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Thermolytic 4-Methylthio-1-butyl Group for Phosphate/Thiophosphate Protection in Solid-Phase Synthesis of DNA Oligonucleotides

Jacek Cieślak,[‡] Andrzej Grajkowski, Victor Livengood,[†] and Serge L. Beaucage*

Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20892, and Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892

beaucage@cber.fda.gov

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The thermolabile 4-methylthio-1-butyl phosphate/thiophosphate protecting group for DNA oligonucleotides has been investigated for its potential application to a "heat-driven" process for either oligonucleotide synthesis on diagnostic microarrays or, oppositely, to the large-scale preparation of therapeutic oligonucleotides. The preparation of phosphoramidites **10a**−**d** is straightforward, and the incorporation of these amidites into oligonucleotides via solid-phase techniques proceeds as efficiently as that achieved with 2-cyanoethyl deoxyribonucleoside phosphoramidites. The versatility of the 4-methylthio-1-butyl phosphate/thiophosphate protecting group is exemplified by its facile removal from oligonucleotides upon heating for 30 min at 55 °C in an aqueous buffer under neutral conditions or within 2 h at 55 °C in concentrated NH_4OH. The deprotection reaction occurs through an intramolecular cyclodeesterification mechanism leading to the formation of sulfonium salt **18**. When mixed with deoxyribonucleosides and *N*-protected 2′-deoxyribonucleosides or with a model phosphorothioate diester under conditions approximating those of large-scale (>50 mmol) oligonucleotide deprotection reactions, the salt **18** did not significantly alter DNA nucleobases or desulfurize the phosphorothioate diester model to an appreciable extent.

Introduction

With the advent of DNA microarrays as powerful diagnostic tools in clinical medicine,[1] our research efforts have recently focused on the development of thermolytic groups for 5′-hydroxyl[2] and phosphate[3] protection toward the implementation of a "heat-driven" method[4] for the synthesis of DNA oligonucleotides on planar glass surfaces. Ideal thermolabile 5′-/3′- hydroxyl and phosphate protecting groups should be quickly and efficiently released from oligonucleotides, when heated under neutral conditions, to avoid the use of harsh chemicals that are normally required for oligonucleotide chain extension and deprotection and incur the risk of an irreversible loss of valuable oligonucleotides from the glass surface. A number of heat-sensitive phosphate/thiophosphate protecting groups exhibiting unique thermolytic properties have already been investigated[3] through incorporation into oligonucleotides via phosphoramidites **1**−**7** (Scheme 1).

Typically, the thermal cleavage of phosphate protecting groups from oligonucleotides that have been prepared using phosphoramidites **1**,[3a] **3**,[3b,d] **4**,[3c,d] and **5** is complete within 3 h, 1 h, 30 min, and 15 min, respectively, upon heating at 90 °C in an aqueous buffer under neutral conditions (pH 7.0). Interestingly, the thermolytic phosphate deprotection of oligonucleotides that have been prepared via phosphoramidites **7**[3e] occurs spontaneously while the oxidative step of the solid-phase oligonucleotide synthesis cycle is progressing. The deprotection mechanism of each phosphate protecting group is consistent with a well-studied intramolecular cyclodeesterification reaction,[5] which has also been experienced by others.[6] Each thermolytic group has its unique attributes in terms of commercial availability and cost-effectiveness, structural features, ease of incorporation into phosphoramidite monomers, solubility and stability properties conferred to phosphoramidites, coupling efficiency of phosphoramidites, and ease of thermal deprotection from oligonucleotides. It seems therefore justified to search for novel thermolytic groups with distinctive properties to

* To whom correspondence should be addressed. Tel: (301) 827-5162. Fax: (301) 480-3256.

† National Institutes of Health.

‡ On leave from the Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland.

(1) (a) Guo, Q. M. *Curr. Opin. Oncol.* **2003**, *15*, 36−43. (b) Rhodes, D. R.; Chinnaiyan, A. M. *J. Invest. Surg.* **2002**, *15*, 275−279.

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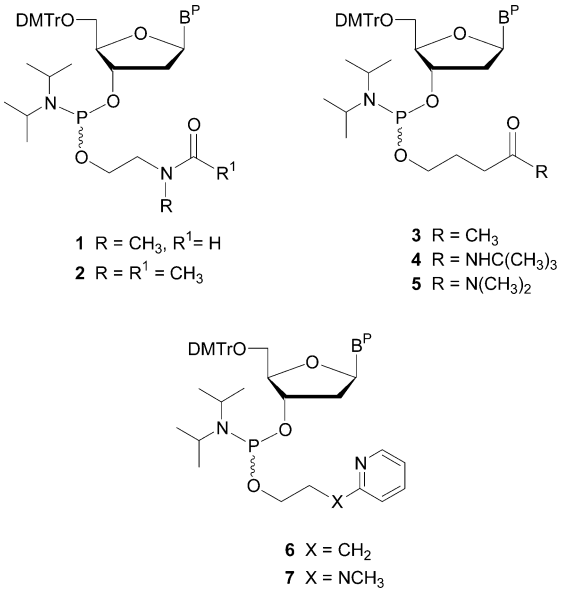
SCHEME 1[a]



