DNA Oligonucleotides Functionalized with Thermolytic 4-Hydroxy-1-butyl or 4-Phosphato/thiophosphato-1-butyl thiophosphate Protecting Groups as Potential Prodrugs†

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Thermolytic Phosphate/Thiophosphate Protecting Groups.

† S.L.B dedicates this paper to his mentor, Prof. Kelvin K. Ogilvie.

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ABSTRACT

Several thermolytic CpG-containing DNA oligonucleotides have been synthesized to enable the preparation of potential immunotherapeutic oligonucleotides prodrug formulations for the treatment of infectious diseases. The CpG motif of each DNA oligonucleotide (Fig. 1) has been functionalized with thermolytic 4-hydroxy-1-butyl or 4-phospho/thiophosphato-1-butyl tiophosphate protecting groups during solid-phase synthesis upon incorporation of deoxyribonucleoside phosphoramidites 8a-d. Hydrazinolysis of the levulinyl groups produced CpG ODN hbu1555 and allowed the phosphitylation of 4-hydroxybutylated thiophosphate protecting groups by treatment with the phosphoramidite 9, leading to CpG ODN pob1555 and CpG ODN psb1555. Dinucleotide models functionalized with 4-hydroxy-1-butyl and 4-phosphato-thiophosphato-1-butyl thiophosphate protecting groups confirmed the thermosensitivity of these groups, as these underwent cyclodeesterification under thermolytic conditions (PBS, pH 7.2, 37 °C). CpG ODNs hbu1555, pob1555, and psb1555 were characterized by ESI mass spectrometry and complete thermolytic conversion to CpG ODN1555, which also substantiated the function of these oligonucleotides as prodrugs toward the development of potential immunotherapeutic oligonucleotide formulations.

Introduction

Oligonucleotide prodrugs have attracted considerable attention over the last decade in an effort to address the cellular uptake of antisense oligonucleotides and the sensitivity of these biomolecules to the hydrolytic nucleases that are present in physiological environments. These limitations are still impeding most therapeutic applications. A strategy to improve cellular permeation of negatively charged oligonucleotide drugs entails masking temporarily the phosphodiester groups of these biopolymers with acyloxyethyl,\(^1\) 2-(S-acylthio)ethyl,\(^2\) 2-, and 4-(acyloxy)benzy\(^3\) protecting groups or with groups derived from bis(hydroxymethyl)-1,3-dicarbonyl compounds.\(^4\) Upon cellular uptake of these
oligonucleotide prodrugs, hydrolysis of the phosphodiester masking groups by intracellular enzymes generates bioactive oligonucleotide drugs. We recently reported a new class of DNA oligonucleotide prodrugs, which did not require intracellular enzyme(s) for prodrug-to-drug conversion. This class of oligonucleotide prodrugs includes oligonucleoside phosphorothioates functionalized with the thermolytic 2-(N-formyl-N-methyl)aminoethyl group for thiophosphate protection (Scheme 1). These DNA oligonucleotides exhibit the characteristics of oligonucleotide prodrugs in that they are uncharged to facilitate cellular delivery and provide stability to hydrolytic nucleases. A distinctive feature of this class of modified oligonucleotides is that only a 37 °C environment is necessary for converting oligonucleoside 2-(N-formyl-N-methyl)aminoethyl phosphorothioate triesters (1) to functionally bioactive oligonucleoside phosphorothioate diesters (2).

Scheme 1. Thermolytic conversion of DNA oligonucleotide prodrugs to bioactive therapeutic oligonucleotides

\[ \text{Scheme 1. Thermolytic conversion of DNA oligonucleotide prodrugs to bioactive therapeutic oligonucleotides}^a \]

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\[ \begin{array}{c}
\text{O} & \text{O} & \text{B} \\
\text{S=P-O-C-H-Me-N-H} \\
\text{O} & \text{O} & \text{B} \\
\end{array} \xrightarrow{37^\circ C, \text{pH } \sim 7.0, 25 \text{ days}} \begin{array}{c}
\text{O} & \text{O} & \text{B} \\
\text{O=P-O-S} \\
\end{array} \]

\[ a \text{ B= thymin-1-yl, cytosin-1-yl, adenin-9-yl or guanin-9-yl} \]

Thus, when synthetic single-stranded DNA oligonucleoside phosphorothioates containing unmethylated CpG motifs (CpG ODNs) are functionalized with 2-(N-formyl-N-methyl)aminoethyl thiophosphate protecting groups and administered to mice, an immunostimulatory response similar to that produced with traditional CpG ODN phosphodiesters in terms of the number of cells secreting cytokines, chemokines and immunoglobulins was generated. However, a delay was observed in the induction of these immunostimulatory events, which is consistent with the thermolytic conversion of 2-(N-formyl-N-methyl)aminoethyl thiophosphate triesters to the biologically active phosphorothioate
diesters, occurring with a half-time of 73 h at 37 °C. A noteworthy outcome of these findings is that the co-administration of CpG ODN produgs of type 1 and conventional CpG ODN of type 2 to mice widened the timetable of therapeutic treatment against specific viral infections. The observation prompted us to design CpG ODN produgs exhibiting shorter and longer half-time of thiophosphate deprotection relative to that of CpG ODN 1 toward the preparation of long-acting immunotherapeutic oligonucleotide formulations against various infectious diseases. However, the design of thermolytic oligonucleotide produgs poses a paradox in that one must consider the criticality of oligonucleotide solubility in biological media, which precludes the use of lipophilic thiophosphate protecting groups, and cellular uptake, which is reportedly commensurate to the relative lipophilicity of oligonucleotides.

Given that CpG ODN fm1555 \([d(G_{PS(FMA)}C_{PS(FMA)}T_{PS(FMA)}A_{PS(FMA)}G_{PS(FMA)}A_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}A_{PS(FMA)}G_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}T)]\), where PS(FMA) stands for the thermolytic 2-(N-formyl-N-methyl)aminoethyl phosphorothioate triester function] is functional as a prodrug in mice, we propose to evaluate the biological implications of replacing the thermolytic thiophosphate protecting groups of the CpG motif \((G_{PS(FMA)}A_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}T_{PS(FMA)}T)\) within CpG ODN fm1555 with thermolytic groups displaying a slower or faster deprotection kinetics than that of the 2-(N-formyl-N-methyl)aminoethyl group. The new thermolytic groups will be designed to impart similar or better solubility properties in biological media than that of the original CpG ODN fm1555. The dinucleoside phosphorothioate derivatives 3-6 shall serve as models to determine the kinetics of thermolytic cleavage of each thiophosphate protecting group. With the exception of the 3-hydroxy-1-propyl group for thiophosphate protection in dinucleotide 3, the thiophosphate protecting groups of dinucleotides 4, 5, and 6 are expected to follow the well-studied intramolecular cyclodeesterification pathway under thermolytic conditions at near neutral pH.
We now report the preparation of deoxyribonucleoside phosphoramidites 7a and 8a-d, which are required for the synthesis of all model dinucleotides and solid-phase assembly of the DNA oligonucleotides shown in Figure 1. We also report the use of bis[S-(4,4’-dimethoxtrityl)-1-mercaptoethyl]-N,N-diisopropylphosphoramidite (9) for the preparation of negatively charged CPG ODN prodrugs (Figure 1).

Results and Discussion
A prerequisite to the synthesis of dinucleoside phosphotriesters 3 and 4, and that of the CpG DNA oligonucleotides shown in Figure 1, is the preparation of deoxyribonucleoside phosphoramidites 7a and 8a-d, which is outlined in Scheme 2. Specifically, 1,3-propanediol or 1,4-propanediol is condensed with an equimolar amount of levulinic acid in the presence of N,N’-dicyclohexylcarbodiimide (DCC) to afford the levulinylated alcohol 10 or 11 in yields averaging 46% after silica gel chromatography. The reaction of 10

**Figure 1.** Potential CpG DNA oligonucleotide prodrugs functionalized with thermolytic thiophosphate protecting groups.
or 11 with and equimolar amount of bis(N,N-diisopropylamino)chlorophosphine and excess i-Pr$_2$NEt in dry CH$_2$Cl$_2$ gave the corresponding phosphorodiamidite 12 or 13, which was purified by silica gel chromatography and isolated in yields of ca. 70%.

