to speculate that the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting group may find application in areas other than nucleic acid chemistry. For example, studies involving phospholipid analogues, sugar phosphates, phosphopeptides, and inositol phosphate analogues may benefit from a phosphate protecting group that is considerably more stable to strong non-nucleophilic bases under anhydrous conditions than the 2-cyanoethyl phosphate protecting group by presenting new synthetic possibilities.

Experimental Section

Materials and Methods. Common chemicals and solvents were purchased from commercial sources and used without further purification. Anhydrous pyridine and benzene were obtained from Aldrich and used as received. Et_2O (Mallinckrodt) was refluxed over sodium under an inert atmosphere and freshly distilled just prior to use. Acetonitrile, dichloromethane, *N*,*N*-diisopropylamine, and petroleum ether were refluxed over calcium hydride and distilled before being used. Phosphorus trichloride (Aldrich) was distilled immediately prior to use. Acrylonitrile, 3-aminopropionitrile fumarate, *N*-methylpyrrolidine, γ-butyrolactone, lithium aluminum hydride, methyl trifluoroacetate, diethyl chlorophosphate, (2-cyanoethyl)-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphordiamidite, sublimed 1*H*-tetrazole, and Stains-all were purchased from Aldrich and used without further purification. Lecture bottles of ammonia and methylamine gases were obtained from Aldrich.

Chromatography on silica gel columns was performed using Merck silica gel 60 (230−400 mesh), whereas analytical thin-layer chromatography (TLC) was conducted on 2.5 cm × 7.5 cm glass plates coated with a 0.25 mm thick layer of silica gel 60 F_{254} (Whatman).

NMR spectra were recorded at 7.05 T (300 MHz for ^{1}H). ^{1}H and proton-decoupled ^{31}P NMR spectra were obtained using deuterated solvents. Unless otherwise indicated, tetramethylsilane (TMS) was used as internal reference for ^{1}H NMR spectra and 85% phosphoric acid in deuterium oxide as an external reference for ^{31}P NMR spectra. Proton-decoupled ^{13}C NMR spectra were recorded in either $CDCl_3$, C_6D_6, or DMSO-d_6 using TMS as an internal reference. Chemical shifts δ are reported in parts per million (ppm). NMR spectra were run at 25 °C or as indicated. Chemical shifts of rotameric ^{1}H NMR signals are reported with a signal percentage approximating the ratio of each rotamer.

Low- and high-resolution FAB mass spectra were acquired from samples dissolved in either 4-nitrobenzyl alcohol or a mixture of dithiothreitol and dithioerythritol (3:1, v/v) and bombarded with 8 keV fast cesium ions. A mass calibration standard of cesium iodide, or a mixture of cesium iodide and sodium iodide, was used. Accurate mass meaurements were performed on $[M + H]^+$ ions or on $[M + Na]^+$ ions, which were obtained by addition of aqueous sodium iodide to the sample matrix.

Procedure for the Alkylation of Deoxyribonucleosides with Acrylonitrile. To an unprotected or an N-protected deoxyribonucleoside (0.5 mmol) in a 7 mL screw-capped glass vial were added acrylonitrile (250 μL, 4 mmol), concentrated NH_4OH (1.1 mL, ca. 8 mmol NH_3), and acetonitrile (3 mL). The screw-capped vial was then heated at 55 °C for 10 h. An aliquot of the reaction mixture was analyzed by RP-HPLC using a 5 μm Supelcosil LC-18S column (25 cm × 4.6 mm) and a linear gradient of 1% MeCN/min, starting from 0.1 M triethylammonium acetate (pH 7.0), at a flow rate of 1 mL/min. This analysis showed that thymidine (t_R = 13.2 min) is alkylated to N^3-(2-cyanoethyl) thymidine **5** (t_R = 19.7 min) in 11% yield based on HPLC peak area. The peak corresponding to **5** was collected and characterized. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.84 (d, ^4J = 1.1 Hz, 3H), 2.12 (m, 2H), 2.83 (t, J = 6.7 Hz, 2H), 3.59 (ddd, J = 11.8, 4.1, 3.8 Hz, 1H), 3.63 (ddd, J = 11.8, 4.1, 3.8 Hz, 1H), 3.79 (q, J = 3.8 Hz, 1H), 4.07 (t, J = 6.7 Hz, 2H), 4.25 (m, 1H), 5.04 (t, J = 4.1 Hz, 1H), 5.24 (d, J = 4.0 Hz, 1H), 6.21 (t, J = 6.7 Hz, 1H), 7.82 (q, ^4J = 1.1 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 12.7, 15.4, 36.1, 39.6, 61.1, 70.1, 84.9, 87.4, 108.4, 118.3, 135.1, 150.1, 162.3. FAB-HRMS: calcd for $C_{13}H_{17}N_3O_5$ $(M + H)^+$ 296.1247, found 296.1225.