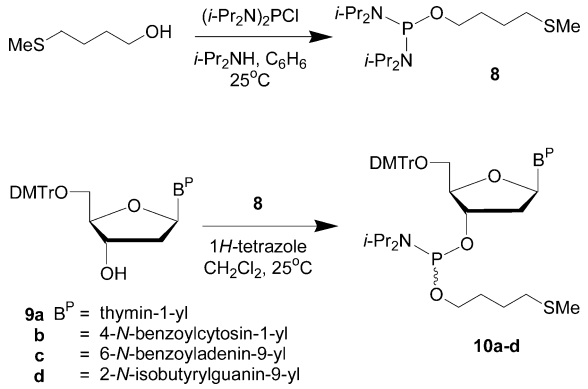
[a] Keys: DMTr, 4,4′-dimethoxytrityl; B[P], thymin-1-yl, 4-*N*-benzoylcytosin-1-yl, 6-*N*-benzoyladenin-9-yl, or 2-*N*-isobutyrylguanin-9-yl.

enable one to select the group that will optimally address specific experimental requirements. We now wish to report the 4-methylthio-1-butyl group as a new thermolabile phosphate/thiophosphate protecting group in the synthesis of oligodeoxyribonucleotides toward the production of high quality diagnostic DNA microarrays and/or therapeutic drugs for clinical studies.

Results and Discussion

As for all thermolytic phosphate/thiophosphate protecting groups studied so far, the alcohol conferring thermolytic properties is first converted to a phosphinylating reagent. Thus, 4-methythio-1-butanol is transformed into phosphorodiamidite **8** (Scheme 2) upon reaction with bis(*N*,*N*-diisopropylamino)chlorophosphine under conditions described earlier.[3] Condensation of **8**[7] with commercially available deoxyribonucleosides **9a**−**d** and 1*H*-tetrazole in anhydrous CH_2Cl_2[8] affords the deoxyribonucleoside phosphoramidites **10a**−**d** (Scheme 2), which are purified by silica gel chromatography and

SCHEME 2. Synthesis of Deoxyribonucleoside Phosphoramidites 10a−d



characterized by ^{31}P NMR spectroscopy[9] and high-resolution mass spectrometry.

To assess the thermolytic deprotection kinetics of the 4-methylthio-1-butyl phosphate/thiophosphate protecting group, the dinucleoside phosphotriester **14** or **15** (Scheme 3) is prepared from the condensation of **10a** with thymidine covalently attached to long-chain alkylamine controlled-pore glass (LCAA-CPG) through a 3′-*O*-succinyl linker and 1*H*-tetrazole in dry MeCN. Oxidation/sulfurization[10] of the phosphite triester **11** (Scheme 3), followed by cleavage of the 5′-*O*-DMTr group under acidic conditions, gives the solid-phase-linked phosphotriester **12** or **13**. The dinucleotide is then released from LCAA-CPG upon a short (∼3 min) exposure to pressurized methylamine gas and eluted from the support with a triethylammonium acetate buffer (pH 7.0) to provide the dinucleoside phosphotriester **14** or **15**. Reversed phase high performance liquid chromatography (RP-HPLC) analysis of **14** reveals a ∼12% postsynthetic loss of the phosphate protecting group.[11] Heating either **14** or **15** in the elution buffer for 30 min at 55 °C results in complete cleavage of the 4-methylthio-1-butyl group to afford the dinucleoside phosphodiester **16** or **17** as the major product (>99%) detected by RP-HPLC analysis of the thermolytic deprotection reaction.[12,13]

Participation of the 4-methylthio functionality to the postulated cyclodeesterification reaction (Scheme 3) is demonstrated via oxidation of **11** with a solution of *tert*-

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(6) (a) Van Houten, K. A.; Heath, D. C.; Pilato, R. S. *J. Am. Chem. Soc.* **1998**, *120*, 12359−12360. (b) Guzaev, A. P.; Manoharan, M. *Tetrahedron Lett.* **2000**, *41*, 5623−5626. (c) Guzaev, A. P.; Manoharan, M. *J. Am. Chem. Soc.* **2001**, *123*, 783−793. (d) Zhang, S.-W.; Swager, T. M. *J. Am. Chem. Soc.* **2003**, *125*, 3420−3421.

(7) Crude **8** is characterized by ^1H and ^{13}C NMR spectrocopies (data shown in Experimental Section and spectra provided as Supporting Information) and used without further purification.

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

(9) ^{31}P NMR spectra are shown as Supporting Information.

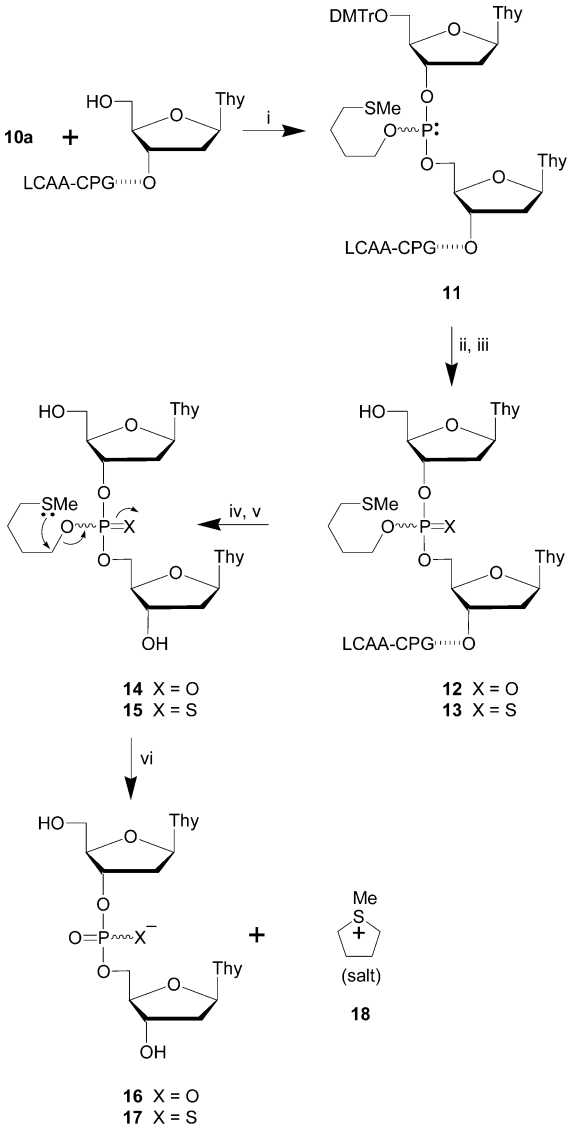
(10) The oxidation reaction is performed using commercial 0.02 M iodine in THF/pyridine/water, whereas the sulfurization reaction is effected using 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN as recommended in the literature.[24]