Dry commercial 2’-deoxyribonucleosides 14a-d were reacted with equimolar amounts of phosphorodiamidite 12 or 13 and 1H-tetrazole in anhydrous MeCN. While a total reaction reaction time of 3 h was optimal for the preparation of 7a or 8a-c, a reaction time of 16 h was necessary to ensure complete formation of 8d. The crude deoxyribonucleoside phosphoramidites were purified by silica gel chromatography employing an eluant containing Et$_3$N to neutralize the inherent acidity of silica gel, thereby preventing dedimethoxytritylation and hydrolysis of the phosphoramidite monomers during purification. It is however critically important to remove excess Et$_3$N from the purified phosphoramidites monomers to avoid neutralization of 1H-tetrazole and reduced coupling efficiency during solid-phase oligonucleotide synthesis. The removal of residual Et$_3$N from purified 7a or 8a-d is preferably achieved by dissolving the phosphoramidites in a minimum amount of dry C$_6$H$_6$ followed by precipitation in a large volume of cold hexane. To ensure complete removal of Et$_3$N, the
phosphoramidite precipitate is dissolved in a substantial amount of dry C\textsubscript{6}H\textsubscript{6} (~10 mL/g) and then freeze-dried under high vacuum over an extended period of time (16-24 h).\textsuperscript{9}

**Scheme 2.** Synthesis of the phosphorodiamidites 12 and 13 leading to the deoxyribonucleoside phosphoramidites 7\(\text{a}\) and 8\(\text{a-d}^a\)

\[
\text{HO(CH}_2\text{)}_n\text{OH} \xrightarrow{\text{DCC, Dioxane}} \text{HO(CH}_2\text{)}_n\text{O} \text{Lev} \\
\text{HO(CH}_2\text{)}_n\text{OH} \xrightarrow{(i-\text{Pr}_2\text{N})_2\text{POCl}} \text{HO(CH}_2\text{)}_n\text{O} \text{Lev} \\
\text{DMTrO} \xrightarrow{} \text{12 or 13} \xrightarrow{1H-tetrazole, MeCN} 7\text{a} \text{ or 8\text{a-d}}
\]

\(\text{14a} \quad \text{B}^p = \text{thymin-yl} \)
\(\text{b} \quad N^\text{b}-\text{benzoylcytosin-1-yl} \)
\(\text{c} \quad N^\text{b}-\text{benzoyladenin-9-yl} \)
\(\text{d} \quad N^\text{b}-\text{isobutyrylguanin-9-yl} \)

\(\text{a} \) Keys: DCC, \(N,N'\)-dicyclohexylcarbodiimide.

The solid-phase-linked dinucleoside thiophosphate triesters 15 and 16 (Scheme 3) were prepared on a 0.2 µmole scale by adding a solution of phosphoramidites 7\(\text{a}\) or 8\(\text{a}\) (20 molar equiv) and 1\(H\)-tetrazole (40 molar equiv) in dry MeCN to a controlled-pore glass support functionalized with thymidine, followed by sulfuration with 0.05 M 3\(H\)-1,2-benzodithiol-3-one 1,1-dioxide\textsuperscript{10} in MeCN. The solid-phase-linked dinucleotide 15 or 16 was then treated, sequentially, with 3\% trichloroacetic acid (TCA) in CH\textsubscript{2}Cl\textsubscript{2} to cleave the 4,4’-dimethoxytrityl (DMTr) group and 0.5 M hydrazine hydrate in pyridine:acetic acid (3:2 v/v) to remove the levulinyl group. Exposure of the solid support to pressurized MeNH\textsubscript{2} gas (~2.5 bar) for 3 min released the hydroxyalkylated dinucleoside thiophosphate triester 3 or 4. RP-HPLC analysis of the crude dinucleotides revealed that the purity of 3 and 4 was better than 97\% (Data shown in the Supporting Information). Parenthetically, a solution phase approach to the preparation of the 4-hydroxy-1-butyl phosphate analogue of 4 was described in the literature more than 25 years ago.\textsuperscript{11}
Scheme 3. Solid-phase synthesis of the dinucleoside thiophosphate triester 3 or 4

\[ \text{Reagents and conditions: (i) 3\% TCA/CH}_2\text{Cl}_2, 2 \text{ min. (ii) 7a or 8a (20 equiv), 0.45 M 1H-tetrazole/MeCN, 3 min. (iii) 0.05 M 3H-1,2-benzodithiol-3-one 1,1-dioxide/MeCN, 2 min. (iv) 0.5 M NH}_2\text{NH}_2\cdot\text{H}_2\text{O in C}_5\text{H}_5\text{N:AcOH (3:2 v/v), 10 min, 25 °C. (v) MeNH}_2\text{ gas (~2.5 bar), 3 min. Keys: CPG-LCAA-Succ, succinyl long chain alkylamine controlled-pore glass.} \]

The thermostability of each thiophosphate protecting group was then evaluated using RP-HPLC-purified 3 and 4. On the basis of our earlier findings in regard to the thermolytic properties of various phosphate/thiophosphate protecting groups, it is anticipated that the 4-hydroxy-1-butyl thiophosphate protecting group in 4 would undergo intramolecular cyclodeesterification under thermolytic conditions with the concomitant formation of tetrahydrofuran (Scheme 4). It is also anticipated that the 3-hydroxy-1-propyl thiophosphate protecting group in 3 would not lead to significant thermolytic cyclodeesterification considering the unfavorable formation of trimethylene oxide (Scheme 4). As predicted, RP-HPLC-purified dinucleotide 4 afforded cleanly and quantitatively the parent dinucleoside phosphorothioate diester \( T_\text{PS}T \) within 6 h when heated in 1X phosphate-buffered saline (PBS, pH 7.2) at 90 °C. A reference sample of \( T_\text{PS}T \) was prepared from commercial thymidine 5′-O-DMTr-3′-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite using standard solid-phase techniques. The \( T_\text{PS}T \) reference sample exhibited a RP-HPLC retention time identical (\( t_R = 20.5 \) and 21.1 min) to that of \( T_\text{PS}T \) generated from the thermolytic deprotection of 4.
Scheme 4. Thermolytic properties of hydroxyalkyl thiophosphate protecting groups in dinucleotides 3 and 4 under near neutral conditions$^a$

$^a$ Keys: PBS, 1X phosphate-buffered saline.

Thermolytic cleavage of the 4-hydroxy-1-butyl group from 4 in PBS (pH 7.2) occurred with a half time of 47 min at 90 °C or 168 h at 37°C. In accordance with our expectations, heating RP-HPLC-purified dinucleotide 3 in PBS (pH 7.2) for 6 h at 90 °C resulted in the production of less than 5% TPST, as indicated by RP-HPLC analysis of the solution. Several unidentified side-products, which were generated presumably through the cleavage of the internucleotidic linkage, were detected and accounted for less than 20% of the total peak area (data shown in the Supporting Information).

To confirm whether the thermal cleavage of the 4-hydroxy-1-butyl group from 4 followed the typical cyclodeesterification pathway, a simpler model was used to monitor the formation of THF by $^1$H NMR spectroscopy during the process. Specifically, the reaction of diphenyl chlorophosphate with an excess
1,4-butanediol and Et$_3$N in dry C$_6$H$_6$ afforded the 4-hydroxy-1-butyl ester of diphenylphosphate (17) in high yield (85%) after purification by silica gel chromatography. $^1$H NMR analysis of pure 17 that was heated in DMSO-$d_6$/D$_2$O (5:1 v/v) for 4 h at 90 °C shows signals at 1.7 and 3.5 ppm that are identical to those obtained from the $^1$H NMR spectrum of commercial THF in terms of chemical shifts and signal multiplicity (Data shown in the Supporting Information). Signals at 7.1-7.4 ppm correspond to those of diphenyl phosphate. This assignment is further supported by $^{31}$P NMR analysis of the deprotection reaction in DMSO/D$_2$O (5:1 v/v), which clearly showed the conversion of 17 ($\delta_P$ −12 ppm) to diphenyl phosphate ($\delta_P$ −13 ppm) (Data shown in the Supporting Information).

Given that the 3-hydroxy-1-propyl group for thiophosphate protection in 3 is relatively stable under thermolytic conditions, we rationalized that phosphorylation/thiophosphorylation of this protecting group would convert it to a thermosensitive group that should undergo cyclodeesterification under elevated temperature conditions. The validity of this rationale depends on whether one can perform the phosphorylation/thiophosphorylation reaction under conditions that will not induce premature thermolytic cleavage of the newly formed phosphato/thiophosphatoalkyl thiophosphate protecting group. As reported earlier,$^8$ bis[S-(4,4′-dimethoxytrityl)-1-mercaptoethyl]-N,N-diisopropylphosphoramidite was designed for this specific purpose. Thus, hydrazinolysis of the levulinylated, solid-phase-linked, dinucleoside phosphorothioate triester 15 was achieved over a period of 10 min without releasing the dinucleotide from the support to give 18 (Scheme 5).

