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The 3-(*N*-*tert*-Butylcarboxamido)-1-propyl Group as an Attractive Phosphate/Thiophosphate Protecting Group for Solid-Phase Oligodeoxyribonucleotide Synthesis

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Among the various phosphate/thiophosphate protecting groups suitable for solid-phase oligonucleotide synthesis, the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group is one of the most convenient, as it can be readily removed, as needed, under thermolytic conditions at neutral pH. The deprotection reaction proceeds rapidly ($t_{1/2}$ ~100 s) through an intramolecular cyclodeesterification reaction involving the amide function and the release of the phosphate/thiophosphate group as a 2-(*tert*-butylimino)tetrahydrofuran salt. Incorporation of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group into the deoxyribonucleoside phosphoramidites **1a**−**d** is achieved using inexpensive raw materials. The coupling efficiency of **1a**−**d** in the solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioate analogue is comparable to that of commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites. These oligonucleotides were phosphate/thiophosphate-deprotected within 30 min upon heating at 90 °C in Phosphate-Buffered Saline (PBS buffer, pH 7.2). Since no detectable nucleobase modification or significant phosphorothioate desulfurization occurs, the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group represents an attractive alternative to the 2-cyanoethyl group toward the large-scale preparation of therapeutic oligonucleotides.

Introduction

With the advent of deoxyribonucleoside phosphoramidites,[1] rapid and highly efficient solid-phase syntheses of DNA oligonucleotides[2] are now available and have, over the past two decades, revolutionized the biomedical sciences. This technology has expanded oligonucleotide syntheses for therapeutic indications[3] from nanomole- to millimole-scale. The magnitude of this up-scaling has seriously challenged the suitability of 2-cyanoethyl deoxyribonucleoside phosphoramidites for oligonucleotide syntheses, since acrylonitrile is produced as a side-product during oligonucleotide deprotection.[4] The potent DNA-alkylating properties of acrylonitrile have been investigated by us[5] and by others.[6] The extent to which nucleobases are alkylated by acrylonitrile during nanomole- or micromole-scale oligonucleotide deprotection is insignificant, presumably due to the low concentration of acrylonitrile being generated. However, nucleobase alkylation during millimole-scale oligonucleotide deprotection is a valid concern[5] as this threatens the desired biological activity of synthetically derived therapeutic oligonucleotides against a variety of human diseases including cancer and viral infections. To prevent the loss of bioactivity, modifications to nucleobases during oligonucleotide synthesis and deprotection must not occur. Thus, synthesis of therapeutic oligonucleotides must utilize phosphate/thiophosphate protecting groups that will not expose nucleobases to alkylating or otherwise modifying agents during oligonucleotide deprotection.

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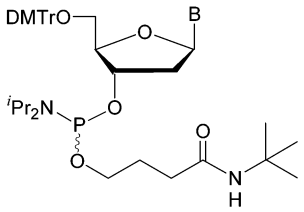
Earlier, we reported the use of deoxyribonucleoside phosphoramidites in the solid-phase synthesis of DNA oligonucleotides having the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl group as a phosphate/thiophosphate protecting group.[5] This protective group is easily removed from oligonucleotides by simple treatment with concentrated ammonium hydroxide or by pressurized ammonia gas.[7] The nonmutagenic side products, 2,2,2-trifluoroacetamide and *N*-methylpyrrolidine, are formed during the course of the deprotection reaction. Although these phosphoramidites lead to alkylation-free DNA oligonucleotide products, 4-(*N*-methylamino)butan-1-ol, which is required in the synthesis of the phosphoramidites, is not commercially available and, consequently, must be synthesized. Because of this added expenditure, the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl deoxyribonucleoside phosphoramidites might not effectively compete with the conventional but less desirable 2-cyanoethyl deoxyribonucleoside phosphoramidites to produce oligonucleotide drugs at the most affordable cost per dose.

In an effort to reduce the cost of therapeutic oligonucleotides, we initiated a search for inexpensive phosphate/thiophosphate protecting groups. Recently, we described the use of the 4-oxopentyl group as a labile phosphate/thiophosphate protecting group in the synthesis of DNA oligonucleotides.[8] The starting material for this protecting group, 4-oxopentan-1-ol, is commercially available and considerably less expensive than 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol. DNA oligonucleotides are readily prepared from 4-oxopentyl deoxyribonucleoside phosphoramidites and the 4-oxopentyl phosphate/thiophosphate protecting group is cleanly removed under very mild conditions by treatment with pressurized ammonia, methylamine gas, or concentrated ammonium hydroxide at ambient temperature.[7,8] Interestingly, the 4-oxopentyl phosphate/thiophosphate protecting group can also be cleaved from oligonucleotides in less than 1 h (in the absence of gaseous or aqueous amines) simply by heating at 90 °C in a neutral aqueous buffer.[8] In this context, the 2-(*N*-formyl-*N*-methylamino)-ethyl group used to protect phosphate/thiophosphate diesters in the solid-phase synthesis of oligodeoxyribonucleotides is also removable under thermolytic conditions at neutral pH.[9] The 2-(*N*-formyl-*N*-methylamino)-ethyl deoxyribonucleoside phosphoramidites are prepared from inexpensive reagents and, hence, are suitable for cost-efficient oligonucleotide syntheses. Since the 2-(*N*-formyl-*N*-methylamino)ethyl phosphate/thiophosphate protecting group cannot be removed reliably by standard ammonolysis, it is cleaved from oligonucleotides only upon heating at 90 °C in an aqueous buffer (pH 7.0) over a period of at least 3 h.[9] Although the group is cleanly removed from phosphate or phosphorothioate triesters, its long deprotection time detracts from an otherwise efficient process. We now propose the use of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group for enhanced phosphate/thiophosphate protection, and report its incorporation into oligonucleotides via the deoxyribonucleoside phosphoramidites **1a**−**d**. The usefulness of these phosphoramidites is demonstrated in the solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioate analogue. The rapid and facile removal of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl phosphate/thiophosphate protecting group under thermolytic conditions at neutral pH is shown, and the factors influencing deprotection kinetics under these conditions are addressed.



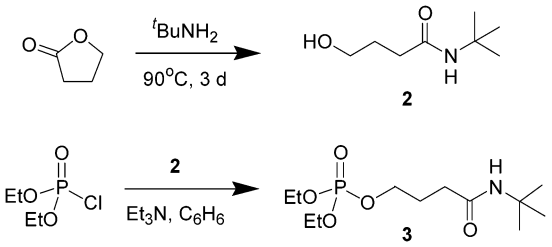
1a B = Thymin-1-yl
b B = 4-*N*-Benzoylcytosin-1-yl
c B = 6-*N*-Benzoyladenin-9-yl
d B = 2-*N*-Isobutyrylguanin-9-yl

DMTr = 4,4'-dimethoxytrityl

Results and Discussion

A direct approach to the preparation of *N*-*tert*-butyl-4-hydroxybutyramide (**2**) in essentially quantitative yields involves heating γ-butyrolactone and *tert*-butylamine at 90 °C in a sealed glass container. Condensation of **2** with *O*,*O*-diethyl chlorophosphate and triethylamine in benzene affords *O*-[3-(*N*-*tert*-butylcarboxamido)-1-propyl]-*O*,*O*-diethyl phosphate (**3**) as a simple phosphotriester model (Scheme 1).

