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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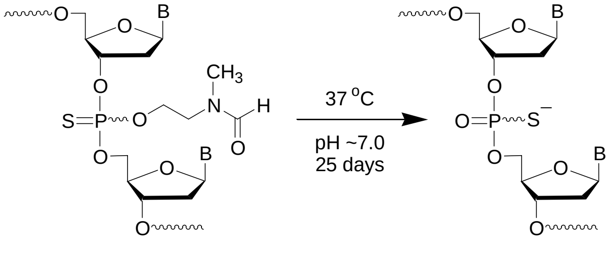
ABSTRACT

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-phosphato/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 *hbu*1555 and allowed the phosphitylation of 4-hydroxybutylated thiophosphate protecting groups by treatment with the phosphoramidite **9**, leading to CpG ODN *pob*1555 and CpG ODN *psb*1555. 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 *hbu*1555, *pob*1555, and *psb*1555 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 acyloxymethyl,[1] 2-(*S*-acylthio)ethyl,[2] 2-, and 4-(acyloxy)benzyl[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.[5] 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**).[5]

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

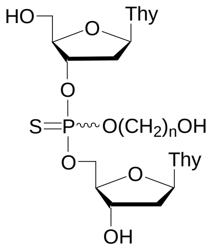


1 **2**

[a] B= thymin-1-yl, cytosin-1-yl, adenin-9-yl or guanin-9-yl

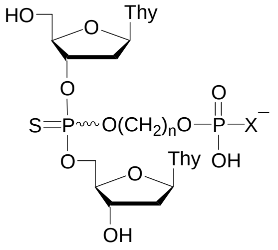
Thus, when synthetic single-stranded DNA oligonucleoside phosphorothioates containing unmethylated CpG motifs (CpG ODNs) are functionalized with 2-(*N*-formyl-*N*-methyl)aminoethyl thiophosphate protecting groups and administered to mice, an immunostimulatory response similar to that produced with traditional CpG ODN phosphodiesters in terms of the number of cells secreting cytokines, chemokines and immunoglobulins was generated.[5] 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.[5] These observations prompted us to design CpG ODN prodrugs 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 prodrugs 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.[2f]

Given that CpG ODN *fma*1555 [d($\mathrm{G_{PS(FMA)}C_{PS(FMA)}T_{PS(FMA)}A_{PS(FMA)}G_{PS(FMA)}A_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}}$-$\mathrm{T_{PS(FMA)}T_{PS(FMA)}A_{PS(FMA)}G_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}T}$), where PS(FMA) stands for the thermolytic 2-(*N*-formyl-*N*-methyl)aminoethyl phosphorothioate triester function] is functional as a prodrug in mice,[5] we propose to evaluate the biological implications of replacing the thermolytic thiophosphate protecting groups of the CpG motif ($\mathrm{G_{PS(FMA)}A_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}T_{PS(FMA)}T}$) within CpG ODN *fma*1555 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 *fma*1555. 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[6] under thermolytic conditions at near neutral pH.[7]



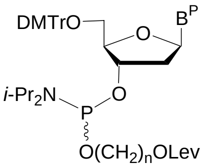
3 n = 3
4 n = 4

Thy = Thymin-1-yl

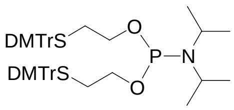


5a n = 3, X = O
5b n = 3, X = S
6a n = 4, X = O
6b n = 4, X = S

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**)[8] for the preparation of negatively charged CPG ODN prodrugs (Figure 1).



7a n = 3
8a-d n = 4



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B^P= thymin-1- yl (**a**), N^4-benzoylcytosin-1-yl (**b**), N^6-adenin-9-yl (**c**), N^2-isobutyrylguanin-9-yl (**d**); DMTr = 4,4'-dimethoxytrityl; Lev, levulinyl

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[a]

$\mathrm{d(G_{PS(FMA)}C_{PS(FMA)}T_{PS(FMA)}A_{PS(FMA)}}\mathbf{G_{PS(HBU)}A_{PS(HBU)}C_{PS(HBU)}G_{PS(HBU)}T_{PS(HBU)}T_{PS(HBU)}}\mathrm{A_{PS(FMA)}G_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}}$
T

[CPG ODN *hbu*1555]