**Scheme 5.** Solid-phase synthesis of the phosphato/thiophosphatoalkylated dinucleoside thiophosphate triesters 23-25$^a$
Reagents and conditions: (i) 0.5 M NH$_2$NH$_2$•H$_2$O in C$_5$H$_5$N:AcOH (3:2 v/v), 10 min, 25 °C. (ii) 9, (20 equiv), 0.45 M 1H-tetrazole/MeCN, 3 min. (iii) 0.1 M 3H-1,2-benzodithiol-3-one 1,1-dioxide/MeCN, 3 min or 0.1 M ethyl(methyl)dioxirane/CH$_2$Cl$_2$, 1 min. (iv) 3% TCA/CH$_2$Cl$_2$, 15 min. (v) 1.2% (w/v) DTT/H$_2$O containing 5% (v/v) Et$_3$N, 60 min. (vi) MeNH$_2$ gas (~2.5 bar), 3 min.

A coupling reaction was then performed using an excess (20 equiv) of activated phosphitylating reagent 9 and was followed by a sulfuration step effected by treatment with 0.1 M 3H-1,2-benzodithiol-3-one-1,1-dioxide in MeCN to produce 20. Removal of all DMTr groups was performed under acidic conditions (3% TCA in CH$_2$Cl$_2$) over a period of 15 min. Treatment of the detritylated solid-phase-linked dinucleotide with an aqueous solution of DL-dithiothreitol (DTT) and Et$_3$N was necessary to minimize the production of side products occurring through the elimination of the 2-mercaptoethyl groups. Release of the thiophosphatoalkylated dinucleotide 23 from the support was achieved upon brief exposure (3 min) to pressurized methylamine gas. RP-HPLC analysis of the crude dinucleotide
confirmed the efficiency of reagent 9 in the preparation of 23, as less than 2% of unreacted dinucleotide 3 was detected. The formation of T₃₅T (<10%) was also detected in spite of the mildness of the conditions employed for the generation of 23.¹³ RP-HPLC-purified 23 was characterized by ³¹P NMR spectroscopy and MALDI-TOF mass spectrometry (Data shown in the Supporting information). The thermolytic properties of 23 were evaluated upon heating the purified dinucleotide in PBS (pH 7.2) at elevated temperatures. The 3-thiophosphato-1-propyl group was cleaved, thermolytically, from 23 to produce T₃₅T; the half time of the cleavage reaction was 155 min at 37 °C. Such relatively rapid deprotection kinetics defeats the purpose of developing oligonucleotide prodrug formulations that might widen the window of therapeutic treatments. Consequently, oligonucleotides functionalized with the 3-thiophosphato-1-propyl group for thiophosphate protection were not given further consideration in the development of oligonucleotide prodrugs.

The solid-phase preparation of dinucleotides 21 and 22 was achieved in a manner identical to that of dinucleotide 20 with the exception of using a solution of 0.1 M ethyl(methyl)dioxirane in CH₂Cl₂¹⁴ as an oxidant in the synthesis of 21. As reported earlier,⁸ the use of this oxidant produced higher yields of deoxyribonucleosine 5’-monophosphates than those obtained when using aqueous iodine or tert-butyl hydroperoxide as oxidants. The conversion of 21 and 22 to 24 and 25, respectively, proceeded under conditions identical to those described for the conversion of 20 to 23. The formation of T₃₅T (~2%) during the preparation of 25 was significantly less than that produced during the preparation of 23 and was consistent with a slower thermolytic cyclodeesterification process. The formation of T₃₅T was not detected during the preparation of 24, which is consistent with the lower nucleophilicity of phosphate monoesters relative to thiophosphate monoesters in cyclodeesterification reactions. RP-HPLC-purified 24 and 25 were characterized by ³¹P NMR spectroscopy (data are presented in the Supporting information) and MALDI-TOF mass spectrometry. The purified dinucleotide 24 was dissolved in PBS (pH 7.2) and heated at elevated temperatures to induce the thermolytic cleavage of the 4-phosphato-1-butyl group and the production of T₃₅T. The thermolytic thiophosphate deprotection of 24 proceeded slowly with a half time of 305 min at 90 °C or 45 d at 37 °C. The deprotection kinetics of the 4-
phosphato-1-butyl group is too slow for practical applications in the context of thermolytic oligonucleotide prodrugs. Interestingly, the dinucleotide 24 served as a substrate for human alkaline phosphatase, which quantitatively dephosphorylated the dinucleotide to its 4-hydroxybutylated derivative 4 within 2 h at 37 °C (data shown in the Supporting information). Thus, oligonucleotides functionalized with the 4-phosphato-1-butyl group for phosphate/thiophosphate protection may conceivably be dephosphorylated to the corresponding 4-hydroxybutylated oligonucleotide prodrugs in the presence of intracellular phosphatases, and then proceed through thermolytic prodrug-to-drug conversion. Unlike 24, the purified dinucleotide 25 is a poor substrate for human alkaline phosphatase, as dethiophosphorylation to dinucleotide 4 occurred to the extent of ca. ~15% within 30 h at 37 °C (data shown in the Supporting information). However, given the nucleophilicity of thiophosphate monoesters, the thermolytic cleavage of the 4-thiophosphato-1-butyl thiophosphate protecting group from dinucleotide 25 occurred smoothly; the half-time of the deprotection reaction was 13 min at 90 °C or 30 h at 37 °C. As discussed above, the thermolytic thiophosphate deprotection of 25 and that of dinucleotide 23 proceeded presumably through a cyclodeesterification mechanism leading to the generation of TpST and concomitant formation of the respective oxathiaphosphorinane and oxathiaphosphepane derivatives 26 and 27.

To confirm the identity of cyclodeesterification product 27 through the use of spectroscopic methods, the structurally simpler thiophosphatoalkylated phosphate triester 29 was prepared from the reaction of 4-hydroxy-1-butyl diethylphosphate (28) with an equimolar amount of dibenzyl-N,N-diisopropylphosphoramidite in the presence of 1H-tetrazole in dry MeCN (Scheme 6). Solid 3H-1,2-benzodithiol-3-one 1,1-dioxide was then added to the reaction mixture up to a concentration of 0.05 M to give the phosphotriester 29, which was purified by silica gel chromatography. Pure 29 was then reacted with sodium in liquid ammonia to give the desired phosphorothioate monoester 30. Upon addition of solid ammonium chloride to quench the reaction, the formation of the oxathiaphosphepane
derivative 27 was detected by $^{31}$P NMR spectroscopy ($\delta_P$ 17 ppm) along with that of diethylphosphate ($\delta_P$ −11 ppm) and, tentatively, 4-mercaptobutyl-1-phosphate monoester ($\delta_P$ −2 ppm), which resulted from ring opening of 27 under these conditions.

**Scheme 6.** Preparation of the 4-thiophosphato-1-butyl phosphotriester 30 and evaluation of its thermolytic cyclodeesterification$^a$

![Scheme 6 Diagram]

$^a$ Reagents and conditions: (i) dibenzyl-$N,N$-diisopropylphosphoramidite (1 equiv), 0.45 M 1H-tetrazole/MeCN, 3 min. (ii) 3H-1,2-benzodithiol-3-one 1,1-dioxide to [0.05 M], 2 min. (iii) Silica gel chromatography ($C_6H_6$:Et$_3$N (9:1 v/v)). (iv) Na/NH$_3$ (l), $-78$ °C, 2 min. (v) H$_2$O, 1 h.

To validate the spectral characteristics of 27, the compound was prepared using an alternate route (Scheme 7). Specifically, the reaction of 2,2'-dithiodipyridine with 4-mercaptobutan-1-ol gave 4-(2-pyridyldithio)butan-1-ol (31) in high yields. Phosphitylation of purified 31 with bis(2-cyanoethyl)-$N,N$-diisopropylphosphoramidite and 1H-tetrazole in MeCN produced the oxathiaphosphepane derivative 32, which after silica gel purification was obtained in a yield of 86%. The oxathiaphosphepane was dissolved in MeCN/Et$_3$N (4:1 v/v) and the solution was heated for 15 h at 55 °C to afford 27 in near quantitative yields. $^{31}$P NMR analysis of crude 27 in aqueous MeCN revealed a chemical shift identical ($\delta_P$ 17 ppm) to that of the side-product obtained from the cyclodeesterification of 30. When a solution of synthetic 27 was heated to 90 °C in aqueous MeCN, the formation of tentatively 4-mercaptobutyl-1-
phosphate monoester was detected by $^{31}$P NMR analysis of the hydrolytic reaction (data shown in the Supporting information).