SCHEME 1



Removal of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group from purified **3** is then monitored by ^{31}P NMR spectroscopy in D_2O at 80 °C.[10] Within 35 min under these conditions, phosphotriester **3** (δ_P 0.8 ppm) is efficiently converted to the diethyl phosphate salt **4** (δ_P 2.0 ppm) via an intramolecular cyclization of the amide as shown in Figure 1. Our findings are consistent with the known intramolecular cyclization of *N*-substituted amides of ω-bromocarboxylic acids, as reported by Stirling more than 40 years ago.[11] The identity of the 2-(*tert*-butylimino)tetrahydrofuran cation in **4** is confirmed through its synthesis by reacting *tert*-butylamine with 4-bromobutyryl chloride, followed by heating the amide product to ∼140 °C.[11] The ^{1}H and proton-decoupled ^{13}C NMR spectra of salt **4** and of 2-(*tert*-butylimino)tetrahydrofuran hydrobromide show diagnostic signals having identical chemical shifts in D_2O.[12]

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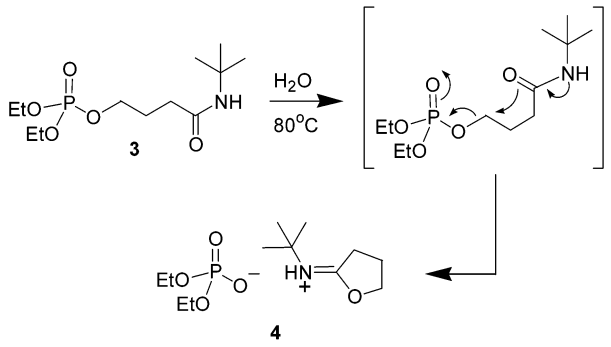
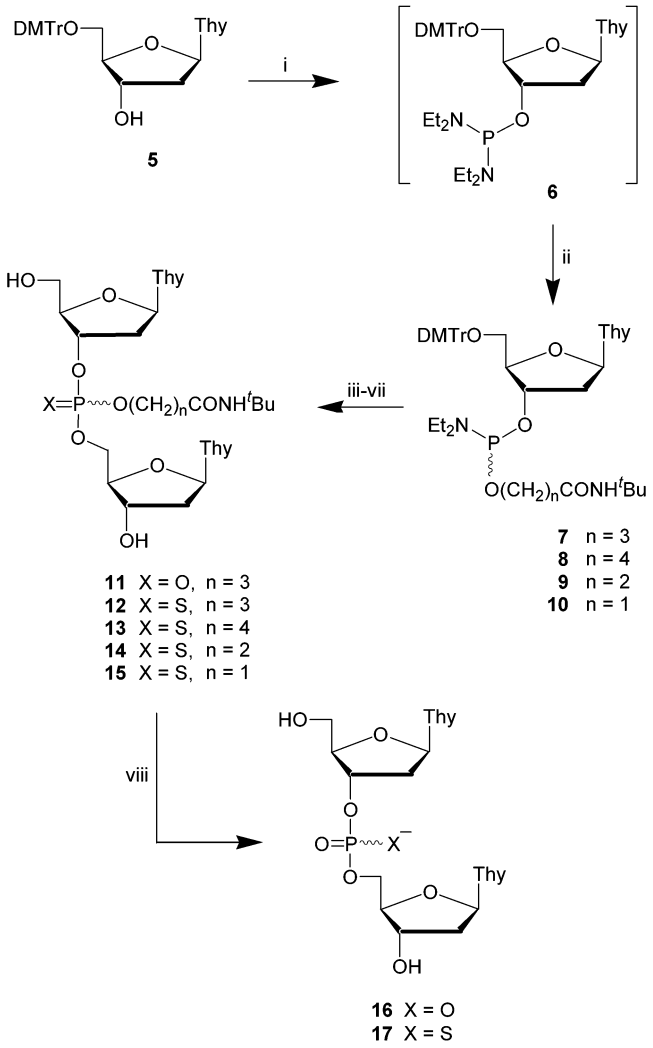


FIGURE 1. Proposed mechanism for the removal of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl phosphate protecting group under thermolytic conditions at neutral pH.

The suitability of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group for phosphate/thiophosphate protection in DNA oligonucleotide syntheses is evaluated first on a dinucleoside phosphate triester and its phosphorothioate analogue. These are prepared from the reaction of 5′-*O*-(4,4′-dimethoxytrityl)thymidine (**5**) with an equimolar amount of tris(diethylamino)phosphine and diethylammonium tetrazolide in dry methylene chloride.[13] The resulting nucleoside 3′-*O*-phosphordiamidite intermediate **6** is not isolated, but is immediately condensed with a slight excess (1.2 equiv) of amido alcohol **2** to afford the desired deoxyribonucleoside phosphoramidite **7** (Scheme 2). The crude phosphoramidite is purified by silica gel chromatography and, then, used in a manual solid-phase synthesis of dinucleoside phosphotriesters **11** and **12** (Scheme 2) according to published procedures.[9] The dinucleotides **11** and **12** are released from the solid support upon treatment with pressurized methylamine gas, and are then purified by reversed-phase (RP) HPLC. Heating either **11** or **12** in 0.1 M triethylammonium acetate (pH 7.0) at 90 °C leads to complete removal of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group to afford either the dinucleoside phosphodiester **16** or ***R*$_P$-17** and ***S*$_P$-17**, respectively, as the sole nucleic acid products. The reaction half-time of the phosphate/thiophosphate deprotection is ∼100 s, as determined by monitoring **16** or **17** by RP-HPLC at various time points during the course of the reaction (see Experimental Section). There is no indication of diastereomeric bias in the removal of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group from either phosphate or thiophosphate triesters under neutral aqueous conditions at 90 °C. Indeed, the thiophosphate deprotection kinetics of RP-HPLC purified ***R*$_P$-12** or ***S*$_P$-12** are, under similar conditions, indistinguishable. As expected, deprotection proceeds with retention of configuration at phosphorus with no significant desulfurization of the phosphorothioate.[9]

Since the cleavage of the 4-oxopentyl phosphate/thiophosphate protecting group occurs under neutral aqueous conditions at elevated temperature,[8] it is tentatively proposed that a suitably located carbonyl group relative to the phosphate/thiophosphate leaving group is all that is necessary for deprotection. Consistent with this postulate, the 2-[*N*-(2-fluoroacetyl)amino]-1-phenylethyl,[14] 2-(*N*-acetylamino)ethyl,[9] 2-(*N*-acetyl-*N*-methylamino)ethyl,[9] 2-(*N*-formyl-*N*-methylamino)ethyl,[9] 2-(*N*,*N*-dimethylaminocarbonyloxy)ethyl,[9] and several 2-benzamidoethyl groups[3] are all cleaved from respective phosphate/thiophosphate triesters under neutral aqueous conditions, and all have a carbonyl group located at a similar distance from the phosphate/thiophosphate leaving group.