(11) RP-HPLC analyses are performed with a 5-μm Supelcosil LC-18S column (25 cm × 4.6 mm) under the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min and then held isocratic for 20 min.

(12) Coincidentally, the thermolytic deprotection kinetics of the 4-methylthio-1-butyl phosphate/thiophosphate group from **14** or **15** are very similar to those determined for the 3-(2-pyridyl)-1-propyl phosphate/thiophosphate protecting group under similar conditions.[3e] Both groups are cleaved from dinucleoside phosphotriesters within 30 min ($t_{1/2}$ ∼225 s) or 5 min ($t_{1/2}$ ∼40 s) upon heating in an aqueous buffer (pH 7.0) at 55 or 90 °C, respectively. By comparison, deprotection of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group from dinucleoside phosphotriesters is much slower ($t_{1/2}$ ∼100 s[3c]) under similar thermolytic conditions (pH 7.0, 90 °C).

(13) Alternatively, treatment of **12** or **13** with concentrated NH_4OH for 30 min at 25 °C followed by heating the ammoniacal solution for 2 h at 55 °C also results in complete thermolytic cleavage of the 4-methylthio-1-butyl group to generate the dinucleoside phosphodiester **16** or **17** as the major (>99%) product.

SCHEME 3. Synthesis of Dinucleoside Phosphotriester 14 or 15 and Thermolytic Cleavage of the 4-Methylthio-1-butyl Phosphate/Thiophosphate Protecting Group under Neutral Conditions[a]

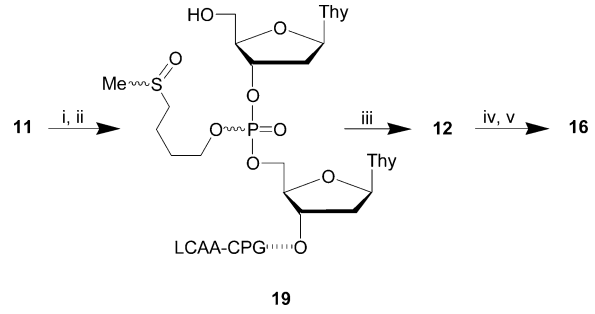


[a] Reagents and conditions: (i) 0.45 M 1*H*-tetrazole in MeCN, 1 min; (ii) 0.02 M I_2 in THF/pyridine/water or 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN, 3 min; (iii) 3% trichloroacetic acid in CH_2Cl_2, 1 min; (iv) $MeNH_2$ gas (2.5 bar), 3 min; (v) 0.1 M TEAA, pH 7.0; (vi) 55 °C, 30 min. Keys: Thy, thymin-1-yl; LCAA-CPG, succinyl long-chain alkylamine controlled-pore glass; TEAA, triethylammonium acetate.

butyl hydroperoxide[14] or (1*S*)-(+)-(10-camphorsulfonyl)-oxaziridine[15] to give **19** (Scheme 4).

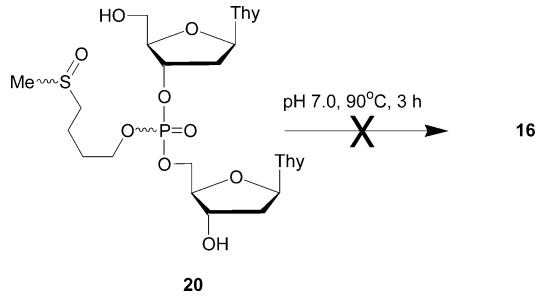
After cleavage of the 5′-*O*-DMTr group and release from the CPG support, the dinucleoside phosphotriester **20** is isolated by RP-HPLC and characterized by mass spectrometry. The phosphate protecting group in **20** is completely stable to the conditions used for thermolytic

SCHEME 4[a]



[a] Reagents and conditions: (i) 0.1 M *t*-BuOOH in decane/CH_2Cl_2 (1:4 v/v), 3 h or 0.5 M (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine in MeCN, 5 min; (ii) 3% TCA in CH_2Cl_2, 1 min; (iii) 0.25 M $(EtO)_3P$ and 0.30 M NBS in MeCN, 2 h; (iv) concentrated NH_4OH, 25 °C, 30 min; (v) concentrated NH_4OH, 55 °C, 2 h. Keys: NBS, *N*-bromosuccinimide.

phosphate/thiophosphate deprotection of **14** or **15**. The phosphate protecting group is also stable when **20** is heated for 3 h at 90 °C in an aqueous buffer (pH 7.0). The nucleophilicity of the 4-methanesulfinyl function is considerably weaker than that of its 4-methylthio precursor and thus validates the nucleophilic participation of the 4-methylthio group in the thermolytic deprotection of **14** or **15**.