**Scheme 7.** Alternate synthesis of the oxathiaphosphepane derivative $27^a$

```
\[
\begin{array}{c}
\text{SS NN} \\
\text{O} \\
\text{OCH}_2\text{CH}_2\text{CN}
\end{array}
\xrightarrow{\text{i}}
\begin{array}{c}
\text{SS} \\
\text{S(CH}_2\text{)_3OH}
\end{array}
\]

\[
\begin{array}{c}
\text{OCH}_2\text{CH}_2\text{CN} \\
\text{O}
\end{array}
\xrightarrow{\text{ii}}
\begin{array}{c}
\text{SS} \\
\text{(CH}_2\text{)_4NOH}
\end{array}
\xrightarrow{\text{iii}}
\begin{array}{c}
\text{SS} \\
\text{OCH}_2\text{CH}_2\text{CN}
\end{array}
\]

$^a$ Reagents and conditions: (i) 4-mercaptobutan-1-ol, Et$_3$N, 25 °C, 7 h. (ii) $i$-Pr$_2$NP(OCH$_2$CH$_2$CN)$_2$, 1$H$-tetrazole, 5 min. (iii) Et$_3$N/MeCN (1:4 v/v), 55 °C, 15 h.

Solid-phase synthesis of oligonucleotide CpG ODN $hbu1555$ (Figure 1) was performed employing deoxyribonucleoside phosphoramidites functionalized with the 2-($N$-formyl-$N$-methyl)aminoethyl group for phosphorus protection and phosphoramidites $8a$-$d$ under conditions identical to those used for the preparation of CpG ODN $fma1555$. Upon completion of the synthesis, the 5’-DMTr-solid-phase-linked oligonucleotide was first subjected to hydrazinolysis to remove the levulinyl groups from the oligonucleotide. Then, pressurized ammonia gas was used to deprotect the nucleobases and release the oligonucleotide from the support. The crude 5’-DMTr-oligonucleotide was purified by reversed-phase high performance liquid chromatography (RP-HPLC) and was then treated with aqueous 80% AcOH to cleave the 5’-DMTr group. Purification of the dedimethoxytritylated oligonucleotide by RP-HPLC gave CpG ODN $hbu1555$, which was characterized satisfactorily by ESI-TOF mass spectrometry. Conversion of purified CpG ODN $hbu1555$ to CpG ODN 1555 was achieved upon heating to 90 °C in PBS (pH 7.2) over a period of 6 h. Analysis of the deprotection reaction by polyacrylamide gel electrophoresis
(PAGE) under denaturing conditions revealed, as expected, an oligonucleotidic band exhibiting an electrophoretic mobility identical to that of CpG ODN 1555 (Data shown in Supporting information). When taken together, these analytical methods confirm the identity of CpG ODN hbu1555 and its function as a prodrug through clean thermolytic conversion to CpG ODN 1555. The solid-phase synthesis of oligonucleotides CpG ODN pob1555 and CpG ODN psb1555 (Figure 1) was achieved in a manner identical to that described for CpG ODN hbu1555 with the following modifications: (i) after hydrazinolysis of the levulinyl groups, the released hydroxyl groups were reacted twice with phosphorylating reagent 9 (5 equiv per hydroxyl) and 0.45 M 1H-tetrazole in MeCN over a period of 3 min; (ii) the newly formed phosphite triesters were treated with 0.1 M 3H-1,2-benzodithiol-3-one 1,1-dioxide in MeCN for 3 min or with 0.1 M ethyl(methyl)dioxirane in CH₂Cl₂ for 1 min; (iii) the DMTr groups were cleaved upon exposure to 3% TCA in CH₂Cl₂ over a period of 15 min; and (iv) the dedimethoxytritylated oligonucleotides were mixed with 1.2% (w/v) DTT/H₂O containing 5% (v/v) Et₃N for 60 min. Pressurized ammonia gas was employed to deprotect the nucleobases and cleave the oligonucleotide from the support. Crude oligonucleotides CpG ODN pob1555 and CpG ODN psb1555 were then purified by anion-exchange HPLC. Desalted CpG ODN pob1555 and CpG ODN psb1555 were characterized by ESI-TOF mass spectrometry. CpG ODN psb1555 was characterized further by conversion to CpG ODN 1555 upon heating at 90 °C in PBS (pH 7.2) over a period of 2 h. Desalted CpG ODN pob1555 was dephosphorylated within 2 h by treatment with human alkaline phosphatase at 37 °C and was then converted to CpG ODN 1555 upon heating in PBS (pH 7.2) for 6 h at 90 °C. PAGE analysis showed complete thermolytic conversion of CpG ODN psb1555 and dephosphorylated CpG ODN pob1555 to CpG ODN 1555 (Data shown in Supporting information) thereby supporting the identity and function of the potential oligonucleotide prodrugs. Noteworthy is the increased solubility of CpG ODN pob1555 and CpG ODN psb1555 relative to that of CpG ODN hbu1555 and CpG ODN fma1555 in aqueous media.

The resistance of oligonucleotide prodrugs CpG ODN hbu1555, CpG ODN pob1555 and CpG ODN psb1555 to nucleases was demonstrated by exposing each of the prodrugs to snake venom
phosphodiesterase (SVP) and S1 nuclease over a period of 24 h at 37 °C. Given that oligonucleoside phosphorothioates are very sensitive to S1 nuclease and moderately sensitive to SVP,\textsuperscript{16} CpG ODN 1555 was subjected to the nucleolytic activity of these enzymes for comparison purposes. On the basis of RP-HPLC analysis of the hydrolysates, all three prodrugs showed minimal hydrolysis (<10%) upon treatment with SVP and S1 nuclease, whereas CpG ODN 1555 was completely hydrolyzed by S1 nuclease and to the extent of 80% by SVP under similar conditions.

To summarize, we have designed and prepared thermolytic CpG oligonucleotide prodrugs, each of which has a CpG motif functionalized with a thiophosphate protecting group exhibiting an increased or decreased thermosensitivity relative to that of the previously studied 2-(N-formyl-N-methyl)aminoethyl thiophosphate protecting group. Although CpG ODN \textit{hbu}1555 and CpG ODN \textit{psb}1555 will not require intracellular enzymes for conversion to the bioactive CpG ODN 1555, the oligonucleotide prodrug CpG ODN \textit{pob}1555 will require intracellular phosphatase activity for conversion to CpG ODN \textit{hbu}1555. Such a two-stage conversion process to CpG ODN 1555 provides an additional degree of control on the prodrug-to-drug conversion. The negatively charged oligonucleotide prodrugs CpG ODN \textit{psb}1555 and CpG ODN \textit{pob}1555 are freely soluble in aqueous media, whereas the solubility of CpG ODN \textit{hbu}1555 and that of the CpG ODN \textit{fma}1555 in similar media is limited to concentrations of ~? µM for chain lengths of no more than 15 nucleobases. Studies assessing cellular uptake and the biological activity of these oligonucleotide prodrugs are ongoing and the results of these studies will be reported elsewhere in due course. Other thermolytic oligonucleotide prodrugs are also currently being developed in our laboratories to provide increasingly effective heat-sensitive therapeutic oligonucleotide formulations.
Experimental Section

3-Hydroxy-1-propyl levulinate (10). To a stirred solution of 1,3-propanediol (7.60 g, 100 mmol), levulinic acid (11.6 g, 100 mmol) and 4-dimethylaminopyridine (500 mg) in 1,4-dioxane (60 mL) was added DCC (20 g, 97 mmol), portionwise, over a period of 2 h at ambient temperature. The reaction mixture was allowed to stir overnight under these conditions. The \(N,N'\)-dicyclohexylurea precipitate was filtered off and washed with 1,4-dioxane (20 mL). The filtrates were collected and evaporated to an oil under reduced pressure. The oily material was dissolved in a minimum volume of CHCl\(_3\)-MeOH (96:4 v/v) and applied to the top of a column packed with silica gel (~150 g) pre-equilibrated CHCl\(_3\)-MeOH (96:4 v/v). Elution of the column with the equilibration solvent afforded pure 3-hydroxy-1-propyl levulinate (10) in 49% yield (8.5 g, 48.8 mmol). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)): \(\delta\) 4.05 (t, \(J = 6.4\) Hz, 2H), 3.45 (t, \(J = 6.1\) Hz, 2H), 2.70 (t, \(J = 6.4\) Hz, 2H), 2.45 (t, \(J = 6.4\) Hz, 2H), 2.10 (s, 3H), 1.7 (tt, \(J = 6.1, 6.4\) Hz, 2H). \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)): \(\delta\) 206.8, 172.3, 61.3, 57.2, 37.4, 31.5, 29.4, 27.6.