SCHEME 2[a,b]



[a] Reaction conditions: (i) $(Et_2N)_3P$/diethylammonium tetrazolide/CH_2Cl_2, 25 °C, 30 min; (ii) **2** or *N*-*tert*-butyl-5-hydroxyvaleramide or *N*-*tert*-butyl-3-hydroxypropionamide or *N*-*tert*-butyl-2-hydroxyacetamide, 25 °C, 8 h followed by purification on silica gel; (iii) T-LCAA-CPG/1*H*-tetrazole/MeCN; (iv) 0.02 M I_2 in THF/pyridine/water or 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN; (v) 3% TCA/CH_2Cl_2; (vi) $MeNH_2$ gas (∼ 2 bar), 1 min; (vii) RP-HPLC purification; (viii) 0.1 M TEAA, pH 7.0, 90 °C. [b] Key: Thy, thymin-1-yl; T, thymidine 3′-*O*-succinyl; LCAA, long chain alkylamine; CPG, controlled-pore glass; TEAA, triethylammonium acetate.

(12) The following NMR signals are found in the spectrum of **4** and that of 2-(*tert*-butylimino)tetrahydrofuran hydrobromide: ^{1}H NMR (300 MHz, D_2O), δ 1.28 (s, 9H), 2.21 (tt, J = 7.3 and 8.4 Hz, 2H), 2.98 (t, J = 8.4 Hz, 2H), 4.79 (t, J = 7.3 Hz, 2H). ^{13}C NMR (75 MHz, D_2O), δ 26.5, 32.4, 36.9, 62.4, 85.6, 185.8.

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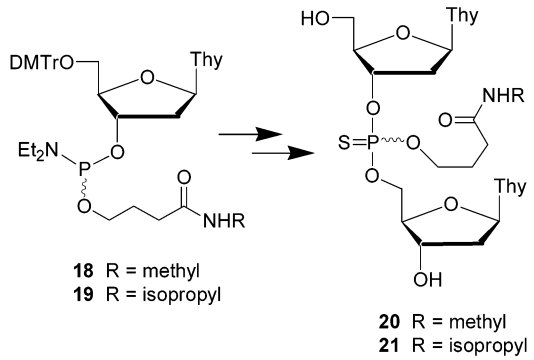
The nature of the spatial arrangement of the carbonyl group for the removal of the 3-(*N-tert*-butylcarboxamido)-1-propyl thiophosphate protecting group was investigated further. Thus, dinucleoside phosphorothioates **13**−**15** carrying a 4-(*N-tert*-butylcarboxamido)-1-butyl group, a 2-(*N-tert*-butylcarboxamido)ethyl group, and a (*N-tert*-butylcarboxamido)methyl group, respectively, were prepared from the corresponding deoxyribonucleoside phosphoramidites **8**−**10** (Scheme 2) in a manner identical to that used for the preparation of **12**.

Heating **13** in 0.1 M triethylammonium acetate (pH 7.0) at 90 °C leads to its complete conversion to **17**. The half time of thiophosphate deprotection was determined as described for **12** (vide supra) and was found to be 42 min. Thus, moving the carbonyl group one bond-length *away* from the thiophosphate leaving group results in a ∼25-fold reduction in the deprotection rate as compared to that determined for **12**. However, when moving the carbonyl group one bond-length *closer* to the thiophosphate leaving group (such as in **14**), deprotection of the 2-(*N-tert*-butylcarboxamido)ethyl group is very sluggish under the conditions used for deprotecting **12**; the half-time of thiophosphate deprotection is estimated to be in excess of 65 h. Since an intramolecular cyclization of the amide leading to the formation of a four-membered iminolactone is very unlikely, a β-elimination mechanism with simultaneous formation of *N-tert*-butylacrylamide may be preferred in this case, as the formation of **17** is pH-dependent. Indeed, heating **14** in 20% NH_4OH (pH 12) at 90 °C produces **17** to the extent of 50% within 25 min, whereas heating **14** in 0.1 M triethylammonium acetate (pH 4) at the same temperature generates **17** very slowly ($t_{1/2}$ > 100 h). These findings strongly suggest that under thermolytic conditions at pH 7.0, the 2-(*N-tert*-butylcarboxamido)ethyl group may predominantly be removed from **14** via a β-elimination mechanism rather than through a cyclodeesterification mechanism.

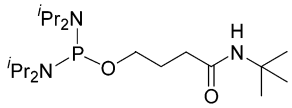
Heating **15** to effect thiophosphate deprotection is also observed to proceed slowly ($t_{1/2}$ > 16 h). This may be expected because of the steric constraints associated with intramolecular cyclization of the amide thiophosphate protecting group to an imino-α-lactone. Thus, phosphate/thiophosphate deprotection experiments performed with the dinucleoside phosphotriesters **11**−**15** have collectively shown a critical relationship between the deprotection reaction rate and the distance separating the amidic carbonyl from the phosphate/thiophosphate function.

We also investigated 3-(*N*-methylcarboxamido)-1-propanol and 3-(*N*-isopropylcarboxamido)-1-propanol as potential protecting groups for phosphates/thiophosphates in solid-phase oligonucleotide synthesis. These amido alcohols were prepared similarly as described in Scheme 1 and then incorporated into model dinucleoside phosphorothioate triesters **20** and **21**, which were synthesized from deoxyribonucleoside phosphoramidites **18** and **19**, respectively, in a manner identical to that shown in Scheme 2. As in the case of **12**, heating RP-HPLC purified **20** or **21** in 0.1 M triethylammonium acetate (pH 7.0) at 90 °C leads to **17** with a reaction half time of ∼300 s. On the basis of RP-HPLC analysis, deprotection of **20** or **21** occurs without significant desulfurization of the phosphorothioate diester.[9]

Thiophosphate deprotection of the model dinucleoside phosphorothioate triesters **12**, **20**, and **21** is an attractive and facile process that strongly suggests its application to solid-phase synthesis of oligodeoxyribonucleotides. Low cost and ease of preparation of a particular amido alcohol are major factors influencing its selection as a phosphate/thiophosphate protecting group for an economical and alkylation-free oligodeoxyribonucleotide synthesis. Considering all factors, the 3-(*N-tert*-butylcarboxamido)-1-propyl group is currently the superior choice, and its utility is now demonstrated for phosphate/thiophosphate protection in the solid-phase synthesis of a 20-mer, d(ATCCGTAGCTAAGGTCATGC), and its phosphorothioate analogue.



Thus, reaction of *N-tert*-butyl-4-hydroxybutyramide (**2**) with bis(*N*,*N*-diisopropylamino)chlorophosphine, which is generated immediately prior to use from phosphorus trichloride and *N*,*N*-diisopropylamine in dry benzene, affords the *O*-[3-(*N-tert*-butylcarboxamido)-1-propyl]-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphordiamidite **22**.



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Crude **22** may then be activated using 1*H*-tetrazole and condensed with 5′-*O*-(4,4′-dimethoxytrityl)-*N*-protected deoxyribonucleosides in dry dichloromethane to give the corresponding deoxyribonucleoside phosphoramidites **1a**−**d**. These phosphoramidites were purified by chromatography on silica gel and then used in the automated solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioate analogue using standard synthesis cycle parameters. Upon completion of the syntheses, each oligonucleotide is treated with pressurized ammonia gas (∼10 bar) for 10 h at 25 °C to effect both its release from the CPG support and *N*-deprotection of the nucleobases. The unmodified 20-mer is eluted from the CPG column with PBS buffer, pH 7.2, and then heated at 90 °C for 30 min to remove the phosphate protecting groups.[15] Using polyacrylamide gel electrophoresis (PAGE), the coupling efficiency of **1a**−**d** in the preparation of the 20-mer can be compared with that of commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites (see Figure 2). The

(15) We noticed that the removal of the 3-(*N-tert*-butylcarboxamido)-1-propyl phosphate protecting group from **3** under thermolytic conditions resulted in an acidic solution. Although this pH drop from neutrality did not affect the outcome of the deprotection reaction, it is nonetheless recommended to use PBS buffer (pH 7.2) for the thermolytic cleavage of 3-(*N-tert*-butylcarboxamido)-1-propyl phosphate/thiophosphate protecting group from DNA oligonucleotides to ensure neutrality throughout the deprotection reaction.