The above HPLC analysis also revealed that N^4-(2-cyanoethyl)-2′-deoxycytidine **6** (t_R = 12.7 min) is produced in 7% yield (based on HPLC peak area) from the reaction of N^4-benzoyl-2′-deoxycytidine with acrylonitrile and concentrated NH_4OH. The HPLC peak corresponding to **6** was collected and characterized. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.95 (ddd, J = 13.2, 7.2, 6.0 Hz, 1H), 2.13 (ddd, J = 13.2, 6.0, 3.4 Hz, 1H), 2.76 (t, J = 6.3 Hz, 2H), 3.49 (m, 2H), 3.78 (m, 1H), 4.21 (m, 1H), 4.98 (bs, 1H), 5.21 (bs, 1H), 5.81 (d, J = 7.5 Hz, 1H), 6.16 (t, J = 6.3 Hz, 1H), 7.82 (d, J = 7.5 Hz, 1H), 8.07 (t, J = 5.7 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 17.1, 36.0, 40.4, 61.3, 70.4, 85.0, 87.3, 94.4, 119.2, 140.5, 140.7, 154.9. FAB-HRMS: calcd for $C_{12}H_{16}N_4O_4$ $(M + H)^+$ 281.1250, found 281.1270.

The structure of **6** was further corroborated by the reaction of N^4-benzoyl-2′-deoxycytidine (166 mg, 0.5 mmol) with 3-aminopropionitrile fumarate (512 mg, 4 mmol), triethylamine (1.4 mL, 10 mmol), and 3 mL of MeCN/H_2O (2:1 v/v). The reaction mixture was heated in a 7 mL screw-capped vial at 55 °C for 10 h. HPLC analysis of the reaction products revealed the presence of **6**, which is indistinguishable, by co-injection, from the alkylated product obtained in the reaction of N^4-benzoyl-2′-deoxycytidine with acrylonitrile and concentrated NH_4OH.

***N*-Methyl-4-hydroxybutyramide (11).** γ-Butyrolactone (17.2 g, 199 mmol) was placed in a 250 mL glass-lined pressure vessel. After a brief evacuation (2 min) to ca. 2 mmHg, the vessel was filled with gaseous methylamine. The reaction mixture was then magnetically stirred overnight under a positive pressure of methylamine (ca. 2.5 bar). Excess methylamine was released to the atmosphere in a well-ventilated fume hood and the reaction product kept under vacuum (2 mmHg) for 1 h. *N*-Methyl-4-hydroxybutyramide was isolated

(24) Lehmann, C.; Xu, Y.-Z.; Christodoulou, C.; Tan, Z.-K.; Gait, M. J. *Nucl. Acids Res.* **1989**, *17*, 2379−2390.

(25) Bergmann, F.; Pfleiderer, W. *Helv. Chim. Acta* **1994**, *77*, 203-215.

(26) Treatment of thymidine with acrylonitrile in the presence of DBU produced a mixture of N^3-(2-cyanoethyl) thymidine **5** and its 5′-O- and 3′-O-alkylated derivatives within 15 min at 25 °C (Srinivasachar, K.; Beaucage, S. L. Unpublished results).

in near-quantitative yield (23.1 g, 197 mmol) as a crystalline mass (mp 32−34 °C). NMR analysis indicated that the purity of **11** was greater than 98%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.63 (dt, J = 7.6, 6.4 Hz, 2H), 2.10 (t, J = 7.6 Hz, 2H), 2.56 (d, J = 4.6 Hz, 3H), 3.37 (t, J = 6.4 Hz, 2H), 3.58 (b, ∼1H), 7.75 (b, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.6, 28.7, 32.2, 60.5, 173.1.