4-Hydroxy-1-butyl levulinate (11). This compound was prepared from 1,4-butanediol and levulinic acid in a manner identical to that of 10 in terms of scale and conditions. Following silica gel chromatography, 4-hydroxy-1-butyl levulinate (11) was isolated as a pure oil (8.0 g, 42.5 mmol) in 43% yield. \(^1\)H NMR (300 MHz, DMSO-\(d_6\)): \(\delta\) 4.40 (b, 1H), 3.99 (t, \(J = 6.6\) Hz, 2H), 3.40 (t, \(J = 6.2\) Hz, 2H), 2.70 (t, \(J = 6.6\) Hz, 2H), 2.45 (t, \(J = 6.6\) Hz, 2H), 2.1 (s, 3H), 1.58 (m, 2H) 1.44 (m, 2H) \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)): \(\delta\) 206.8, 172.2, 63.8, 60.2, 37.3, 29.3, 28.7, 27.6, 24.9.

\(O\)-[3-(2,4-dioxopent-1-yl)oxy]-1-propyl-N,N,N',N'-tetraisopropylphosphorodiamidite (12). To a stirred solution of 3-hydroxy-1-propyl levulinate (0.87 g, 5.0 mmol) and \(i\)-Pr\(_2\)NEt (4.15 g, 34.6 mmol) in dry CH\(_2\)Cl\(_2\), was added bis\((N,N\)-diisopropylamino)chlorophosphine (1.35 g, 5.06 mmol) by syringe over a period of 2 h at ~25 °C. \(^{31}\)P NMR analysis of the reaction showed complete conversion of bis\((N,N\)-diisopropylamino)chlorophosphine (\(\delta_p\) 135.5) to the phosphorodiamidite 12 (\(\delta_p\) 122.0). The suspension was filtered and the filtrate was evaporated to an oil under reduced pressure. The crude
phosphorodiamidite was purified by silica gel chromatography using a solution of C\textsubscript{6}H\textsubscript{6} and Et\textsubscript{3}N (9:1 v/v) as the eluant. Yield: 75% (1.52 g, 3.75 mmol). \textsuperscript{1}H NMR (300 MHz, C\textsubscript{6}D\textsubscript{6}): \( \delta \) 4.23 (t, \( J = 6.6 \) Hz, 2H), 3.60 (dt, \( J = 6.2 \) Hz, \( J_{HP} = 7.1 \) Hz, 2H), 3.51 (sept, \( J = 6.8 \) Hz, 2H), 3.47 (sept, \( J = 6.8 \) Hz, 2H), 2.36 (t, \( J = 6.5 \) Hz, 2H), 2.17 (t, \( J = 6.5 \) Hz, 2H), 1.80 (m, 2H), 1.62 (s, 3H), 1.21 (d, \( J = 6.8 \) Hz, 12H), 1.17 (d, \( J = 6.8 \) Hz, 12H). \textsuperscript{13}C NMR (75 MHz, C\textsubscript{6}D\textsubscript{6}): \( \delta \) 204.4, 172.3, 61.9, 61.0 (d, \( J_{CP} = 21.5 \) Hz), 44.8, 44.6, 37.6, 31.1 (d, \( J_{CP} = 8.4 \) Hz), 29.1, 28.1, 24.7, 24.6, 24.1, 24.0. \textsuperscript{31}P NMR (121 MHz, C\textsubscript{6}D\textsubscript{6}): \( \delta \) 123.5.

\textbf{O-[4-(2,4-dioxopent-1-yl)oxy]-1-butyl-\( N,N,N',N' \)-tetraisopropylphosphorodiamidite} (13). The preparation of this phosphorodiamidite was performed on a 15 mmole scale under conditions identical to those used for the synthesis and purification of 12. Yield: 72% (4.5 g, 10.8 mmol). \textsuperscript{1}H NMR (300 MHz, C\textsubscript{6}D\textsubscript{6}): \( \delta \) 4.04 (t, \( J = 6.4 \) Hz, 2H), 3.54 (dt, \( J = 5.9 \) Hz, \( J_{HP} = 7.4 \) Hz, 2H), 3.53 (sept, \( J = 6.8 \) Hz, 2H), 3.49 (sept, \( J = 6.8 \) Hz, 2H), 2.35 (t, \( J = 6.5 \) Hz, 2H), 2.15 (t, \( J = 6.5 \) Hz, 2H), 1.61 (s, 3H), 1.60 (m, 4H), 1.23 (d, \( J = 6.8 \) Hz, 12H), 1.19 (d, \( J = 6.8 \) Hz, 12H). \textsuperscript{13}C NMR (75 MHz, C\textsubscript{6}D\textsubscript{6}): 204.5, 172.3, 64.5, 64.2 (d, \( J_{CP} = 21.5 \) Hz), 44.7, 44.6, 37.6, 29.1, 28.4 (d, \( J_{CP} = 8.4 \) Hz), 28.1, 26.0, 24.8, 24.7, 24.1, 24.0. \textsuperscript{31}P NMR (121 MHz, C\textsubscript{6}D\textsubscript{6}): \( \delta \) 122.8.

\textbf{General procedure for the preparation deoxyribonucleoside phosphoramidites 7a and 8a-d.} Commercial 2’-deoxyribonucleosides (14a-d, 1.0 mmol) were dried by co-evaporation with anhydrous pyridine (2 × 5 mL) and dry toluene (5 mL) under reduced pressure. The foamy nucleoside was then dissolved in anhydrous MeCN (5 mL) and phosphorodiamidite 12 or 13 (1.0 mmol) was added by syringe, under a positive pressure of argon. Sublimed \( 1H \)-tetrazole (70 mg, 1.0 mmol) was added to the stirred solution, portionwise, over a period of 1 h. The reaction mixture was allowed to stir at ambient temperature for 3 h\textsuperscript{17} and was then rotoevaporated to a foam under vacuum. The material was dissolved in a minimal volume of C\textsubscript{6}H\textsubscript{6}:Et\textsubscript{3}N (9:1 v/v) and added to the top of a chromatography column packed with silica gel (~30 g) that was equilibrated in C\textsubscript{6}H\textsubscript{6}:Et\textsubscript{3}N (9:1 v/v). The column was eluted with the equilibration solvent and fractions containing the product were identified by TLC. These fractions were pooled together and ratoevaporated under low pressure to a white foam. The material was dissolved in
dry C₆H₆ (~5 mL). The resulting solution was frozen and then lyophilized under high vacuum to give 7a or 8a-d as white powders in yields ranging from 70% to 90%.

5’-O-(4,4’-Dimethoxytrityl)-3’-O-[(N,N-diisopropylamino)(3-(2,4-dioxopent-1-yl)oxy-1-propyloxy)] phosphinyl-2’-deoxythymidine (7a). ³¹P NMR (121 MHz, C₆H₆): δ 148.8, 148.4. APESI-HRMS: calcd for C₄₅H₅₉N₃O₁₁P (M + H)⁺ 848.3887, found...???

5’-O-(4,4’-Dimethoxytrityl)-3’-O-[(N,N-diisopropylamino)(4-(2,4-dioxopent-1-yl)oxy-1-butyloxy)] phosphinyl-2’-deoxythymidine (8a). ³¹P NMR (121 MHz, C₆H₆): δ 148.3, 147.8. APESI-HRMS: calcd for C₄₆H₆₁N₃O₁₁P (M + H)⁺ 862.4044, found 862.4036.


N⁶-Benzoyl-5’-O-(4,4’-dimethoxytrityl)-3’-O-[(N,N-diisopropylamino)(4-(2,4-dioxopent-1-yl)oxy-1-butyloxy)] phosphinyl-2’-deoxyadenosine (8c). ³¹P NMR (121 MHz, C₆H₆): δ 148.0. APESI-HRMS: calcd for C₅₃H₆₄N₆O₁₀P (M + H)⁺ 975.4422, found 975.4413.


Preparation of the dinucleoside thiophosphate triesters 3 and 4. A commercial synthesis column packed with succinyl long chain alkylamine controlled-pore glass functionalized with 5’-O-DMTr-2’-deoxythymidine (0.2 µmol/g of support) was treated with 3% TCA in CH₂Cl₂ for 1 min. After washing the support extensively with CH₂Cl₂ and MeCN, the coupling reaction was performed by shaking the support in a solution composed of phosphoramidite 7a or 8a (0.03 mmol) and 0.45 M 1H-tetrazole in MeCN (0.3 mL) for 10 min. Excess reagents were washed off the synthesis column with MeCN and the support was then treated with 0.05 M 3H-1,2-benzodithiol-1,1-dioxide in MeCN (1 mL) for 2 min. Excess oxidant was washed off the support with MeCN. The levulinyl group was then removed by hydrazinolysis using 0.5 M NH₂NH₂H₂O in pyridine/acetic acid (3:2 v/v) for 10 min at ~25 °C.
Following extensive washing of the CPG support with CH\textsubscript{2}Cl\textsubscript{2} and MeCN, removal of the 5'-DMTr groups was effected by the use of 3% TCA in CH\textsubscript{2}Cl\textsubscript{2} over a period of 1 min. After careful washing of the support with CH\textsubscript{2}Cl\textsubscript{2}, the synthesis column was exposed to pressurized methylamine gas for 3 min to release the dinucleoside thiophosphate triester 3 or 4. The purity of the crude dinucleoside triesters was assessed by RP-HPLC and was determined to be ~97% (data shown in the Supporting Information).