FIGURE 2. Polyacrylamide gel electrophoresis analysis of d(ATCCGTAGCTAAGGTCATGC) under denaturing conditions (7 M urea, 1X TBE buffer, pH 8.3). Left lane: crude oligomer synthesized from commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites and deprotected by treatment with concentrated NH_4OH for 10 h at 55 °C. Right lane: crude oligomer synthesized from **1a**−**d** and deprotected under pressurized ammonia gas (∼10 bar) for 10 h at 25 °C followed by heating in PBS buffer (pH 7.2) for 30 min at 90 °C. Oligonucleotides are visualized as blue bands upon staining the gel with Stains-all. Bromophenol blue is used as a marker and appears as a large band, in each lane, at the bottom of the gel.

PAGE profiles of these two syntheses are very similar in terms of product yields and distribution of shorter than full-length oligonucleotides.

The likelihood of nucleobase modification under the conditions used for chain assembly and complete oligonucleotide deprotection when using the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group for phosphate protection deserves further evaluation. Thus, after subjecting the crude deprotected oligonucleotide to snake venom phosphodiesterase and bacterial alkaline phosphatase, RP-HPLC analysis of the digest indicates that the oligonucleotide is completely converted to the expected nucleosides, free of any detectable nucleobase modifications.[10]

The possibility of nucleobase modification occurring under conditions mimicking large-scale oligonucleotide deprotection was also investigated. Specifically, a solution composed of thymidine, 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine (125 mM each), and 1 M 2-(*tert*-butylimino)tetrahydrofuran hydrobromide in 0.1 M triethylammonium acetate (pH 7.0) or PBS buffer (pH 7.2) was heated to 90 °C and maintained at that temperature for 1 h. RP-HPLC analysis of the reaction mixture shows that each 2′-deoxyribonucleoside is unaffected under such conditions and demonstrates the suitability of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group for phosphate/thiophosphate protection in large-scale oligonucleotide syntheses.

The stability of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl phosphate/thiophosphate protecting group to pressurized ammonia gas under the conditions used for deprotecting nucleobases and releasing oligonucleotides from the CPG support (10 bar, 10 h, 25 °C) was evaluated using the phosphorothioated 20-mer as a model. ^{31}P NMR analysis of the oligomer eluted from the CPG column with

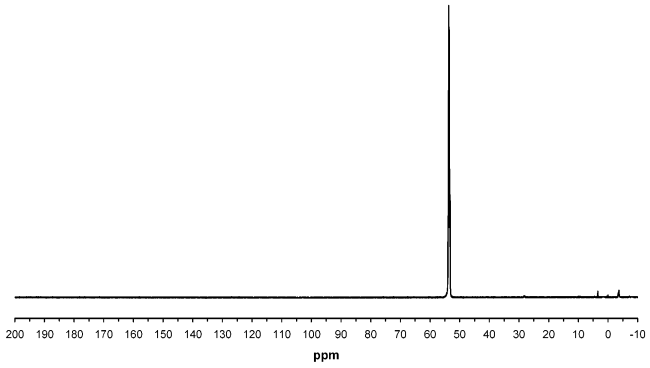

FIGURE 3. 121 MHz ^{31}P NMR spectrum of d($A_{PS}T_{PS}C_{PS}C_{PS}$-$G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}T_{PS}A_{PS}A_{PS}G_{PS}G_{PS}T_{PS}C_{PS}$ $A_{PS}T_{PS}G_{PS}C$) in D_2O.

deuterated water indicated that ∼60% of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl thiophosphate protecting groups are cleaved under these conditions, as estimated by the ratio of the integrated signals corresponding to phosphotriester (δ_P 64 ppm) and phosphodiester (δ_P 53 ppm), respectively.[10] Complete thiophosphate deprotection is accomplished within 25 min upon heating the oligonucleotide solution at 60 °C in the NMR probe (see Figure 3). Desulfurized material is observed ($\delta_P \sim 4$ to −4 ppm) in amounts comparable to that obtained when using the 2-cyanoethyl group for thiophosphate protection.

To further evaluate phosphorothioate desulfurization in the context of large-scale oligonucleotide deprotection, a solution of 0.5 M potassium *O*,*O*-diethylthiophosphate and 0.75 M 2-(*tert*-butylimino)tetrahydrofuran hydrobromide in 0.1 M triethylammonium acetate (pH 7.0) or PBS buffer (pH 7.2) was heated for 1 h at 90 °C. Analysis of the reaction mixture by ^{31}P NMR did not show any significant conversion of *O*,*O*-diethylthiophosphate (δ_P ∼55 ppm) to *O*,*O*-diethyl phosphate (δ_P ∼2 ppm).[10]

Conclusion

We have shown that the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group provides convenient phosphate/thiophosphate protection in solid-phase syntheses of oligonucleotides and their phosphorothioated analogues. The protecting group is prepared from inexpensive and readily available starting materials and is incorporated into the deoxyribonucleoside phosphoramidites **1a**−**d** in a very straightforward manner. Small-scale (0.2 μmol) solid-phase synthesis of oligodeoxyribonucleotides using either **1a**−**d** or commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites produces unmodified and phosphorothioated oligonucleotides in similar yields and purity. When a large-scale oligonucleotide deprotection reaction is simulated, no detectable nucleobase modification and no significant desulfurization of the phosphorothioates are observed. The 2-(*tert*-butylimino)tetrahydrofuran side-product that is generated during the removal of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group is innocuous toward the reaction mixture, as there are no unwanted secondary reactions resulting from its presence. Thus, use of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group for phosphate/thiophosphate protection in the large-scale preparation of therapeutic oligonucleotides is an obvious

choice and therefore recommended to ensure economic manufacture and optimal potency of the drugs.

One may speculate that the 3-(*N-tert*-butylcarboxamido)-1-propyl group will find further application as a phosphate/thiophosphate protecting group in the solid-phase synthesis of ribonucleotides, or in the preparation of phosphosugars, phosphopeptides, and inositol phosphates or thiophosphates. Currently, we are searching for novel thermolytic groups that can be used for hydroxyl protection in nucleic acid chemistry, the results of which will be the subject of future reports.