$\mathrm{d(G_{PS(FMA)}C_{PS(FMA)}T_{PS(FMA)}A_{PS(FMA)}}\mathbf{G_{PS(POB)}A_{PS(POB)}C_{PS(POB)}G_{PS(POB)}T_{PS(POB)}T_{PS(POB)}}\mathrm{A_{PS(FMA)}G_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}T}$

[CPG ODN *pob*1555]

$\mathrm{d(G_{PS(FMA)}C_{PS(FMA)}T_{PS(FMA)}A_{PS(FMA)}}\mathbf{G_{PS(PSB)}A_{PS(PSB)}C_{PS(PSB)}G_{PS(PSB)}T_{PS(PSB)}T_{PS(PSB)}}\mathrm{A_{PS(FMA)}G_{PS(FMA)}C_{PS(FMA)}G_{PS(FMA)}T}$

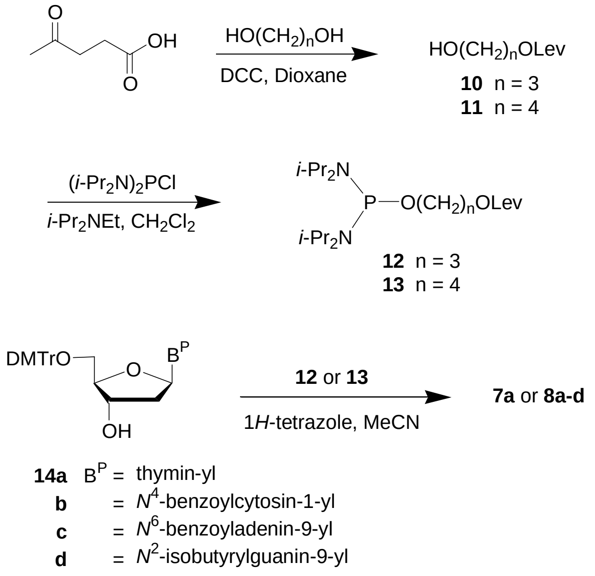
[CPG ODN *psb*1555]

[a] Keys: PS(FMA), internucleotidic 2-(*N*-formyl-*N*-methyl)aminoethyl phosphorothioate triester; PS(HBU), internucleotidic 4-hydroxy-1-butyl phosphorothioate triester; PS(POB), internucleotidic 4-phosphato-1-butyl phosphorothioate triester; PS(PSB), internucleotidic 4-thiophosphato-1-butyl phosphorothioate triester.

or **11** with and equimolar amount of bis(*N*,*N*-diisopropylamino)chlorophosphine and excess i-$\mathrm{Pr_2NEt}$ in dry $\mathrm{CH_2Cl_2}$ 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 1*H*-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 $\mathrm{Et_3N}$ 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 $\mathrm{Et_3N}$ from the purified phosphoramidites monomers to avoid neutralization of 1*H*-tetrazole and reduced coupling efficiency during solid-phase oligonucleotide synthesis. The removal of residual $\mathrm{Et_3N}$ from purified **7a** or **8a-d** is preferably achieved by dissolving the phosphoramidites in a minimum amount of dry $\mathrm{C_6H_6}$ followed by precipitation in a large volume of cold hexane. To ensure complete removal of $\mathrm{Et_3N}$, the phosphoramidite precipitate is dissolved in a substantial amount of dry C_6H_6 (~10 mL/g) and then freeze-dried under high vacuum over an extended period of time (16-24 h).[9]

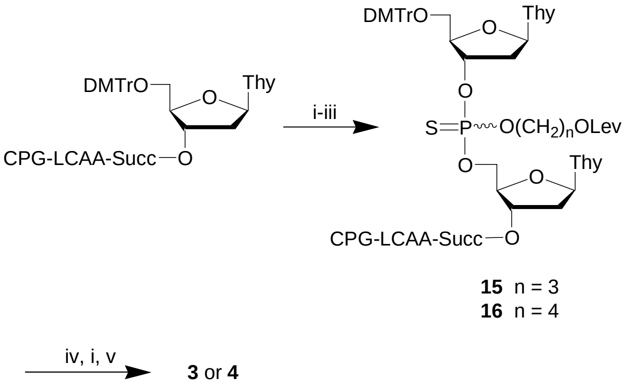
Scheme 2. Synthesis of the phosphorodiamidites **12** and **13** leading to the deoxyribonucleoside phosphoramidites **7a** and **8a-d**[a]