**Thymidylyl-(3’→5’)-thymidine thiophosphate 3-hydroxy-1-propyl ester (3).** ESI-MS: calcd for C\textsubscript{23}H\textsubscript{33}N\textsubscript{4}O\textsubscript{12}PS (M)\textsuperscript{+} 620.16, found...?..

**Thymidylyl-(3’→5’)-thymidine thiophosphate 4-hydroxy-1-butyl ester (4).** ESI-MS: calcd for C\textsubscript{24}H\textsubscript{35}N\textsubscript{4}O\textsubscript{12}PS (M)\textsuperscript{+} 634.17, found...?..

**Kinetic analysis of the thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group from 4.** RP-HPLC-purified 4 (~10 OD\textsubscript{260}) were dissolved in PBS (pH 7.2, 500 µL) and was heated to 90 ± 2 °C. Aliquots were taken periodically over a period of 6 h. Each aliquot was analyzed by RP-HPLC using a 5 µm Supelcosil LC-18S column (25 cm × 4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min. Under these chromatographic conditions the retention times (t\textsubscript{R}) for 4 and T\textsubscript{PS}T were 30.4 min and 20.5, 21.1 min, respectively. The half time for the thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group at 90 °C was determined to be 47 min. The thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group was also investigated at 37 ± 2 °C using ~20 OD\textsubscript{260} of RP-HPLC-purified 4 in PBS (pH 7.2, 1 mL). Aliquots were taken periodically over a period of 200 h and were analyzed by RP-HPLC under the conditions used for the kinetic analysis performed at 90 °C. The thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group at 37 °C proceeded with a half time of 168 h.

**Kinetic analysis of the thermolytic cleavage of the 3-hydroxy-1-propyl thiophosphate protecting group from 3.** This analysis was performed at 90 ± 2 °C under conditions identical to those described for the thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group. RP-HPLC analysis of the deprotection reaction indicated that the 3-hydroxy-1-propyl group was thermolytically
cleaved from 3 to the extent of less than 5% over a period of 6 h at 90 °C. Unidentified side-products accounting for less than 20% of the total peak area were also detected.

**Preparation of O,O-diphenyl-O-(4-hydroxy-1-butyl)phosphate (17).** To a stirred solution 1,4-butanediol (2.25 g, mmol) and i-Pr<sub>2</sub>NEt (1.0 g, mmol) in dry C<sub>6</sub>H<sub>6</sub> (?? mL) was added diphenyl chlorophosphate (1.35 g, mmol), dropwise, over a period of 15 min. The reaction mixture was left stirring at ambient temperature for 1 h. The reaction mixture was rotoevaporated to dryness under reduced pressure. The material left was suspended in a minimal amount of CHCl<sub>3</sub> (2 mL) and applied to the top of a chromatography column packed with silica gel (40 g) equilibrated in CHCl<sub>3</sub>. The product was eluted from the column with CHCl<sub>3</sub> affording 17 (??g, ??mmol) as a colorless oil. Yield: 80%.

**1H NMR (300 MHz, DMSO-d<sub>6</sub>):** δ 7.38 (m, 4H), 7.20 (m, 6H), 4.23 (dt, <i>J</i><sub>HP</sub> = <i>J</i> = 6.4 Hz, 2H), 3.95 (s, 1H), 3.38 (t, <i>J</i><sub>HP</sub> = 6.4 Hz, 2H), 1.66 (m, 2H), 1.45 (m, 2H).<br>

**13C NMR (75 MHz, DMSO-d<sub>6</sub>):** δ 150.0 (d, <i>J</i><sub>CP</sub> = 7.2 Hz), 130.0, 125.4, 119.8 (d, <i>J</i><sub>CP</sub> = 4.8 Hz), 69.2 (d, <i>J</i><sub>CP</sub> = 6.0 Hz), 60.0, 28.2, 26.4 (d, <i>J</i><sub>CP</sub> = 6.0 Hz).<br>

**31P NMR (121 MHz, DMSO-d<sub>6</sub>):** δ −12.1.

**Generation of THF during thermal cyclodeesterification of O,O-diphenyl-O-(4-hydroxy-1-butyl)phosphate.** Pure 17 (??mg, ??mmol) was dissolved in DMSO-d<sub>6</sub>/D<sub>2</sub>O (5:1 v/v, 0.5 mL) and heated for 4 h at 90 °C. **1H NMR analysis of the reaction revealed an almost complete disappearance of signals at 1.4 and 1.6 ppm and the appearance of new signals at 1.7 and 3.5 ppm, which are identical to those obtained from the **1H NMR spectrum of commercial THF.** Signals at 7.1-7.4 ppm correspond to those of diphenyl phosphate. **31P NMR analysis of the reaction shows an almost complete conversion (>95%) of 17 (δ<sub>p</sub> = −12 ppm) to diphenyl phosphate (δ<sub>p</sub> = −13 ppm). Chemical shifts are upfield relative to an external standard of phosphoric acid in D<sub>2</sub>O (δ<sub>p</sub> 0 ppm).**

**Preparation of the thiophosphatoalkylated dinucleoside thiophosphate triesters 23 and 25.** A commercial synthesis column packed with succinyl long chain alkylamine controlled-pore glass functionalized with 5’-O-DMTr-2’-deoxythymidine (0.2 µmol/g of support) was treated with 3% TCA in CH<sub>2</sub>Cl<sub>2</sub> for 1 min. After carefully washing the support with CH<sub>2</sub>Cl<sub>2</sub> and MeCN, the coupling reaction
was performed by agitating the support in a solution composed of phosphoramidite 7a or 8a (0.03 mmol) and 0.45 M 1H-tetrazole in MeCN (0.3 mL) for 10 min. Excess reagents were washed off the synthesis column with MeCN and the support was then treated with 0.05 M 3H-1,2-benzodithiol-3-one 1,1-dioxide in MeCN (1 mL) for 2 min. Excess oxidant was washed off the support with MeCN affording the dinucleoside phosphotriesters 15 or 16. The levulinyl group was then removed from 15 or 16 by treatment with 0.5 M NH₂NH₂•H₂O in pyridine/acetic acid (3:2 v/v) for 10 min at ~25 °C. Following extensive washing of the CPG support 18 or 19 with CH₂Cl₂ and MeCN, a coupling reaction was performed using an excess of the phosphorylating reagent 9 (27 mg, 0.03 mmol) activated with 0.45 M 1H-tetrazole in MeCN (0.3 mL) over a period of 3 min. Excess reagents were washed off the synthesis column with MeCN and the support was then treated with 0.05 M 3H-1,2-benzodithiol-3-one 1,1-dioxide in MeCN (1 mL) for 2 min. Excess oxidant was washed off the support with MeCN to give the dinucleotides 20 or 22. Removal of all the DMTr groups was effected by the use of 3% TCA in CH₂Cl₂ over a period of 10 min. The support was then washed with CH₂Cl₂ and MeCN followed by a solution of 1.2 % (w/v) DTT in H₂O (1 mL) containing 5% (v/v) Et₃N over a period of 30 min. After extensive washing of the support with CH₂Cl₂, the synthesis column was exposed to pressurized methylamine gas (~2.5 bar) for 3 min to release the crude thiophosphatoalkylated dinucleoside thiophosphate triesters 23 or 25. Each dinucleotide was purified by RP-HPLC using a 5 µm Supelcosil LC-18S column (25 cm × 4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min. Under these chromatographic conditions, the retention times (t_R) of diastereomeric 23 or 25 are 25.3 and 25.6 min or 26.5 min, respectively. Thymidylyl-(3’→5’)thymidine thiophosphate 3-thiophosphato-1-propyl ester (23).³¹P NMR (121 MHz, D₂O): δ_p ? +MALDI-TOF MS: calcd for C₂₃H₃₄N₄O₁₄P₂S₂ (M + 2H)⁺ 716.10, found ? . Thymidylyl-(3’→5’)thymidine thiophosphate 4-thiophosphato-1-butyl ester (25).³¹P NMR (121 MHz, H₂O): δ 67.1, 66.9, 51.9. −MALDI-TOF MS: calcd for C₂₄H₃₅N₄O₁₄P₂S₂ (M – H) − 729.63, found 729.66.