4-(*N*-Methylamino)butan-1-ol (12). To a flame-dried three-necked 2 L round-bottom flask equipped with a mechanical stirrer, an addition funnel, and a reflux condenser were added under an argon atmosphere 1 L of diethyl ether freshly distilled from sodium and lithium aluminum hydride (36 g, 0.94 mol). *N*-Methyl-4-hydroxybutyramide (**11**, 58.5 g, 0.50 mol) was melted and the neat supercooled liquid added to the mechanically stirred suspension, dropwise, over a period of 2 h at 25 °C. The reaction mixture was refluxed for 6 h and allowed to stir for an additional 6 h at 25 °C. The reduction was quenched by first adding triethanolamine[27] (125 mL, 0.94 mol) dropwise over a period of 1 h followed by the dropwise addition of water (50 mL, 2.8 mol) over a period of 1 h. The resulting slurry was mechanically stirred for 12 h and then filtered through a glass-sintered funnel (coarse porosity). The solid cake was carefully triturated on the filter with diethyl ether (3 × 200 mL); the etheral filtrates were pooled together and rotoevaporated under reduced pressure. The residue was distilled under reduced pressure to give **12** (34.3 g, 0.33 mol, 67%) as a colorless liquid. Bp 47 °C/0.25 mmHg. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.64 (m, 4H), 2.42 (s, 3H), 2.62 (dd, J = 6.0, 5.3 Hz, 2H), 3.57 (dd, J = 5.3, 4.6 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.3, 32.3, 35.7, 51.7, 62.5.

4-[*N*-Methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol (8). Methyl trifluoroacetate (7.8 g, 60 mmol) was added by syringe, through a septum, to 4-(*N*-methylamino)butan-1-ol **12** (5 g, 48 mmol) in a 50 mL round-bottom flask. Upon completion of the addition, the reaction mixture was stirred at 25 °C for 12 h. Methanol and excess methyl trifluoroacetate were distilled off at atmospheric pressure. The material left was then distilled under reduced pressure to afford **8** (8.9 g, 45 mmol, 94%) as a slightly viscous colorless liquid. Bp: 71 °C/0.36 mmHg. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.56 (m, 2H), 1.70 (m, 2H), 3.00 (q, $^5J_{HF}$ = 0.7 Hz, 3H) (33%) and 3.14 (q, $^5J_{HF}$ = 1.6 Hz, 3H) (67%), 3.46 (m, J = 7.4 Hz, 2H), 3.65 (t, J = 6.1 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.8, 24.7, 29.2, 29.3, 34.1, 34.6, 49.1, 49.4, 61.7, 61.8, 116.5 (q, $^1J_{CF}$ = 288 Hz), 116.6 (q, $^1J_{CF}$ = 288 Hz), 156.7 (q, $^2J_{CF}$ = 36.0 Hz), 156.8 (q, $^2J_{CF}$ = 36.0 Hz).