[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 **7a** or **8a** (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[10] in MeCN. The solid-phase-linked dinucleotide **15** or **16** was then treated, sequentially, with 3% trichloroacetic acid (TCA) in CH_2Cl_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 $MeNH_2$ 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.[11]

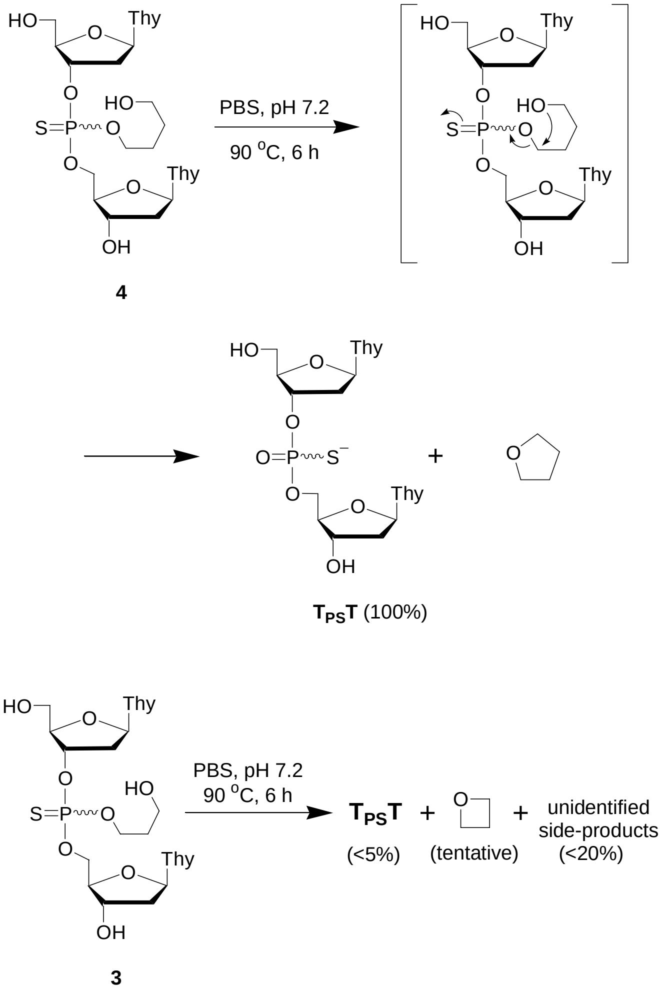
Scheme 3. Solid-phase synthesis of the dinucleoside thiophosphate triester **3** or **4**[a]



[a] Reagents and conditions: (i) 3% TCA/CH$_2$Cl$_2$, 2 min. (ii) **7a** or **8a** (20 equiv), 0.45 M 1*H*-tetrazole/MeCN, 3 min. (iii) 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide/MeCN, 2 min. (iv) 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. (v) MeNH$_2$ 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,[7] it is anticipated that the 4-hydroxy-1-butyl thiophosphate protecting group in **4** would undergo intramolecular cyclodeesterification[6] 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$_{PS}$T within 6 h when heated in 1X phosphate-buffered saline (PBS, pH 7.2) at 90 °C.[12] A reference sample of T$_{PS}$T was prepared from commercial thymidine 5’-*O*-DMTr-3’-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite using standard solid-phase techniques. The T$_{PS}$T reference sample exhibited a RP-HPLC retention time identical (t_R = 20.5 and 21.1 min) to that of T$_{PS}$T generated from the thermolytic deprotection of **4**.[12]

Scheme 4. Thermolytic properties of hydroxyalkyl thiophosphate protecting groups in dinucleotides **3** and **4** under near neutral conditions[a]

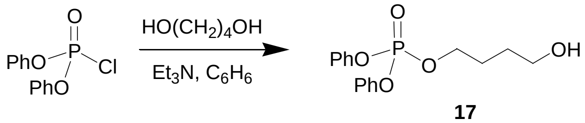


[a] Keys: PBS, 1X phosphate-buffered saline.

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% $T_{PS}T$, 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_3N in dry C_6H_6 afforded the 4-hydroxy-1-butyl ester of diphenylphosphate (**17**) in high yield (85%) after purification by silica gel chromatography. ^1H NMR analysis of pure **17** that was heated in DMSO-d_6/D_2O (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 ^1H 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_2O (5:1 v/v), which clearly showed the conversion of **17** (δ_P −12 ppm) to diphenyl phosphate (δ_P −13 ppm) (Data shown in the Supporting Information).