Preparation of the phosphatoalkylated dinucleoside thiophosphate triester 24. The preparation of the dinucleotide 24 was achieved in a manner identical to that described for the preparation of dinucleotides 23 and 25 with the following exceptions: (i) upon phosphitylation of 19 with 9, a solution of 0.1 M ethyl(methyl)dioxirane in CH₂Cl₂, instead of 3H-1,2-benzodithiol-3-one 1,1-dioxide, was used over a period of 1 min to give the dinucleotide 21; and (ii) following removal of all the DMTr groups, a solution of 1.2% (w/v) DTT in H₂O containing 5% (v/v) Et₃N was employed over a period of 60 min prior to release of 24 from the support. The RP-HPLC purification of dinucleotide 24 was done under conditions identical to those described for the purification of 23 and 25. Under such chromatographic conditions, the retention time (tᵣ) of diastereomeric 24 is 27.5 and 27.6 min. Thymidylyl-(3′→5′)-thymidine thiophosphate 4-phosphato-1-butyl ester (24). ³¹P NMR (121 MHz, H₂O): δ 68.0, 1.8. +MALDI-TOF MS: calcd for C₂₄H₃₆N₄O₁₅P₂S (M + 2H)⁺ 714.14, found...?.

Kinetic analysis of the thermolytic cleavage of the thiophosphatoalkylated dinucleoside thiophosphate triesters 23 and 25. This analysis was performed 37 ± 2 °C under conditions identical to those described for the thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group from dinucleotide 4. Aliquots were taken periodically over a period of 200 h. RP-HPLC analysis of the deprotection reaction indicated that the thermolytic cleavage of the 3-thiophosphato-1-propyl protecting group from dinucleotide 23 to produce T₃P₂T occurred with a half time of 95 min. By comparison, the thermolytic cleavage of the 4-thiophosphato-1-butyl protecting group from dinucleotide 25 to produce T₄P₂T proceeded with a half time of 30 h.

Kinetic analysis of the thermolytic cleavage of the phosphatoalkylated dinucleoside thiophosphate triester 24. This analysis was performed 37 ± 2 °C under conditions identical to those described for the thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group from dinucleotide 4. Aliquots were taken periodically over a period of 45 d. RP-HPLC analysis of the deprotection reaction indicated that the thermolytic cleavage of the 4-phosphato-1-butyl protecting group from dinucleotide 24 to produce T₄P₂T occurred with a half time of 45 d.
Enzymatic dephosphorylation of dinucleotide 24 catalyzed by human alkaline phosphatase. To a solution of RP-HPLC purified dinucleotide 24 (OD$_{260}$) in 10 mM glycine buffer (pH 10.4, 200 µL) was added human alkaline phosphatase (1 U, 15 µL). The solution was incubated at 37 ± 2 °C for 2 h. An aliquot was analyzed by RP-HPLC using a 5 µm Supelcosil LC-18S column (25 cm × 4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min. The analysis revealed complete dephosphorylation of 24 ($t_R = ?$ min) to dinucleotide 4 ($t_R = ?$ min). When dinucleotide 25 was used as substrate under identical conditions, less than 5% dethiophosphorylation to dinucleotide 4 was detected after an incubation time of 2 h, whereas % dethiophosphorylation was detected after an incubation time of 30 h.

Preparation of $O$-(4-hydroxy-1-butyl)-$O,O$-diethylphosphate (28). To a stirred solution of 1,4-butanediol (11.3 g, 125 mmol) and Et$_3$N (204 mmol) in dry benzene (15 mL) was added diethyl chlorophosphate (8.66 g, 50.2 mmol), dropwise under an inert atmosphere, over a period of 1.5 h at ~25 °C. The suspension was filtered and the hydrochloride salt was washed with benzene (50 mL). The filtrates were combined and then evaporated under reduced pressure. The residue was dissolved in a minimal amount of CH$_2$Cl$_2$ and purified by silica gel chromatography. The product was eluted from the column using a gradient of CH$_2$Cl$_2$:MeOH (0 → 5%) affording 9.98 g of pure 28 (44.2 mmol, 88%). $^1$H NMR (300 MHz, DMSO-$d_6$): δ ??.$^1$C NMR (75 MHz, DMSO-$d_6$): δ ??.$^{31}$P NMR (121 MHz, CH$_2$Cl$_2$): δ −0.31.

Preparation of $O$-4-($O'$,$O'$-dibenzylthiophosphoryl)oxy-1-butyl)-$O,O$-diethylphosphate (29). To a stirred solution of $O$-(4-hydroxy-1-butyl)-$O,O$-diethylphosphate (28, 1.00 g, 4.42 mmol) and dibenzyl-$N,N$-diisopropyl phosphoramidite (1.53 g, 4.42 mmol) in dry MeCN (5 mL) was added 0.45 M 1$H$-tetrazole in anhydrous MeCN (10 mL) slowly under an inert atmosphere at ~25 °C. The kinetics of the reaction was monitored by $^{31}$P NMR spectroscopy, which showed a complete reaction within 2 h. 3$H$-1,2-Benzodithiol-3-one 1,1-dioxide (1.00 g, 5.00 mmol) in MeCN (35 mL) was added to the reaction
mixture, which was stirred for period of 2 min. The solvent was removed by rotoevaporation under reduced pressure and the material left was dissolved in CH$_2$Cl$_2$ (50 mL) and washed twice with brine (50 mL). The organic layer was then rotoevaporated under vacuum and the crude product was dissolved with a minimum amount of CH$_2$Cl$_2$ and purified by silica gel chromatography. The product was eluted from the column using a gradient of CH$_2$Cl$_2$:MeOH (0 → 5%) to give pure 29 (1.04 g, 2.07 mmol, 47%) as a pale yellow oil. $^1$H NMR (300 MHz, DMSO-$d_6$): δ ??. $^{13}$C NMR (75 MHz, DMSO-$d_6$): δ ??. $^{31}$P NMR (121 MHz, CH$_2$Cl$_2$): δ 70.2, 0.19.

**Preparation of 4-(2-pyridyldithio)butan-1-ol (31).** This compound was synthesized essentially as described for the preparation of 3-(2-pyridyldithio)propan-1-ol.$^{18}$ To a stirred solution of 2,2-dithiopyridine (8.3 g, 37.7 mmol) and glacial acetic acid (0.55 mL) in MeOH (143 mL) was added, dropwise over 10 min, 4-mercapto-1-butanol (2.00 g, 18.9 mmol) in MeOH (7 mL). The solution was stirred for 3 h at ambient temperature and was then concentrated under reduced pressure to an oil. The crude product was purified by silica gel chromatography. The product was eluted from the column using a gradient of EtOAc:hexane (1:2 → 1:1) affording 1.59 g of pure 30 (7.40 mmol, 39%). $^1$H NMR (300 MHz, DMSO-$d_6$): δ 8.4 (ddd, $J = 0.8, 1.6, 4.7$ Hz, 1H), 7.77 (m, 2H), 7.2 (ddd, $J = 1.4, 4.8, 6.9$ Hz, 1H), 4.43 (bs, 1H), 3.39 (t, $J = 6.2$ Hz, 2H), 2.83 (t, $J = 7.2$ Hz, 2H), 1.67 (m, 2H), 1.50 (m, 2H). $^{13}$C (75 MHz, DMSO-$d_6$): δ 25.1, 31.0, 38.0, 60.1, 119.0, 120.8, 137.5, 149.3, 159.4.

**Preparation of 2-(2-cyanoethyl)oxy-2-oxo-1,3,2-oxathiaphosphepane (32).** To a solution of 4-(2-pyridyldithio)butan-1-ol (31, 476 mg, 2.20 mmol) and bis(2-cyanoethyl)-N,N-diisopropylphosphoramidite (600 mg, 2.20 mmol) in dry MeCN (5.0 mL) was added by syringe, under an inert atmosphere, 0.45 M 1$H$-tetrazole (4.8 mL, 2.2 mmol) in anhydrous MeCN. The reaction mixture was left stirring over a period of 3 h at ambient temperature and was then diluted with CH$_2$Cl$_2$ (80 mL). The solution was extracted with brine (20 mL). The organic layer was washed further with H$_2$O (20 mL) and then dried over anhydrous Na$_2$SO$_4$. The suspension was filtered and evaporated under reduced pressure. The residue was purified by silica gel chromatography using CH$_2$Cl$_2$/MeOH (0 → 15%) as the eluant. Pure 32 (420 mg, 1.90 mmol, 86%) was isolated as an oil after removal of the
solvent upon rotoevaporation under vacuum. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ ?? $^{13}$C (75 MHz, DMSO-$d_6$): $\delta$ ?? $^{31}$P NMR (121 MHz, DMSO-$d_6$): $\delta$ 32.8.

**Preparation of the triethylammonium salt of 2-hydroxy-2-oxo-1,3,2-oxathiaphosphepane (27).**

To a solution of 2-(2-cyanoethyl)oxy-2-oxo-1,3,2-oxathiaphosphepane (32, 160 mg, 0.72 mmol) in MeCN was added Et$_3$N (500 µL, 3.9 mmol). The solution was allowed to stir at 55 °C for 15 h.