***O,O*-Diethyl-*O*-[4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)-amino]butyl]phosphate (9).** In a flame-dried 4 mL glass vial containing 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol **8** (200 mg, 1 mmol) were added, by syringe through a serum cap, dry benzene (2 mL), anhydrous pyridine (89 μL, 1.1 mmol), and *O,O*-diethyl phosphorochloridate **7** (158 μL, 1.1 mmol). The reaction mixture was occasionally shaken at 25 °C for 30 min. The pyridinium hydrochloride salt was then filtered off, and the filtrates were evaporated to dryness under reduced pressure. The colorless and viscous oil was kept under high vacuum for 4 h. The oily phosphate **9** (332 mg, 0.99 mmol, 99%) was pure enough to be used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ 1.25 (dt, J = 7.1 Hz, $^4J_{PH}$ = 0.9 Hz, 6H), 1.62 (m, 4H), 2.97 (q, $^5J_{HF}$ = 0.8 Hz, 3H) (67%) and 3.10 (q, $^5J_{HF}$ = 1.8 Hz, 3H) (33%), 3.44 (m, 2H), 3.91 (m, 2H), 4.02 (dq, J = 7.1 Hz, J_{PH} = 8.3 Hz, 4H). ^{13}C NMR (75 MHz, DMSO-d_6, 25 °C): δ 20.8 (d, J_{PC} = 6.3 Hz), 27.6, 29.4, 32.2 (d, J_{PC} = 6.3 Hz), 32.4 (d, J_{PC} = 6.3 Hz), 39.0, 39.6, 53.8, 54.0, 68.9 (d, $^2J_{PC}$ = 6.3 Hz), 72.1 (d, $^2J_{PC}$ = 6.3 Hz), 121.9 (q, $^1J_{CF}$ = 288 Hz), 122.0 (q, $^1J_{CF}$ = 288 Hz), 161.5. (q, $^2J_{CF}$ = 33.9 Hz), 161.7 (q, $^2J_{CF}$ = 33.9 Hz). ^{1}H NMR (300 MHz, DMSO-d_6, 90 °C): δ 1.29 (t, J = 7.1 Hz, 6H), 1.68 (m, 4H), 3.08 (bs, 3H), 3.47 (t, J = 6.5 Hz, 2H), 4.00 (m, 2H), 4.07 (q, J = 7.1 Hz, 4H). ^{13}C NMR (75 MHz, DMSO-d_6, 90 °C): δ 21.2 (d, J_{PC} = 6.3 Hz), 28.0, 32.6 (d, J_{PC} = 6.3 Hz), 39.7, 54.1, 68.6 (d, $^2J_{PC}$ = 6.3 Hz), 72.1 (d, $^2J_{PC}$ = 6.3 Hz), 122.0 (q, $^1J_{CF}$ = 288 Hz), 161.5. (q, $^2J_{CF}$ = 33.9 Hz). ^{31}P NMR (121 MHz, CD_3CN): δ −3.5.

***O*-[4-[*N*-Methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl]-phosphordichloridite (17).** 4-[*N*-Methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol **8** (8 g, 40 mmol) and anhydrous acetonitrile (10 mL) were placed in a flame-dried addition funnel. The resulting solution was added dropwise over a period of 2 h to a 250 mL flame-dried round-bottom flask containing freshly distilled phosphorus trichloride (6.8 g, 50 mmol) dissolved in anhydrous acetonitrile (50 mL). During the course of the addition, a slight vacuum was applied through the dropping funnel to keep an internal pressure of ca. 750 mmHg. This lower than atmospheric pressure ensured the removal of gaseous hydrogen chloride that was generated. The reaction mixture was then stirred for an additional 3 h at ca. 40 mmHg. The material left was distilled under reduced pressure to give the colorless phosphordichloridite **17** (11 g, 37 mmol, 92%). Bp: 110 °C/0.1 mmHg. ^{1}H NMR (300 MHz, C_6D_6): δ 1.01(bm, 4H) (33%) and 1.11 (bm, 4H) (67%) 2.38 (bs, 3H) (67%) and 2.45 (bs, 3H) (33%), 2.75 (m, 2H) (33%) and 2.88 (m, 2H) (67%), 3.77 (m, 2H). ^{13}C NMR (75 MHz, C_6D_6): δ 22.3, 23.9, 26.0, 26.3, 33.2, 33.5, 47.9, 48.0, 67.5 (d, $^2J_{PC}$ = 10.6 Hz), 67.7 (d, $^2J_{PC}$ = 10.6 Hz), 116.9 (q, $^1J_{CF}$ = 288 Hz), 117.0 (q, $^1J_{CF}$ = 288 Hz), 156.6 (q, $^2J_{CF}$ = 36.0 Hz), 156.7 (q, $^2J_{CF}$ = 36.0 Hz). ^{31}P NMR (121 MHz, C_6D_6): δ 174.2, 174.3.