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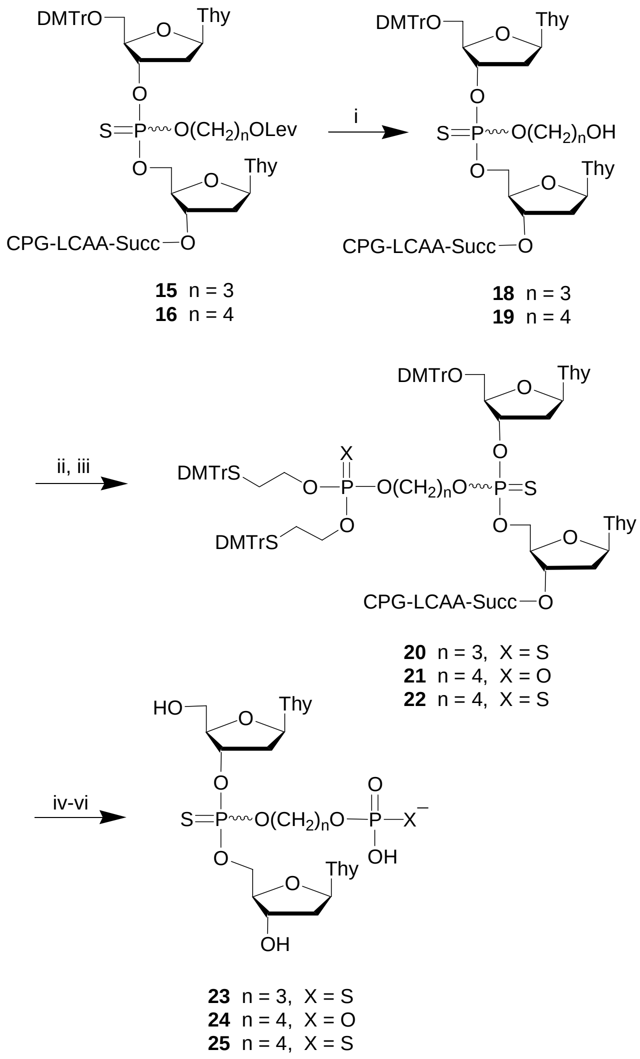
Given that the 3-hydroxy-1-propyl group for thiophosphate protection in **3** is relatively stable under thermolytic conditions, we rationalized that phosphorylation/thiophosphorylation of this protecting group would convert it to a thermosensitive group that should undergo cyclodeesterification under 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'-dimethoxtrityl)-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}

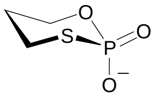


[a] Reagents and conditions: (i) 0.5 M $NH_2NH_2 \bullet H_2O$ in C_5H_5N:AcOH (3:2 v/v), 10 min, 25 °C. (ii) **9**, (20 equiv), 0.45 M 1*H*-tetrazole/MeCN, 3 min. (iii) 0.1 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide/MeCN, 3 min or 0.1 M ethyl(methyl)dioxirane/CH_2Cl_2, 1 min. (iv) 3% TCA/CH_2Cl_2, 15 min. (v) 1.2% (w/v) DTT/H_2O containing 5% (v/v) Et_3N, 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 3*H*-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_2Cl_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_3N 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 $T_{PS}T$ (<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 ^{31}P 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 $T_{PS}T$; 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 CH_2Cl_2[14] as an oxidant in the synthesis of **21**. As reported earlier,[8] the use of this oxidant produced higher yields of deoxyribonucleosise 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 $T_{PS}T$ (~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 $T_{PS}T$ 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 ^{31}P 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 $T_{PS}T$. 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 $T_{PS}T$ and concomitant formation of the respective oxathiaphosphorinane and oxathiaphosphepane derivatives **26** and **27**.