Experimental Section

Materials and Methods. Common chemicals and solvents were purchased from commercial sources and used without further purification. Lecture bottles of ammonia and methylamine gases, anhydrous pyridine, benzene, δ-valerolactone, γ-butyrolactone, 4-bromobutyryl chloride, aluminum chloride, *O*,*O*-diethyl chlorophosphate, *O*,*O*-diethyl thiophosphate (potassium salt), 3-hydroxypropionitrile, 1,1′-carbonyldiimidazole, methyl glycolate, *tert*-butylamine, isopropylamine, tris(diethylamino)phosphine, sublimed 1*H*-tetrazole, and Stains-all were obtained commercially and used as received. Acetonitrile, dichloromethane, *N*,*N*-diisopropylamine, triethylamine, 1,2-dichloroethane, and petroleum ether were refluxed over calcium hydride, distilled, and stored over 4 Å molecular sieves. Phosphorus trichloride (Aldrich) was distilled immediately prior to use. 4,4′-Dimethoxytrityl chloride and suitably protected 2′-deoxyribonucleosides were purchased from ChemImpex International and used without further purification. Unprotected 2′-deoxynucleosides were obtained from Sigma and used as received.

Preparative chromatographic purifications were performed on columns packed with 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 $CDCl_3$, 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.

Low and high-resolution FAB mass spectra were acquired from samples dissolved in a thioglycerol matrix containing sodium iodide as the reference. Samples were then subjected to bombardment with 8 keV fast cesium ions and accurate mass measurements ($n = 3$) were obtained by peak matching.

Gas chromatogaphy−mass spectrometry analyses were performed using a gas chromatograph equipped with a capillary inlet and a mass selective detector, controlled through a DOS-series MS ChemStation. Sample separations were performed on a 15 m × 0.25 mm fused-silica capillary column, wall-coated with 0.25 μm HP-1 cross-linked methyl siloxane (Hewlett-Packard). Helium was employed as the carrier gas at a linear velocity of 52 cm/s using flow rates of 3 and 50 mL/min at the septum purge and split vents, respectively. Temperatures were 250 °C at the injection port and 280 °C at the transfer line to the detector. Injections were made at an initial oven temperature of 60 °C. The inlet purge was activated at 2.0 min postinjection. The oven temperature was held isothermally at 60 °C for 2 min and then increased linearly to 275 °C at 20 °C/min. The final temperature was maintained for 5.0 min. Mass spectral detection (electron−ionization, 70 eV) was performed by scanning ions between *m*/*z* 50 and 550 at the rate of 1.5 scan/s. Data were collected between 2.5 and 17.8 min postinjection.

***N-tert*-Butyl-4-hydroxybutyramide (2).** γ-Butyrolactone (1.64 g, 19.0 mmol) and *tert*-butylamine (5.00 g, 68.4 mmol) are placed in a 20 mL thick-walled glass ampule. The container is flame-sealed and then heated at 90 °C for 3 days (**Caution! The glass container must be shielded as pressure develops**). The glass ampule is cooled to ambient temperature and opened. The reaction mixture is concentrated under reduced pressure until a crystalline material is obtained. The solid is recrystallized from chloroform/hexane affording **2** (2.96 g, 18.6 mmol, 98%) as white crystals (mp 73 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.23 (s, 9H), 1.59 (dt, $J = 6.6$, 7.5 Hz, 2H), 2.04 (t, $J = 7.5$ Hz, 2H), 3.34 (t, $J = 6.6$ Hz, 2H), 3.38 (s, 1H), 7.35 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 28.5, 28.7, 32.9, 49.8, 60.3, 171.8. EI-MS (70 eV): *m*/*z* (% relative abundance), 159 (M^+, 4), 144 (7), 115 (31), 87 (11), 86 (14), 59 (19), 58 (100), 57 (29).

***O*-[3-[(*N-tert*-Butyl)carboxamido]-1-propyl]-*O*,*O*-diethyl Phosphate (3).** *O*,*O*-Diethyl chlorophosphate (180 mg, 1.04 mmol) is added to a suspension of (*N-tert*-butyl)-4-hydroxybutyramide (**2**, 160 mg, 1.00 mmol) in 1 mL of dry benzene: triethylamine (1:1 v/v). The reaction mixture is stirred at ambient temperature for 12 h. The reaction mixture is then filtered to remove triethylamine hydrochloride and extracted with water (3 mL). The organic phase is collected and evaporated to dryness under reduced pressure. The residue is dissolved in chloroform (0.5 mL) and loaded onto a silica gel column (1.3 cm × 5 cm) preequilibrated in chloroform. The product is eluted from the column using a gradient of methanol (0−10% v/v) in chloroform. The desired fractions are collected and concentrated under reduced pressure affording 210 mg of **3** (0.71 mmol, 71%) as a colorless viscous oil. ^{1}H NMR (300 MHz, D_2O): δ 1.10 (s, 9H), 1.14 (dt, $J = 7.1$ Hz, $^4J_{PH} = 1.0$ Hz, 6H), 1.75 (tt, $J = 6.3$, 7.1 Hz, 2H), 2.08 (t, $J = 7.1$ Hz, 2H), 3.90 (dt, $J = J_{PH} = 6.3$ Hz, 2H), 3.97 (dq, $J = 7.1$ Hz, $J_{PH} = 8.1$ Hz, 4H). ^{13}C NMR (75 MHz, C_6D_6): δ 27.2 (d, $J_{PC} = 6.4$ Hz), 28.8, 33.1, 50.8, 63.5 (d, $^2J_{PC} = 6.4$ Hz), 64.9, 66.5 (d, $J_{PC} = 6.4$ Hz), 170.9. ^{31}P NMR (121 MHz): δ 1.44 (C_6D_6) or 0.69 (D_2O).

2-(*N-tert*-Butylimino)tetrahydrofuran Hydrobromide. This compound is prepared according to the procedure of Sterling.[11] A solution of *tert*-butylamine (2.0 g, 27 mmol) in ether (10 mL) is added over a period of 15 min to a vigorously stirred etheral solution of 4-bromobutyryl chloride (2.0 g, 11 mmol). The reaction mixture is then immediately washed with water (3 × 20 mL), and the organic phase is dried over anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. Upon further drying under high vacuum, the gummy mass crystallized. Crude *N-tert*-butyl-4-bromobutyramide (1.00 g, 4.25 mmol) is heated in a flame-sealed glass ampule until it melted at ∼140 °C. The melt resolidified within minutes to give a crystalline product. Slow diffusion of ether into an ethanol solution of the crude product gave pure 2-(*N-tert*-butylimino)tetrahydrofuran hydrobromide as colorless crystals (mp 174−175 °C) in essentially quantitative yield. ^{1}H NMR (300 MHz, D_2O), δ 1.28 (s, 9H), 2.21 (tt, $J = 7.3$ and 8.4 Hz, 2H), 2.98 (t, $J = 8.4$ Hz, 2H), 4.79 (t, $J = 7.3$ Hz, 2H). ^{13}C NMR (75 MHz, D_2O), δ 26.5, 32.4, 36.9, 62.4, 85.6, 185.8.

***N-tert*-Butyl-5-hydroxyvaleramide.** This compound is prepared according to a literature procedure.[16] Aluminum chloride (13.0 g, 97.5 mmol) is suspended in 1,2-dichloroethane (50 mL) and cooled to 5 °C in an ice bath. Anhydrous triethylamine (10.0 g, 98.8 mmol) in 1,2-dichloroethane (30 mL) is added to the stirred suspension. The reaction mixture is left stirring for 15 min and then allowed to warm to room temperature. A solution of δ-valerolactone (7.0 g, 70 mmol) and *tert*-butylamine (5.7 g, 78 mmol) in 1,2-dichloroethane (20 mL) is then added to the suspension, dropwise, over 30 min.