***N,N,N′,N′*-Tetraisopropyl-*O*-[4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl]phosphordiamidite (18).** To a 1 L flame-dried round-bottom flask equipped with a reflux condenser were added anhydrous petroleum ether (300 mL) and *O*-[4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl] phosphordichloridite **17** (11 g, 37 mmol). Anhydrous *N,N*-diisopropylamine (28 mL, 200 mmol) in dry petroleum ether (100 mL) was added in portions, through the reflux condenser, to the vigorously stirred solution of **17**. The reaction mixture was then left to react for 48 h at 25 °C. The precipitated *N,N*-diisopropylammonium hydrochloride salt was filtered off. The filtrate was concentrated under reduced pressure and kept under high vacuum for 12 h to give **18** as a slightly yellow syrup (15 g, 35 mmol, 95%). The compound was pure enough for the phosphinylation of suitably protected deoxyribonucleosides. ^{1}H NMR (300 MHz, C_6D_6): δ 1.21 (m, 24H), 1.40 (m, 4H), 2.41 (q, $^5J_{HF}$ = 1.6 Hz, 3H) (67%) and 2.50 (q, $^5J_{HF}$ = 0.7 Hz, 3H) (33%), 2.89 (tq, J = 7.4 Hz, $^5J_{HF}$ = 1.0 Hz, 2H) (33%) and 3.04 (t, J = 6.9 Hz, 2H) (67%), 3.43 (m, 2H), 3.50 (m, 4H). ^{13}C NMR (75 MHz, C_6D_6): δ 23.2, 23.7, 23.8, 24.3, 24.5, 25.0, 28.3, 28.4, 28.5, 28.6 33.3, 33.5 (q, $^4J_{CF}$ = 4.2 Hz), 44.3, 44.5, 48.5, 48.7 (q, $^4J_{CF}$ = 4.2 Hz), 63.4 (d, $^2J_{CP}$ = 6.4 Hz), 63.7 (d, $^2J_{CP}$ = 6.4 Hz), 117.0 (q, $^1J_{CF}$ = 288 Hz), 117.1 (q, $^1J_{CF}$ = 288 Hz), 156.1 (q, $^2J_{CF}$ = 36 Hz). ^{31}P NMR (121 MHz, C_6D_6): δ 117.9, 118.2. FAB-MS: calcd for $C_{19}H_{39}F_3N_3O_2P$ $(M + H)^+$ 430, found 430.

General Procedure for the Preparation of the Deoxyribonucleoside Phosphoramidites 4a−d. A suitably protected deoxyribonucleoside (2 mmol) was dried under high vacuum for 2 h in a 50 mL round-bottom flask. Anhydrous methylene chloride (10 mL) was added to the dried nucleoside followed by *N,N,N′,N′*-tetraisopropyl-*O*-[4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl]phosphordiamidite **18** (900 mg, 2.1 mmol). To this solution was added 1*H*-tetrazole (140 mg, 2 mmol), in small batches, over a period of 0.5 h. The rates of the reaction were monitored by TLC using benzene/triethylamine (9:1 v/v) as an eluent. Phosphinylation of suitably protected 2′-deoxynucleosides was usually complete within 1 h at 25 °C (for best results, phosphinylation of properly protected 2′-deoxyguanosine was allowed to proceed for 12 h). The reaction mixture was then concentrated under reduced pressure, dissolved in benzene/triethylamine (9:1 v/v), and chromatographed on a silica gel column (4 cm × 10 cm) using the same solvent for equilibration and elution. Appropriate fractions were pooled and concentrated, and each of the deoxyribonucleoside phosphoramidites **4a−d** was isolated as a white amorphous powder in yields ranging from 92 to 98%.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N,N*-diisopropylamino)-[4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butoxy]-phosphinyl-2′-deoxythymidine (4a). ^{31}P NMR (121 MHz,

(27) Powell, J.; James, N.; Smith, S. J. *Synthesis* **1986**, 338−340. See also: Gardrat, C.; Latxague, L.; Picard, J. P. *J. Heterocycl. Chem.* **1990**, *27*, 811−812.