Given the stability of the 4-methanesulfinyl-1-butyl group to thermolytic and standard deprotection conditions, the use of *tert*-butyl hydroperoxide or (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine as an oxidant in the synthesis of oligonucleotides functionalized with the thermolabile 4-methylthio-1-butyl phosphate protecting group should therefore be avoided. This apparent limitation can, however, be circumvented by converting **19** to **12** through reduction of the methanesulfinyl group by treatment with a solution of triethyl phosphite and *N*-bromosuccinimide in MeCN over a period of 2 h at 25 °C (Scheme 4).[16]

Treatment of **12** with concentrated NH_4OH to release the dinucleotide from the support followed by heating the ammoniacal solution for 2 h at 55 °C gives **16** as essentially the sole nucleotidic product (>99%) detected by RP-HPLC analysis of the reduction reaction.

(14) Hayakawa, Y.; Uchiyama, M.; Noyori, R. *Tetrahedron Lett.* **1986**, *27*, 4191−4194.

(15) (a) Davis, F. A.; Towson, J. C.; Weismiller, M. C.; Lal, S.; Carroll, P. J. *J. Am. Chem. Soc.* **1988**, *110*, 8477−8482. (b) Ugi, I.; Jacob, P.; Landgraf, B.; Rupp, C.; Lemmen, P.; Verfürth, U. *Nucleosides Nucleotides* **1988**, *7*, 605−608.

(16) Reverse engineering of the oxidation of oligonucleosidic phosphite triesters effected by NBS-DMSO in MeCN[23] led to the development of this mild reduction reaction. Even though the conversion of **19** to **12** went smoothly and near quantitatively (>99%), it should be understood that the reduction reaction has not, as yet, been optimized and thoroughly investigated in terms of potential nucleobase modifications and/or other side reactions that might occur with representative oligonucleotides. Investigations addressing these concerns are currently underway in our laboratory, and our findings on the scope and limitations of this reduction reaction will be reported soon.

To confirm the formation of sulfonium salt **18** as a consequence of the thermolytic deprotection of nucleotidic 4-methylthio-1-butyl phosphate/thiophosphate protecting groups (Scheme 3), we set out to produce the salt from **12** on a 2 μmol scale and characterize it by ^{1}H NMR spectroscopy. Specifically, the solid-phase-linked dinucleoside phosphotriester is suspended in a solution of 0.1 M NaCl in D_2O and heated for 30 min at 55 °C. ^{1}H NMR analysis of the supernatant reveals signals identical to those of synthetic **18**,[17] when compared under similar conditions.[18] These data unambiguously demonstrate the cyclodeesterification of 4-methylthio-1-butyl phosphate/thiophosphate protecting groups under thermolytic deprotection conditions with concomitant formation of the sulfonium salt **18**.

Given the sensitivity of the 4-methylthio-1-butyl phosphate protecting group to certain oxidants (vide supra), its reactivity toward the standard iodine oxidation reagent for oligonucleotide synthesis was reexamined. Typically, RP-HPLC analysis of **14** that is produced from exposure of **11** to 0.02 M I_2 in THF/pyridine/water for a period of time ranging from 3 to 180 min revealed the presence of **20** to the extent of less than 0.5%. While the concentration of **20** after a 180 min oxidation is not different than that obtained after a 3 min oxidation, one can safely conclude that 0.02 M I_2 in THF/pyridine/water does not oxidize the 4-methylthio functional group in **14** to a significant level during solid-phase oligonucleotide synthesis.[19] Altogether, our investigations on the preparation of **14** or **15** and on the facile thermolytic phosphate/thiophosphate deprotection of these dinucleotides to **16** or **17** were encouraging and prompted us to employ phosphoramidites **10a**−**d** in the solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) along with that of its fully phosphorothioated analogue.[20] Commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites are also used to synthesize identical oligonucleotides to compare the coupling efficiency of these monomers with that of **10a**−**d** under similar conditions.[21]

The solid-phase-linked 5′-*O*-DMTr-oligonucleotides are released from the LCAA-CPG support upon treatment with concentrated NH_4OH for 30 min at 25 °C. Each oligonucleotidic solution is then heated for 10 h at 55 °C to thermally remove phosphate or thiophosphate protecting groups from the respective oligonucleotide and complete nucleobase deprotection. Comparative analysis of the crude 5′-*O*-DMTr-oligonucleotides with each other in terms of synthesis efficiency by assessing the ratio of full-length 5′-*O*-DMTr-20-mers to shorter oligonucleotide sequences is accomplished using RP-HPLC. Results of the RP-HPLC analyses show that syntheses of the 20-mer (Chart 1 in Supporting Information) and that of its phosphorothioated analogue (Chart 2 in Supporting Information) via phosphoramidites **10a**−**d** are very comparable to those performed with commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites. Consistent with these findings is the analysis of the same but 5′-*O*-dedimethoxytritylated oligonucleotides by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (Figure 1). Careful examination of the stained gel reveals that the intensity of bands corresponding to shorter than full-length sequences relative to that of the band corresponding to each full-length oligonucleotide is similar regardless of whether the oligonucleotide was synthesized using phosphoramidites **10a**−**d** or 2-cyanoethyl deoxyribonucleoside phosphoramidites.