(16) (a) Lesimple, P.; Bigg, D. C. H. *Synthesis* **1991**, 306−308. (b) Bigg, D. C. H.; Lesimple, P. *Synthesis* **1992**, 277−278.

After additional stirring (2 h) at room temperature, the reaction mixture is quenched with ice/water (~200 mL). Upon addition of sodium carbonate (100 g), the mixture is extracted with ethyl acetate (10 × 30 mL). The organic fractions are combined, dried over magnesium sulfate, and concentrated under reduced pressure. The residue is dissolved in dichloromethane (30 mL) and filtered through a 45 μm nylon membrane filter. The solution is brought to a boil, and hexane is added until opalescence of the solution, which is left to cool slowly to room temperature and then stored at 4 °C for 12 h. The crystalline product that is formed is filtered off, washed with hexane, and air-dried to give *N*-*tert*-butyl-5-hydroxyvaleramide (6.7 g, 39 mmol, 56%) as white crystals (mp 70 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.34 (s, 9H), 1.60 (m, 2H), 1.70 (m, 2H), 2.15 (t, J = 7.0 Hz, 2H), 3.64 (t, J = 6.2 Hz, 2H), 4.09 (s, 1H), 5.33 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.5, 28.9, 32.1, 36.9, 51.2, 62.1, 172.5. EI-MS (70 eV): *m*/*z* (% relative abundance), 173 (M^+, 2), 158 (3), 155 (11), 115 (6), 101 (15), 100 (15), 83 (6), 72 (6), 59(15), 58 (100).

***N*-*tert*-Butyl-3-hydroxypropionamide.** 3-Hydroxypropionic acid[17] (1.00 g, 11.1 mmol) is coevaporated twice with pyridine (50 mL) and then concentrated to volume of ~20 mL. 4,4′-Dimethoxytrityl chloride (4.00 g, 11.8 mmol) is added to the solution, and the reaction mixture is left stirring at room temperature for 12 h. The reaction mixture is then poured into a saturated solution of sodium bicarbonate (100 mL) and extracted with ethyl acetate (3 × 50 mL). The organic fractions are collected and evaporated under reduced pressure, affording a foam. The product is then dissolved in chloroform and loaded onto a silica gel column (2.5 cm × 5 cm) preequilibrated in chloroform. The desired product is eluted from the column using a gradient of methanol (0−10% v/v) in chloroform. It is important to ensure that the chloroform used for column equilibration and the preparation of various eluents contains pyridine (1% v/v) to prevent premature cleavage of the 4,4′-dimethoxytrityl ether. Appropriate fractions are pooled, concentrated, and dried under high vacuum to give 4.3 g of pyridinium 3-(4,4′-dimethoxytrityloxy)propionate as a viscous oil. The dry material is mixed with 1,1′-carbonyldiimidazole (3.5 g, 22 mmol) and dry THF (20 mL), and the resulting solution is heated at 50 °C for 2 h. Then, *tert*-butylamine (2.0 g, 27 mmol) and triethylamine (2 mL) are added to the solution, which is left stirring at 50 °C for an additional 3 h. The reaction mixture is evaporated to dryness and kept under high vacuum overnight. The material is triturated in ethyl acetate:hexane (1:1 v/v), and the sludge is applied onto a silica gel column (6 cm × 5 cm) preequilibrated in ethyl acetate:hexane (1:1 v/v). The product is eluted from the column using a gradient of ethyl acetate (50−70% v/v) in hexane. Selected fractions are collected, concentrated, and dried under high vacuum, affording 1.47 g of *N*-*tert*-butyl-3-(4,4′-dimethoxytrityloxy)propionamide as an amorphous solid. The 4,4′-dimethoxytrityl ether derivative is dissolved in dichloromethane (10 mL), and solid *p*-toluenesulfonic acid monohydrate (650 mg, 3.42 mmol) is added in one portion to the solution. After 15 min, the reaction mixture is loaded onto a silica gel column (13 mm × 5 cm) preequilibrated in chloroform. The product is eluted from the column using a gradient of methanol (0−5% v/v) in chloroform. Elution of the desired product is monitored by thin-layer chromatography and phosphomolybdic acid staining. Product-containing fractions are combined, concentrated, and dried under high vacuum. *N*-*tert*-Butyl-3-hydroxypropionamide is recrystallized from methylene chloride:hexane (1:4 v/v) to produce large white crystals (180 mg) melting at 93 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.36 (s, 9H), 2.35 (t, J = 5.5 Hz, 2H), 3.39 (s, 1H), 3.85 (t, J = 5.5 Hz, 2H), 5.70 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.0, 38.9, 51.5, 59.2, 172.1. EI-MS (70 eV): *m*/*z* (% relative abundance), 145 (M^+, 9), 130 (26), 90 (8), 73 (12), 58 (100), 57 (19).

(17) Read, R. R. *Organic Syntheses*; Wiley: New York, 1941; Collect. Vol. I, pp 321−322.

***N*-*tert*-Butylglycolamide.** Methyl glycolate (9.00 g, 100 mmol) is mixed with *tert*-butylamine (16.0 g, 219 mmol) in a 50 mL round-bottom flask equipped with a reflux condenser and a drying tube. The reaction mixture is refluxed for 16 h. Excess amine is evaporated under reduced pressure, and the residue is further purified by recrystallization from chloroform:hexane. *N*-*tert*-Butylglycolamide (4.8 g, 37 mmol) is obtained as white crystals (mp 75 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.39 (s, 9H), 3.97 (s, 2H), 6.19 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.9, 51.2, 62.4, 170.6. EI-MS (70 eV): *m*/*z* (% relative abundance), 131 (M^+, 20), 116 (51), 100 (7), 76 (16), 58 (85), 57 (100).

***N*-Methyl-4-hydroxybutyramide.** This compound is prepared and characterized as reported earlier.[5a] EI-MS (70 eV): *m*/*z* (% relative abundance), 117 (M^+, 0.3), 99 (5), 87 (42), 86 (10), 73 (100), 69 (14), 59 (9), 58 (69).

***N*-Isopropyl-4-hydroxybutyramide.** This compound is prepared in a manner identical to that described for the preparation of **2** and isolated as a viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.15 (d, J = 6.6 Hz, 6H), 1.86 (m, 2H), 2.32 (t, J = 6.4 Hz, 2H), 3.29 (bs, 1H), 3.68 (t, J = 5.8 Hz, 2H), 4.06 (m, 1H), 5.94 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.7, 28.4, 34.2, 41.6, 62.3, 172.6. EI-MS (70 eV): *m*/*z* (% relative abundance), 145 (M^+, 7), 130 (7), 115 (21), 114 (22), 112 (59), 101 (100), 86 (50), 70 (26).