**Solid-phase oligonucleotide synthesis.** Solid-phase synthesis of the modified oligonucleotides shown in Figure 1 was performed on a scale and under conditions identical to that described earlier for the preparation of CpG ODN $fma$1555 and CpG ODN 1555. However, upon completion of the first four automated synthesis cycles, the four glass bottles each containing one of the four $fma$ phosphoramidites were replaced with four glass bottles each containing 8a, 8b, 8c, or 8d as a 0.1 M solution in dry MeCN. The phosphoramidite-feeding lines on the synthesizer were properly flushed with MeCN to prevent cross-contamination of 8a-d and were primed with amidites to ensure high coupling efficiency. Six synthesis cycles were performed with phosphoramidites 8a-d and were followed with the last four synthesis cycles using the four $fma$ phosphoramidites after a phosphoramidite bottle change, and a flush and prime of phosphoramidite-feeding lines.

Upon completion of the synthesis, the support was treated with 0.5 M NH$_2$NH$_2$•H$_2$O in pyridine:AcOH (3:2 v/v) by pushing the solution (0.5 mL) back and forth through the synthesis column over a period of 10 min using two 1 mL syringes, one of each being connected to one end of the column. The excess reagent was discarded and the column was flushed, repeatedly, with CH$_2$Cl$_2$ and MeCN. Approximately two-thirds of the support were transferred to a new empty synthesis column and a solution of phosphorylating reagent 9 (?M) in 0.45 M 1H-tetrazole/MeCN (? mL) was syringed back and forth through the column for 3 min. The excess reagent was pushed off the synthesis column with dry MeCN (? mL) and the phosphorylation step was performed again. After thoroughly washing the support with MeCN (? mL), approximately half of it was transferred to a new empty synthesis column.
The support of one of the two columns was reacted with 0.05 M 3H-1,2-benzodithiol-3-one 1,1-dioxide in MeCN (\text{? mL}) for 2 min, whereas the support of the other column was treated 0.1 M ethyl(methyl)dioxirane in CH$_2$Cl$_2$ (\text{? mL}) for 1 min. The excess oxidant was flushed off the column with MeCN and the crude support-bound oligonucleotides were ready for final deprotection.

**Oligonucleotide deprotection and purification.** The solid-phase-linked 5’-DMTr-oligonucleotide corresponding to CpG ODN *hbu*1555 was deprotected and purified under conditions identical to those reported for the deprotection and purification of CpG ODN *fma*1555.$^5$ The concentration of CpG ODN *hbu*1555 was determined by UV spectrophotometry at 260 nm upon reconstitution of the purified oligonucleotide in ddH$_2$O (1 mL). The recovered yield of CpG ODN *hbu*1555 was \text{? OD$_{260}$ units.}

Pure CpG ODN *hbu*1555 was stored frozen at −20 °C.

The solid-phase-linked 5’-DMTr-oligonucleotides corresponding to CpG ODN *psb*1555 and CpG ODN *pob*1555 were first dedimetoxytritylated by treatment with 3% TCA in CH$_2$Cl$_2$ for 10 min. Excess TCA was washed off the synthesis column with CH$_2$Cl$_2$ (3 mL) and MeCN (3 mL). The solid-phase bound oligonucleotides were then agitated for \text{? min in 1.2% (w/v) DTT/H$_2$O containing 5% (v/v) Et$_3$N.}

The synthesis columns were flushed with H$_2$O and then with MeCN. The columns were placed into a stainless steel pressure vessel and exposed to pressurized ammonia (10 bar at 25 °C) for 12 h.$^{19}$ Upon removal of residual ammonia from the pressure container, the oligonucleotides were eluted off their respective synthesis column with 40% MeCN in 0.1 M triethyl ammonium acetate (TEAA, pH 7.0) (1 mL). The purification of each oligonucleotide was accomplished by anion-exchange (AE)-HPLC employing a UV-detection system and an analytical Dionex PA-100 anion-exchange column (4.6 mm × 250 mm). Describe here the gradient used… The product peaks were collected and the eluates were evaporated using a stream of air without heating. The product was desalted by RP-HPLC using UV-detection system and a 5 µm Supelcosil LC-18S column (25 cm × 4.6 mm) according to the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min and then held isocratically for 10 min. The product peaks
were collected and the eluates were evaporated using a stream of air without heating. The concentration of CpG ODN \textit{psb}1555 and CpG ODN \textit{pob}1555 was determined by UV spectrophotometry at 260 nm upon reconstitution of the purified oligonucleotides in ddH\textsubscript{2}O (1 mL). The recovered yields of CpG ODN ODN \textit{psb}1555 and CpG ODN \textit{pob}1555 were \textit{?} and \textit{?} OD\textsubscript{260} units, respectively. Pure CpG ODN \textit{psb}1555 and CpG ODN \textit{pob}1555 were stored frozen at −20 °C.

**Oligonucleotide characterization.** RP-HPLC analysis of purified CpG ODN \textit{hbu}1555, CpG ODN \textit{psb}1555 and CpG ODN \textit{pob}1555 was performed using an analytical 5 μm Supelcosil LC-18S column (4.6 mm × 25 cm) according to the following conditions: starting from 0.1 M TEAA (pH 7.0), a linear gradient of 1% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min. Under these chromatographic conditions, CpG ODN \textit{hbu}1555 had a retention time ($t_R$) of \textit{?} min, whereas CpG ODN \textit{psb}1555 and CpG ODN \textit{pob}1555 displayed $t_R$s of \textit{?} min and \textit{?} min, respectively. RP-HPLC profiles of the purified oligonucleotides are shown in the Supporting information. Samples of the purified oligonucleotides were characterized by Electrospray Ionization Time of Flight mass spectrometry (ESI-TOF MS) operating in the positive ion mode (technical and operational details are provided in the Supporting information) and resulted in the following mass determinations: CpG ODN \textit{hbu}1555 (+ESI-TOF MS): calcd for $C_{203}H_{289}N_{65}O_{89}P_{14}S_{14}$ (M)$^+$ 5946, found………. CpG ODN \textit{psb}1555 (+ESI-TOF MS): calcd for $C_{203}H_{295}N_{65}O_{101}P_{20}S_{20}$ (M + 12H)$^+$ 6523, found………. CpG ODN \textit{pob}1555 (+ESI-TOF MS): calcd for $C_{203}H_{295}N_{65}O_{107}P_{20}S_{14}$ (M + 12H)$^+$ 6426, found……….

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**Supporting Information Available.** Experimental Section including materials and methods; $^1$H and $^{13}$C NMR spectra of 10-13; $^{31}$P NMR spectra of 7a, 8a-d, 12, 13, 24, and 25; $^1$H and $^{31}$P NMR analysis of the thermolytic cyclodeesterification of 17; RP-HPLC analysis of the thermolytic thiophosphate deprotection of 3, 4, 23, 24, and 25; RP-HPLC analysis of the digestion of 24 and 25 catalyzed by human alkaline phosphatase. This material is available free of charge via the internet at [http://pubs.acs.org](http://pubs.acs.org).

References and Footnotes


(9) Pure deoxyribonucleoside phosphoramidites 7a and 8a-d were characterized by $^{31}$P NMR spectroscopy and by high-resolution mass spectrometry. $^{31}$P NMR spectra of 7a and 8a-d are presented in the Supporting Information.


(12) RP-HPLC chromatograms of the thermolytic deprotection of thymidylyl-(3’→5’)-thymidine thiophosphate 4-hydroxy-1-butyl ester (4) to produce T$_{PS}$T are shown in the Supporting Information.

(13) RP-HPLC chromatogram of the crude dinucleotide is shown in the Supporting Information.


(17) To ensure optimal formation of 7d or 8d, the reaction time was extended to 16 h at ~25 °C.