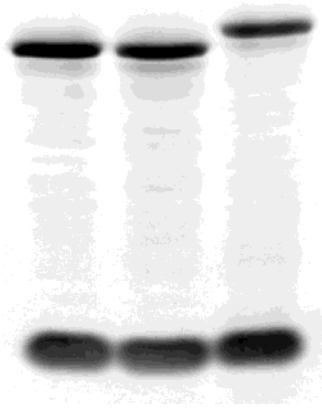


FIGURE 1. Polyacrylamide gel electrophoresis analysis of d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioated analogue under denaturing conditions (7 M urea, 1X TBE buffer, pH 8.3). Left lane: crude oligomer synthesized from commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites and deprotected by treatment with concentrated NH_4OH for 10 h at 55 °C. Middle lane: crude oligomer synthesized from phosphoramidites **10a**−**d** and deprotected under conditions identical to those used for the 20-mer shown in the left lane. Right lane: crude phosphorothioated oligomer synthesized from **10a**−**d** and deprotected under conditions identical to those used for the 20-mer shown in the left lane. Unmodified oligonucleotides are visualized as blue bands and fully thioated oligonucleotides as purple bands, upon staining the gel with Stains-all. Bromophenol blue is used as a marker and shows as a large band, in each lane, at the bottom of the gel.

To further demonstrate the comparability of d(ATCCGTAGCTAAGGTCATGC) prepared via **10a**−**d** or 2-cyanoethyl deoxyribonucleoside phosphoramidites, these 20-mers were subjected to enzymatic digestion catalyzed by bacterial alkaline phosphatase and snake venom phosphodiesterase to assess whether any nucleobase modifications might have been caused by the use of **10a**−**d**. RP-HPLC analyses of the enzymatic digests show complete digestion of the oligonucleotides with no detectable nucleobase modification, thereby demonstrating that phosphoramidites **10a**−**d** can produce oligonucleotides of quality comparable to those prepared using 2-cyanoethyl deoxyribonucleoside phosphoramidites (Chart 3 in Supporting Information).

(17) Compound **18** (as its chloride salt) is prepared from the reaction of tetrahydrothiophene with methyl chloroformate. See: Byrne, B.; Lafleur Lawter, L. M. *Tetrahedron Lett.* **1986**, *27*, 1233−1236.

(18) ^{1}H NMR spectra of **18** produced from the thermolytic deprotection of **12** in D_2O containing 0.1 M NaCl and that of synthetic **18** in D_2O are shown as Supporting Information.

(19) The origin of trace amounts of **20** contaminating **14** is still unclear and is currently under investigation.

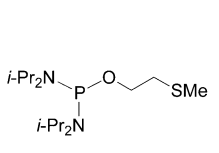
(20) Solid-phase synthesis of the fully thioated 20-mer involves replacing the standard 0.02 M solution of iodine in THF/pyridine/water with a 0.05 M solution of 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN and performing the capping reaction after the sulfurization step.[24]

(21) Phosphoramidites **10a**−**d** and the corresponding 2-cyanoethyl deoxyribonucleoside phosphoramidites were used as 0.1 M solutions in dry MeCN in accordance with a standard automated solid-phase oligonucleotide synthesis program executed by a commercial DNA/RNA synthesizer.

Whereas the 4-methylthio-1-butyl group for phosphate/thiophosphate protection has been adequately tested in the solid-phase synthesis of oligonucleotides on a small scale (0.2 μmol), the protecting group may also be suitable for the preparation of therapeutic oligonucleotides on a large-scale (>50 mmol) to support clinical studies. In this context, one must investigate whether oligonucleotides synthesized via **10a**−**d** would undergo nucleobase modification[5b] under deprotection conditions similar to those used for large-scale oligonucleotide syntheses. Thus, thymidine, 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine, N^4-benzoyl-2′-deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, and N^2-isobutyryl-2′-deoxyguanosine are mixed with the sulfonium salt **18** in concentrations approximating those existing in large-scale (>50 mmol) oligonucleotide deprotection reactions and heated in NH_4-OH for 10 h at 55 °C. The model reaction is then analyzed by RP-HPLC to determine the extent of nucleobase modification, if any (Chart 4 in Supporting Information). No significant nucleobase alkylation products or other nucleoside modifications are detected under these conditions, thereby indicating that the use of phosphoramidites **10a**−**d** is suitable for large-scale preparations of therapeutic oligonucleotides.

Given that small-scale (0.2 μmol) solid-phase synthesis of phosphorothioated oligonucleotides via **10a**−**d** does not generate substantial amounts of partially desulfurized products (<5%) under postsynthesis processing conditions on the basis of ^{31}P NMR data (Chart 5 in Supporting Information), it is nonetheless questionable as to whether the sulfonium salt **18** can desulfurize phosphorothioated oligonucleotides when produced in concentrations comparable to those prevailing under large-scale oligonucleotide deprotection conditions. To assess this concern, the potassium salt of *O*,*O*-diethyl thiophosphate is used as a model mimicking thioated oligonucleotides and is mixed with **18** in concentrations and under conditions simulating large-scale oligonucleotide deprotection reactions (see Experimental Section). ^{31}P NMR analysis of the reaction did not indicate any conversion of *O*,*O*-diethyl thiophosphate (δ_P ~55 ppm) to *O*,*O*-diethyl phosphate (δ_P ~2 ppm) induced by the sulfonium salt **18** (Chart 6 in Supporting Information). Consequently, it is likely that **18** will not cause significant desulfurization of thioated oligonucleotides under a large-scale deprotection process.