General Procedure for the Preparation of the Deoxyribonucleoside Phosphoramidites 7−10, 18, and 19. 5′-*O*-(4,4′-Dimethoxytrityl)thymidine (**5**, 270 mg, 0.50 mmol) and diethylammonium tetrazolide (34 mg, 0.49 mmol) are dried under high vacuum for 5 h. Dry methylene chloride (5 mL) is added under a dry argon atmosphere followed by tris(diethylamino)phosphine (125 mg, 0.50 mmol). After 20 min, the appropriate amido alcohol (0.60 mmol) is added to the solution, and the reaction mixture is stirred at room temperature for 8 h. The reaction mixture is then evaporated to dryness under reduced pressure, resuspended in 1 mL benzene:triethylamine (9:1 v/v), and loaded onto a silica gel chromatography column (13 mm × 50 mm). The reaction product is eluted from the column using benzene:triethylamine (9:1 v/v) as the eluent. Selected fractions are pooled and concentrated to dryness under vacuum. The deoxyribonucleoside phosphoramidites **7**−**10**, **18**, and **19** are isolated as white amorphous solids in yields ranging from 75 to 95%.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diethylamino)[3-(*N*-*tert*-butylcarboxamido)-1-propyloxy]phosphinyl-2′-deoxythymidine (7). ^{31}PNMR (121 MHz, C_6D_6): δ 148.6, 149.3.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diethylamino)-[4-(*N*-*tert*-butylcarboxamido)butyloxy]phosphinyl-2′-deoxythymidine (8). ^{31}P NMR (121 MHz, C_6D_6): δ 149.1, 149.4.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diethylamino)-[2-(*N*-*tert*-butylcarboxamido)ethyloxy]phosphinyl-2′-deoxythymidine (9). ^{31}P NMR (121 MHz, C_6D_6): δ 148.9, 149.5.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diethylamino)-[(*N*-*tert*-butylcarboxamido)methyloxy]phosphinyl-2′-deoxythymidine (10). ^{31}P NMR (121 MHz, C_6D_6): δ 149.8, 150.7.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diethylamino)[3-(*N*-methylcarboxamido)-1-propyloxy]phosphinyl-2′-deoxythymidine (18). ^{31}PNMR (121 MHz, C_6D_6): δ 144.8, 145.2.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diethylamino)[3-(*N*-isopropylcarboxamido)-1-propyloxy]phosphinyl-2′-deoxythymidine (19). ^{31}PNMR (121 MHz, C_6D_6): δ 148.6, 149.2.

General Procedure for the Preparation of the Dinucleoside Phosphate Triester 11 and Phosphorothioate Triesters 12−15, 20, 21. Manual solid-phase dinucleotide syntheses are performed on a 0.2 μmol scale under conditions recommended for standard automated DNA synthesis. The sulfurization reaction required in the preparation of dinucleoside phosphorothiates is effected by treatment with 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Glen Research) in ac-

TABLE 1. RP-HPLC Retention Time (R_T) of Dinucleoside Phosphotriesters

dinucleotide	R_T (min)
11	26.4, 26.7
12	32.7, 33.1
13	34.6, 35.1
14	31.3, 31.7
15	24.6, 25.1
20	28.2, 28.4
21	32.2[a]

[a] R_P and S_P diastereomers are not resolved under the chromatographic conditions used.

etonitrile as recommended in the literature.[18] Cleavage of the dinucleoside phosphotriesters from the solid support is achieved upon exposure to gaseous methylamine (~2 bar) for 1 min.[7] The phosphotriesters **11**−**15**, **20**, and **21** are then eluted from the respective columns with 0.1 M triethylammonium acetate (pH 7.0, 0.3 mL). Each of the dinucleoside phosphotriesters are purified 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. Peaks corresponding to either the R_P or S_P diastereomer of dinucleoside phosphotriesters **11**−**15**, **20**, and **21** are collected individually. The RP-HPLC retention time (R_T) of each pair of diastereomers are shown in Table 1.

Phosphate/Thiophosphate Deprotection Studies of the RP-HPLC Purified Dinucleoside Phosphotriesters 11−15, 20, and 21. These studies are aimed at providing relatively accurate assessments of the deprotection rates of selected nucleotidic phosphate/phosphorothioate protecting groups under thermolytic conditions. Typically, each of five 150 μL-aliquots of a RP-HPLC-purified and, when possible, *P*-stereopure dinucleoside phosphate/phosphorothioate triester in 0.1 M triethylammonium acetate (pH 7.0) is flame-sealed in a 1 mL glass ampule. The samples are then placed in a thermostated heat block at 90 ± 2 °C. At appropriate time-points, samples are taken out the heat block and immediately frozen in dry ice. Reference samples (t = 0) are handled identically without being heated. Thawed samples are analyzed by RP-HPLC under the conditions used for the purification of the dinucleoside phosphotriesters (see Experimental Section). Phosphate/thiophosphate deprotection during RP-HPLC analysis at 25 °C is negligible as evidenced by the reference samples (t = 0). The amounts of dinucleoside phosphodiester formation recorded for each time point is calculated from the integrated peak area of **16** or $\boldsymbol{R_P}$/$\boldsymbol{S_P}$**-17** relative to that determined for unreacted dinucleoside phosphotriesters. The half time ($t_{1/2}$) of each deprotection reaction is determined from the best fit of the first-order curve and is reported, where appropriate, in the Results and Discussion section. The idendity of **16** or $\boldsymbol{R_P}$/$\boldsymbol{S_P}$**-17** is corroborated with authentic commercial samples or with samples that were synthesized using 2-cyanoethyl deoxyribonucleoside phosphoramidites under standard conditions.

***O*-[3-(*N*-*tert*-Butylcarboxamido)-1-propyl] *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (22).** Anhydrous diisopropylamine (19.6 mL, 140 mmol) is added dropwise under an argon atmosphere to a solution of freshly distilled phosphorus trichloride (1.75 mL, 20 mmol) in dry benzene (100 mL). The reaction mixture is left stirring at 25 °C until *complete* transformation of (*N*,*N*-diisopropylamino)dichlorophosphine (δ_P 168.2 ppm in C_6D_6) to bis(*N*,*N*-diisopropylamino)chlorophosphine (δ_P 134.1 ppm in C_6D_6) is achieved (~3−4 d), as indicated by ^{31}P NMR spectroscopy. A solution of *N*-*tert*-butyl-4-hydroxybutyramide (**2**, 3.18 g, 20 mmol) in benzene: acetonitrile (1:1 v/v, 20 mL) is then added to the stirred suspension and allowed to react for 2 h at 25 °C. Diisopropylammonium hydrochloride is filtered off, and the filtrate is concentrated under reduced pressure affording **22** as a yellowish oil. Crude **22** (δ_P 123.2 ppm in C_6D_6)[10] is immediately used without further purification in the synthesis of deoxyribonucleoside phosphoramidites **1a**−**d**. For analytical purposes, **22** (0.6 g) is dissolved in benzene/triethylamine (9:1 v/v, 3 mL) and chromatographed on a silica gel column (2.5 cm × 6 cm) using benzene/triethylamine (9:1 v/v) as an eluent. Fractions are collected, and those containing the product are pooled and evaporated to dryness under reduced pressure affording **22** (0.5 g) as a white crystalline solid (mp 71−72 °C). ^{1}H NMR (300 MHz, C_6D_6):[10] δ 1.19 (d, J = 6.8 Hz, 12H), 1.22 (d, J = 6.8 Hz, 12H), 1.25 (s, 9H), 2.00 (m, 2H), 2.05 (m, 2H), 3.48 (sept, J = 6.8 Hz, 2H), 3.52 (sept, J = 6.8 Hz, 2H), 3.63 (dt, J = 6.0 Hz, $^3J_{PH}$ = 7.3 Hz, 2H). ^{13}C NMR (75 MHz, C_6D_6):[10] δ 24.0, 24.1, 24.7, 24.8, 28.1 (d, $^3J_{PC}$ = 8.5 Hz), 28.8, 34.0, 44.6, 44.7, 50.7, 63.7 (d, $^2J_{PC}$ = 21.2 Hz), 171.1. ^{31}P NMR (121 MHz, C_6D_6): δ 123.3.