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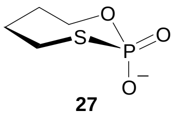

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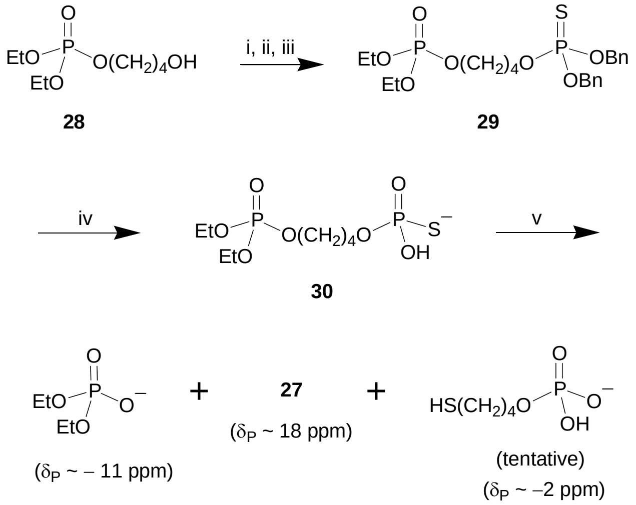
To confirm the identity of cyclodeesterification product **27** through the use of spectroscopic methods, the structurally simpler thiophosphatoalkylated phosphate triester **29** was prepared from the reaction of 4-hydroxy-1-butyl diethylphosphate (**28**) with an equimolar amount of dibenzyl-*N*,*N*-diisopropylphosphoramidite in the presence of 1*H*-tetrazole in dry MeCN (Scheme 6). Solid 3*H*-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[15] 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 (δ_P 17 ppm) along with that of diethylphosphate (δ_P −11 ppm) and, tentatively, 4-mercaptobutyl-1-phosphate monoester (δ_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]

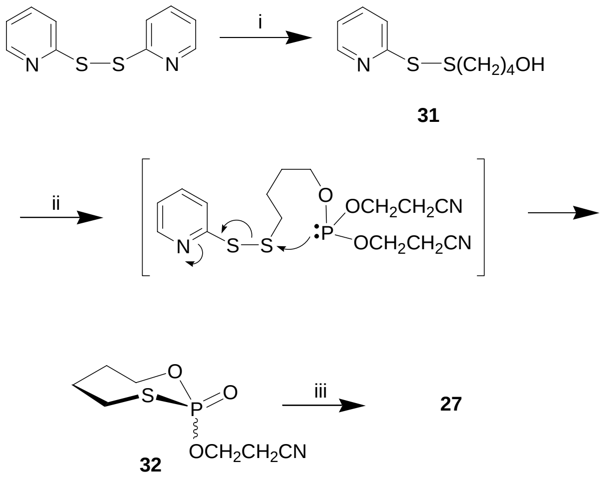


[a] Reagents and conditions: (i) dibenzyl-*N*,*N*-diisopropylphosphoramidite (1 equiv), 0.45 M 1*H*-tetrazole/MeCN, 3 min. (ii) 3*H*-1,2-benzodithiol-3-one 1,1-dioxide to [0.05 M], 2 min. (iii) Silica gel chromatography (C_6H_6:Et_3N (9:1 v/v)). (iv) Na/NH_3 (*l*), −78 °C, 2 min. (v) H_2O, 1 h.

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 1*H*-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_3N (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 (δ_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]



[a] Reagents and conditions: (i) 4-mercaptobutan-1-ol, Et_3N, 25 °C, ? h. (ii) $i\text{-}Pr_2NP(OCH_2CH_2CN)_2$, 1*H*-tetrazole, 5 min. (iii) Et_3N/MeCN (1:4 v/v), 55 °C, 15 h.

Solid-phase synthesis of oligonucleotide CpG ODN *hbu*1555 (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 *fma*1555.[5] 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 *hbu*1555, which was characterized satisfactorily by ESI-TOF mass spectrometry. Conversion of purified CpG ODN *hbu*1555 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 *hbu*1555 and its function as a prodrug through clean thermolytic conversion to CpG ODN 1555. The solid-phase synthesis of oligonucleotides CpG ODN *pob*1555 and CpG ODN *psb*1555 (Figure 1) was achieved in a manner identical to that described for CpG ODN *hbu*1555 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 1*H*-tetrazole in MeCN over a period of 3 min; (ii) the newly formed phosphite triesters were treated with 0.1 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN for 3 min or with 0.1 M ethyl(methyl)dioxirane in CH_2Cl_2 for 1 min; (iii) the DMTr groups were cleaved upon exposure to 3% TCA in CH_2Cl_2 over a period of 15 min; and (iv) the dedimethoxytritylated oligonucleotides were mixed with 1.2% (w/v) DTT/H_2O containing 5% (v/v) Et_3N for 60 min. Pressurized ammonia gas was employed to deprotect the nucleobases and cleave the oligonucleotide from the support. Crude oligonucleotides CpG ODN *pob*1555 and CpG ODN *psb*1555 were then purified by anion-exchange HPLC. Desalted CpG ODN *pob*1555 and CpG ODN *psb*1555 were characterized by ESI-TOF mass spectrometry. CpG ODN *psb*1555 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 *pob*1555 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 *psb*1555 and dephosphorylated CpG ODN *pob*1555 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 *pob*1555 and CpG ODN *psb*1555 relative to that of CpG ODN *hbu*1555 and CpG ODN *fma*1555 in aqueous media.