A logical extension of our studies is to assess the thermolytic properties of the 2-(methylthio)ethyl group for potential phosphate/thiophosphate protection in oligonucleotide synthesis. Specifically, the deoxyribonucleoside phosphoramidite **22** is synthesized from the condensation of **9a** with crude phosphorodiamidite **21** under conditions identical to those described for the preparation of **10a**−**d** (Scheme 2).



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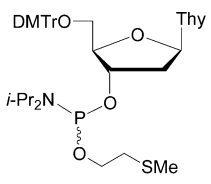

22

The phosphorodiamidite **21** is prepared, much like **8**, from the reaction of commercial 2-(methylthio)ethanol with bis(*N*,*N*-diisopropylamino)chlorophosphine.[3] Replacement of **10a** with **22** in Scheme 3 also led to the preparation of **16** or **17** under identical conditions. Accurate RP-HPLC analysis of the thermolytic deprotection kinetics of the 2-(methylthio)ethyl group from dinucleotides homologous to **14** or **15** indicates that the 2-(methylthio)ethyl group is removed faster ($t_{1/2}$ ~115 s at 55 °C) than the 4-methylthio-1-butyl group ($t_{1/2}$ ~225 s) under identical deprotection conditions.[22] The thermolytic deprotection mechanism appears consistent with that of a cyclodeesterification process, although no attempts have been made at this time to characterize the cyclodeesterification side product and evaluate the DNA-modifying or phosphorothioate desulfurization properties of such a species.

Conclusion

The 4-methylthio-1-butyl group for phosphate/thiophosphate protection in the solid-phase synthesis of DNA oligonucleotides is distinctive in its structural simplicity. This protecting group is not much different than the 2-cyanoethyl group in terms of steric considerations, and yet its versatility allows its removal from DNA oligonucleotides under neutral conditions in an aqueous buffer or under the basic conditions used to cleave the 2-cyanoethyl group. We have demonstrated that the thermolytic deprotection of 4-methylthio-1-butyl phosphate/thiophosphate protecting groups occurred through a cyclodeesterification reaction, which resulted in the simultaneous formation of a sulfonium salt side product. This salt is essentially inert to DNA nucleobases and does not cause significant desulfurization of phosphorothioate diesters. A limitation in the use of the 4-methylthio-1-butyl phosphate protecting group is the selection of the oxidant necessary for converting phosphite to phosphate triesters during oligonucleotide assembly via phosphoramidite chemistry. Whereas the methylthio function is not noticeably oxidized upon treatment with the standard iodine solution routinely used in solid-phase oligonucleotide synthesis, the functional group is particularly sensitive to (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine and *tert*-butyl hydroperoxide. However, this limitation is negated by the facile conversion of oxidized methylthio species to the original methylthio functional group by treatment with *N*-bromosuccinimide and triethyl phosphite. Although the scope and limitations of this reduction method are currently under investigation, the method is innovative in its ability to reverse the condition caused by unwanted oxidation of methylthio groups.

Extension of this work has led us to evaluate the 2-(methylthio)ethyl group for phosphate/thiophosphate protection in solid-phase oligonucleotide synthesis. Our preliminary results indicate that the 2-(methylthio)ethyl group is even faster-deprotecting than the 4-methylthio-1-butyl group under identical thermolytic deprotection conditions and thus deserves further investigations.

The thermosensitivity of 4-methylthio-1-butyl and that of other previously studied phosphate/thiophosphate

(22) In agreement with our findings is the relative instability of bis(*S*-β-D-glucopyranosidyl-2-thioethyl) 3′-azido-3′-deoxythymidin-5′-yl phosphate in aqueous media reported earlier by others. See: Schlienger, N.; Périgaud, C.; Gosselin, G.; Imbach, J.-L. *J. Org. Chem*. **1997**, *62*, 7216−7221.

protecting groups,[3] along with the recent development of heat-sensitive carbonates as 5′-hydroxyl protecting groups,[2] bring closer to reality the conceptual heat-driven approach to the synthesis of DNA oligonucleotides on planar glass surfaces proposed earlier.[4]

Finally, the application of the 4-methylthio-1-butyl phosphate/thiophosphate protecting group to the large-scale preparation of therapeutic DNA or RNA oligonucleotides is likely given the mild thermolytic conditions used for its deprotection and the lack of side products being produced during oligonucleotide deprotection that would modify DNA nucleobases and/or compromise the resistance of these oligonucleotides to nucleases through partial desulfurization.