C_6D_6): δ 143.3, 143.4, 143.66, 143.75. FAB-HRMS: calcd for $C_{44}H_{56}F_3N_4O_9P$ $(M + Na)^+$ 895.3635, found 895.3599.

***N*⁴-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)[4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butoxy]phosphinyl-2′-deoxycytidine (4b).** ^{31}P NMR (121 MHz, C_6D_6): δ 143.82, 143.87, 143.92. FAB-HRMS: calcd for $C_{50}H_{59}F_3N_5O_9P$ $(M + Na)^+$ 984.3900, found 984.3908.

***N*⁶-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)[4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butoxy]phosphinyl-2′-deoxyadenosine (4c).** ^{31}P NMR (121 MHz, C_6D_6): δ 143.5, 143.6, 143.8, 144.0. FAB-HRMS: calcd for $C_{51}H_{59}F_3N_7O_8P$ $(M + Na)^+$ 1008.4010, found 1008.3970.

***N*²-Isobutyryl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)[4-[(*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butoxy]phosphinyl-2′-deoxyguanosine (4d).** ^{31}P NMR (121 MHz, C_6D_6): δ 142.9, 143.1, 143.5, 143.7. FAB-HRMS: calcd for $C_{48}H_{61}F_3N_7O_9P$ $(M + Na)^+$ 990.4118, found 990.4099.

Preparation of Oligonucleotides. Solid-phase oligonucleotide syntheses were performed using a DNA synthesizer according to the manufacturer's recommendations. 2-Cyanoethyl deoxyribonucleoside phosphoramidites **24a**−**d** and all the reagents pertaining to the automated preparation of oligonucleotides were purchased from Perkin-Elmer and used as recommended by the manufacturer. The sulfurization step required for the preparation of oligodeoxyribonucleoside phosphorothioates was effected by a 0.05 M solution of 3*H*-1,2-benzodithiol-3-one 1,1-doxide (Glen Research) in acetonitrile as recommended in the literature.[28]

Purification and Characterization of Oligonucleotides. Fully deprotected oligomers were electrophoresed on 20% polyacrylamide−7 M urea gels (40 cm × 20 cm × 0.75 mm) prepared, as described by Maniatis et al.,[29] using electrophoresis purity reagents (Bio-Rad). Gels were stained by soaking in a solution composed of 10 mL of Stains-all (1 mg/mL in formamide) and 250 mL of a staining buffer. The staining buffer solution was composed of formamide (40 mL), 2-propanol (200 mL), ddH_2O (800 mL), and 3 M Tris pH 8.8 (4 mL). Gel staining was performed in the dark for 30 min with occasional shaking. The staining solution was then discarded and the gel rinsed twice with H_2O (300 mL). The gel was ready for photography as soon as the pink background faded away upon exposure to light.

Enzymatic digestion of crude DNA oligomers was effected by svPDE (*Crotalus durissus*, Boehringer) and bAP (Sigma) according to a published procedure.[30] An aliquot of the digest was analyzed by reversed-phase HPLC using a 5 μm Supelcosil LC-18S column (25 cm × 4.6 mm) and a linear gradient of 1% MeCN/min, starting from 0.1 M triethylammonium acetate pH 7.0, at a flow rate of 1 mL/min.

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Supporting Information Available: ^1H and ^{13}C NMR spectra of **5**, **6**, **8**, **11**, **12**, **17**, and **18**. ^1H, ^{13}C, and ^{31}P NMR mechanistic study of the deprotection of **9** effected by NH_4OH. ^{31}P NMR spectra of **4a**−**d**, **17**, and **18**. RP-HPLC chromatograms of hydrolysates resulting from svPDE and bAP digestion of d(ATCCGTAGCTAAGGTCATGC) prepared from either phosphoramidites **4a**−**d** or **24a**−**d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(28) 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.

(29) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: New York, 1982; pp 173−177.

(30) Scremin, C. L.; Zhou, L.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem*. **1994**, *59*, 1963−1966.