General Procedure for the Preparation of the Deoxyribonucleoside Phosphoramidites 1a−d. A suitably protected 2′-deoxyribonucleoside (2.0 mmol) is dried under high vacuum for 4 h in a 50 mL round-bottom flask. Anhydrous methylene chloride (10 mL) is added under an argon atmosphere followed by crude **22** (818 mg, ~2.1 mmol). To the magnetically stirred solution is added sublimed 1*H*-tetrazole (140 mg, 2.0 mmol), portionwise, over a period of 1 h. The progress of the reaction is monitored by TLC using either benzene:triethylamine (9:1 v/v) or methylene chloride:hexane:triethylamine (3:6:1 v/v/v) as eluents. Phoshinylation of protected 2′-deoxyribonucleosides is usually complete within 3 h at 25 °C. However, in the case of N^2-isobutyryl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyguanosine, the phosphinylation reaction time is extended to 12 h to ensure optimum yields of **1d**. The reaction mixture is then evaporated to dryness under vacuum, resuspended in 1 mL of methylene chloride:hexane:triethylamine (1:8:1 v/v/v), and chromatographed on a silica gel column (2.5 cm × 10.0 cm) preequilibrated in methylene chloride:hexane:triethylamine (1:8:1 v/v/v). The column is first eluted with methylene chloride:hexane:triethylamine (1:8:1 v/v/v), and then the concentration of methylene chloride is increased until final solutions composed of 3:6:1 (**1a**), 5:4:1 (**1b**), 7:2:1 (**1c**), and 8:1:1 (**1d**) (v/v/v) methylene chloride:hexane:triethylamine elute pure phosphoramidites. Appropriate fractions are collected, pooled together, and evaporated to dryness under vacuum. Phosphoramidites **1a**−**d** are obtained as white amorphous solids in yields ranging from 70 to 90%.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)-[3-(*N*-*tert*-butylcarboxamido)-1-propyloxy]phosphinyl-2′-deoxythymidine (1a). ^{31}P NMR (121 MHz, C_6D_6): δ 148.3, 148.5. FAB−HRMS: calcd for $C_{45}H_{61}N_4O_9P$ $(M + Na)^+$ 855.4074, found 855.4083.

***N*^{4}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)[3-(*N*-*tert*-butylcarboxamido)-1-propyloxy]-phosphinyl-2′-deoxycytidine (1b).** ^{31}P NMR (121 MHz, C_6D_6): δ 148.3, 149.1. FAB−HRMS: calcd for $C_{51}H_{64}N_5O_9P$ $(M + Cs)^+$ 1054.3496, found 1054.3480.

***N*^{6}-Benzoyl-5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)[3-(*N*-*tert*-butylcarboxamido)-1-propyloxy]-phosphinyl-2′-deoxyadenosine (1c).** ^{31}P NMR (121 MHz, C_6D_6): δ 148.6, 148.7. FAB−HRMS: calcd for $C_{52}H_{64}N_7O_8P$ $(M + Na)^+$ 968.4453, found 968.4430.

***N*^{2}-Isobutyryl-5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)[3-(*N*-*tert*-butylcarboxamido)-1-propyloxy]phosphinyl-2′-deoxyguanosine (1d).** ^{31}P NMR (121 MHz, C_6D_6): δ 146.3, 148.3. FAB−HRMS: calcd for $C_{49}H_{66}N_7O_9P$ $(M + Na)^+$ 950.4559, found 950.4560.

Preparation of Oligonucleotides. Solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioate analogue is performed using a DNA synthesizer according to the manufacturer's recommendations. 2-Cyanoethyl deoxyri-

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bonucleoside phosphoramidites and all the reagents pertaining to the automated preparation of oligonucleotides were purchased from Perkin-Elmer and used as recommended by the manufacturer. 2-Cyanoethyl deoxyribonucleoside phosphoramidites and phosphoramidites **1a**−**d** are used as 0.1 M solutions in dry acetonitrile. The sulfurization reaction required in the preparation of oligodeoxyribonucleoside phosphorothioates is effected by treatment with 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Glen Research) in acetonitrile as recommended in the literature.[18]

Purification and Characterization of Oligonucleotides. Crude oligomers synthesized from **1a**−**d** that are still covalently linked to the CPG support are treated with pressurized ammonia gas (∼10 bar) for 10 h at 25 °C. The crude oligonucleotides are then eluted from the support with PBS buffer pH 7.2 (1 mL). The solutions are heated in a closed vial at 90 °C for 30 min. Crude oligonucleotides synthesized from commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites are released from CPG and deprotected by treatment with concentrated ammonium hydroxide for 10 h at 55 °C. Fully deprotected oligomers are then electrophoresed on 20% polyacrylamide-7 M urea gels (40 cm × 20 cm × 0.75 mm), which were prepared using electrophoresis purity reagents (Bio-Rad).

Gels are stained by soaking in a solution of Stains-all as described elsewhere.[5a] Figure 2 shows a photograph of such a gel.

Oligonucleoside phosphorothioates are analyzed for desulfurization by ^{31}P NMR spectroscopy as shown in Figure 3 and in Supporting Information.

Enzymatic digestion of crude d(ATCCGTAGCTAAGGTCATGC) synthesized using either **1a**−**d** or conventional 2-cyanoethyl deoxyribonucleoside phosphoramidites is performed with snake venom phosphodiesterase (*Crotalus durissus*, Boehringer) and bacterial alkaline phosphatase (Sigma) according to a published procedure.[19] An aliquot of either digest is 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. RP-HPLC profiles of the digests are shown in Supporting Information. These did not reveal detectable amounts of nucleobase modification or incomplete oligonucleotide deprotection.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of **2**, *N*-*tert*-butyl-5-hydroxyvaleramide, *N*-*tert*-butyl-3-hydroxypropionamide, *N*-*tert*-butylglycolamide, *N*-methyl-4-hydroxybutyramide, *N*-isopropyl-4-hydroxybutyramide, **3**, **4**, 2-(*tert*-butylimino)tetrahydrofuran hydrobromide, and **22**. ^{31}P NMR spectra of **1a**−**d**, **7**−**10**, **18**, **19**, and **22**. RP-HPLC chromatograms of hydrolysates resulting from snake venom phosphodiesterase and bacterial alkaline phosphatase digestion of crude d(ATCCGTAGCTAAGGTCATGC), which was prepared from either phosphoramidites **1a**−**d** or commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites. Kinetic studies of the cleavage of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group from purified **3** and crude d($A_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}G_{PS}$-$C_{PS}T_{PS}A_{PS}A_{PS}G_{PS}G_{PS}T_{PS}C_{PS}A_{PS}T_{PS}G_{PS}$C) in D_2O at 80 °C and 60 °C, respectively. ^{31}P NMR spectrum of the interaction of *O*,*O*-diethyl phosphorothioate with 2-(*tert*-butylimino)tetrahydrofuran hydrobromide in D_2O at 90 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

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