Experimental Section

***O*-(4-Methylthio-1-butyl)-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (8).** 4-Methylthio-1-butanol (2.7 mL, 22 mmol) is added by syringe to a stirred solution of bis(*N,N*-diisopropylamino)chlorophosphine[3c−e] (20 mmol) in dry benzene (100 mL). Formation of the phosphorodiamidite **8** at 25 °C is monitored by ^{31}P NMR spectroscopy. Complete conversion of bis(*N,N*-diisopropylamino)chlorophosphine (δ_P 135.5 ppm) to **8** (δ_P 118.5 ppm) is achieved within 2 h. The suspension is filtered, and the filtrate is evaporated to an oil under reduced pressure. The crude phosphorodiamidite is used without further purification in the preparation of **10a**−**d**. ^{1}H NMR (300 MHz, C_6D_6): δ 3.56 (m, 2H), 3.53 (sept, J = 6.9 Hz, 2H), 3.49 (sept, J = 6.9 Hz, 2H), 2.30 (m, 2H), 1.80 (s, 3H), 1.63 (m, 4H), 1.23 (d, J = 6.9 Hz, 12H), 1.19 (d, J = 6.9 Hz, 12H). ^{13}C NMR (75 MHz, C_6D_6): δ 15.2, 24.0, 24.1, 24.7, 24.8, 26.2, 31.1 (d, J_{PC} = 9.6 Hz), 34.2, 44.6, 44.7, 64.1 (d, $^2J_{PC}$ = 21.5 Hz). ^{31}P NMR (121 MHz, C_6D_6): δ 118.5.

***O*-2-(Methylthio)ethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (21).** This phosphinylating reagent is prepared in a manner identical to that of **8** and used without further purification in the synthesis of phosphoramidite **22**.^{1}H NMR (300 MHz, $CDCl_3$): δ 3.71 (dt, J = 7.2 Hz, $^3J_{PH}$ = 7.7 Hz, 2H), 3.52 (sept, J = 6.9 Hz, 2H), 3.49 (sept, J = 6.9 Hz, 2H), 2.71(t, J = 7.2 Hz, 2H), 2.13 (s, 3H), 1.15 (t, J = 6.4 Hz, 24H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.9, 23.6, 23.7, 24.5, 24.6, 35.4 (d, J_{PC} = 8.4 Hz), 44.2, 44.4, 63.5 (d, $^2J_{PC}$ = 22.7 Hz). ^{31}P NMR (121 MHz, $CDCl_3$): δ 121.5.

General Procedure for Preparation of Deoxyribonucleoside Phosphoramidites 10a−d. To a stirred solution of **9a**−**d** (2.2 mmol) in dry CH_2Cl_2 (10 mL) is added by syringe, under a positive pressure of argon, crude **8** (~700 mg, 2 mmol) followed by sublimed 1*H*-tetrazole (112 mg, 1.6 mmol). ^{31}P NMR analysis of the reaction indicates that **8** is completely consumed within 2 h. Upon addition of triethylamine (1 mL), the solution is then immediately concentrated to a syrup under reduced pressure. The material is purified by silica gel chromatography using benzene/triethylamine (9:1 v/v) as the eluent. Fractions that are identified by TLC as containing the product are pooled together and rotoevaporated under low pressure to a white foam. The material is dissolved in dry benzene, frozen, and then lyophilized under high vacuum to give **10a**−**d** as white powders in yields ranging from 72% to 85%.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(*N,N*-diisopropylamino)(4-methylthio-1-butyloxy)]phosphinyl-2′-deoxythymidine (10a). ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.6, 147.9. FAB−HRMS: calcd for $C_{42}H_{56}N_3O_8PS$ (M + Cs)$^+$ 926.2580, found 926.2537.

***N*^{4}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-[(*N,N*-diisopropylamino)(4-methylthio-1-butyloxy)]phosphinyl-2′-deoxycytidine (10b).** ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.5, 147.9. FAB−HRMS: calcd for $C_{48}H_{59}N_4O_8PS$ (M + Cs)$^+$ 1015.2846, found 1015.2870.

***N*^{6}-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-[(*N,N*-diisopropylamino)(4-methylthio-1-butyloxy)]phosphinyl-2′-deoxyadenosine (10c).** ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.3, 147.7. FAB−HRMS: calcd for $C_{49}H_{59}N_6O_7PS$ (M + Cs)$^+$ 1039.2958, found 1039.2996.

***N*^{2}-Isobutyryl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-[(*N,N*-diisopropylamino)(4-methylthio-1-butyloxy)]phosphinyl-2′-deoxyguanosine (10d).** ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.4, 147.8. FAB−HRMS: calcd for $C_{46}H_{61}N_6O_8PS$ (M + Cs)$^+$ 1021.3064, found 1021.3011.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(*N,N*-diisopropylamino)-2-(methylthio)ethyloxy]phosphinyl-2′-deoxythymidine (22). This compound is prepared, purified, and isolated in a manner identical to that used for the preparation of **10a**−**d**. ^{31}P NMR (121 MHz, C_6H_6): δ 147.6, 148.0. FAB−HRMS: calcd for $C_{40}H_{52}N_3O_8PS$ (M + Cs)$^+$ 898.2267, found 898.2256.

Isolation and Characterization of *S*-Methyltetrahydrothiophenium Chloride (18). Dry phosphoramidite **10a** (40 mg, 50 μmol) is dissolved with 0.45 M 1*H*-tetrazole in MeCN (250 μL) and added by syringe to a commercial synthesis column containing thymidine covalently attached to LCAA-CPG through a 3′-*O*-succinyl linker (50 mg, 2 μmol). The suspension is then manually agitated, and after 3 min, the phosphitylation reaction is stopped by flushing activated **10a** off the column with MeCN (10 mL). A 0.02 M solution of I_2 in THF/pyridine/water (1 mL) is mixed with the CPG support for 3 min. Excess oxidant is pushed through the column with MeCN (10 mL), and a solution of 3% trichloroacetic acid (TCA) in CH_2Cl_2 (2 mL) is added to the column to cleave the 5′-*O*-DMTr group over an exposure time of 1 min. Excess TCA is washed off the column with MeCN (10 mL), and the CPG support is air-dried prior to being transferred to a 4-mL glass vial. A 0.1 M solution of NaCl in D_2O is added to the solid support, and the suspension is heated for 30 min at 55 °C in a heating block. The supernatant is syringed into a 5 mm NMR tube for analysis. ^{1}H NMR spectrum of the solution shows signals identical to those of **18**, which has been synthesized according to a published method.[17] ^{1}H NMR (300 MHz, D_2O): δ 3.49 (m, 2H), 3.25 (m, 2H), 2.69 (s, 3H), 2.23 (m, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.0, 27.6, 44.3. FAB−HRMS: calcd for $C_5H_{11}S$ (M^+) 103.0581, found 103.0576.

Preparation of Thymidilyl-(3′→5′)-thymidine [4-(methanesulfinyl)-1-butyl]phosphate (20). Dry phosphoramidite **10a** (20 mg, 25 μmol) is dissolved with 0.45 M 1*H*-tetrazole in MeCN (250 μL) and added by syringe to a commercial synthesis column containing thymidine covalently attached to LCAA-CPG through a 3′-*O*-succinyl linker (8 mg, 0.2 μmol). The suspension is manually agitated, and after 3 min, the column is flushed with MeCN (10 mL). The CPG support is then exposed to a 0.1 M solution of *tert*-butyl hydroperoxide in decane/CH_2Cl_2 (1:4 v/v, 1 mL) over a period of 3 h. Excess oxidant is washed off the column with MeCN (10 mL), and the 5′-*O*-DMTr group is removed by treatment with a solution of 3% TCA in CH_2Cl_2 (2 mL) for 1 min. Excess TCA is pushed through the column with MeCN (10 mL). The dinucleotide is then released from the CPG support upon treatment with pressurized methylamine gas (2.5 bar, 3 min) and elution of crude **20** from the column is accomplished with 0.1 M triethylammonium acetate buffer (pH 7.0, 1 mL). The dinucleoside phosphotriester is then purified by RP-HPLC with a 5 μm Supelcosil LC-18S column (25 cm × 4.6 mm) under the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min. Under these chromatographic conditions, **20** appears as a mixture of diastereomers exhibiting a retention time (t_R) of 19.1 min. The material corresponding to the peak is collected, and the eluates are evaporated to dryness under reduced pressure. The dry material is analyzed by high-resolution mass spectrometry. FAB−HRMS: calcd for $C_{25}H_{37}N_4O_{13}PS$ (M + Cs)$^+$ 797.0870, found 797.0899.

Procedure for Determining the DNA-Modifying Properties of 18. To thymidine, 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine, N^4-benzoyl-2′-deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, and N^2-isobutyryl-2′-deoxyguanosine (0.5 mmol each) in a 7 mL screw-capped glass vial are added **18** (556 mg, 4 mmol), concentrated NH_4OH (1.1 mL), and MeCN (3 mL). The suspension solubilizes within 5 min when heated at 55 °C. The solution is then kept at 55 °C for a total time of 10 h. For comparison purposes, a control reaction is performed in the absence of **18** under otherwise identical conditions. Aliquots of the reaction mixtures are analyzed by RP-HPLC under the chromatographic conditions used for the purification of **20**. Two negligible unidentified peaks exhibiting a t_R of 11.4 min (0.1%) and 14.8 min (0.2%), respectively, are detected along with peaks corresponding to thymidine, 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine, benzamide, and their individual trace amount impurities when compared with the peaks observed from the analysis of the control reaction (Supporting Information).

Procedure for Determining the Phosphorothioate Desulfurization Properties of 18. A solution of 0.5 M potassium *O*,*O*-diethyl thiophosphate and 0.75 M **18** in concentrated NH_4OH is heated for 10 h at 55 °C. Analysis of the reaction mixture by ^{31}P NMR spectroscopy does not reveal any conversion of *O*,*O*-diethyl thiophosphate ($\delta_P \sim 55$ ppm) to *O*,*O*-diethyl phosphate ($\delta_P \sim 2$ ppm) (Supporting Information).

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Supporting Information Available: Materials and methods; preparation and characterization of oligonucleotides; ^1H, ^{13}C, and ^{31}P NMR spectra of crude **8** and **21**; ^{31}P NMR spectra of **10a**−**d** and **22**; ^1H NMR spectra of **18**; RP-HPLC chromatograms of crude 5′-DMTr-d(ATCCGTAGCTAAGGTCATGC) and that of its fully phosphorothioated analogue synthesized using **10a**−**d**; RP-HPLC chromatograms of the snake venom phosphodiesterase and bacterial alkaline phosphatase digestion of crude d(ATCCGTAGCTAAGGTCATGC); ^{31}P NMR spectra of crude phosphorothioated d(ATCCGTAGCTAAGGTCATGC); RP-HPLC chromatograms of analyses aimed at determining the DNA-modifying properties of **18** under conditions mimicking those of large-scale oligonucleotide deprotection reactions; and ^{31}P NMR spectra of the reaction of *O*,*O*-diethyl thiophosphate with **18** under conditions simulating those of large-scale oligonucleotide deprotections. This material is available free of charge via the Internet at http://pubs.acs.org.

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