The resistance of oligonucleotide prodrugs CpG ODN *hbu*1555, CpG ODN *pob*1555 and CpG ODN *psb*1555 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,[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 *hbu*1555 and CpG ODN *psb*1555 will not require intracellular enzymes for conversion to the bioactive CpG ODN 1555, the oligonucleotide prodrug CpG ODN *pob*1555 will require intracellular phosphatase activity for conversion to CpG ODN *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 *psb*1555 and CpG ODN *pob*1555 are freely soluble in aqueous media, whereas the solubility of CpG ODN *hbu*1555 and that of the CpG ODN *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): δ 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): δ 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): δ 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): δ 206.6, 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_2NEt (4.15 g, 34.6 mmol) in dry CH_2Cl_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 (δ_P 135.5) to the phosphorodiamidite **12** (δ_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_6H_6 and Et_3N (9:1 v/v) as the eluant. Yield: 75% (1.52 g, 3.75 mmol). ^{1}H NMR (300 MHz, C_6D_6): δ 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). ^{13}C NMR (75 MHz, C_6D_6): δ 204.4, 172.3, 61.9, 61.0 (d, $^2J_{CP}$ = 21.5 Hz), 44.8, 44.6, 37.6, 31.1 (d, $^3J_{CP}$ = 8.4 Hz), 29.1, 28.1, 24.7, 24.6, 24.1, 24.0. ^{31}P NMR (121 MHz, C_6D_6): δ 123.5.

***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). ^{1}H NMR (300 MHz, C_6D_6): δ 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). ^{13}C NMR (75 MHz, C_6D_6): 204.5, 172.3, 64.5, 64.2 (d, $^2J_{CP}$ = 21.5 Hz), 44.7, 44.6, 37.6, 29.1, 28.4 (d, $^3J_{CP}$ = 8.4 Hz), 28.1, 26.0, 24.8, 24.7, 24.1, 24.0. ^{31}P NMR (121 MHz, C_6D_6): δ 122.8.

General procedure for the preparation deoxyribonucleoside phosphoramidites 7a and 8a-d. Commercial 2'-deoxyribonucleosides (**14a-d**, 1.0 mmol) were dried by coevaporation 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 1*H*-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[17] and was then rotoevaporated to a foam under vacuum. The material was dissolved in a minimal volume of C_6H_6:Et_3N (9:1 v/v) and added to the top of a chromatography column packed with silica gel (~30 g) that was equilibrated in C_6H_6:Et_3N (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 rotoevaporated under low pressure to a white foam. The material was dissolved in dry C_6H_6 (~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). ^{31}P NMR (121 MHz, C_6H_6): δ 148.8, 148.4. APESI-HRMS: calcd for $C_{45}H_{59}N_3O_{11}P$ $(M + H)^+$ 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). ^{31}P NMR (121 MHz, C_6H_6): δ 148.3, 147.8. APESI-HRMS: calcd for $C_{46}H_{61}N_3O_{11}P$ $(M + H)^+$ 862.4044, found 862.4036.

***N*^{4}-Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-[(*N*,*N*-diisopropylamino)(4-(2,4-dioxopent-1-yl)oxy-1-butyloxy)]phosphinyl-2'-deoxycytidine (8b).** ^{31}P NMR (121 MHz, C_6H_6): δ 148.3, 148.2. APESI-HRMS: calcd for $C_{52}H_{64}N_4O_{11}P$ $(M + H)^+$ 951.4309, found 951.4306.

***N*^{6}-Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-[(*N*,*N*-diisopropylamino) (4-(2,4-dioxopent-1-yl)oxy-1-butyloxy)]phosphinyl-2'-deoxyadenosine (8c).** ^{31}P NMR (121 MHz, C_6H_6): δ 148.0. APESI-HRMS: calcd for $C_{53}H_{64}N_6O_{10}P$ $(M + H)^+$ 975.4422, found 975.4413.

***N*^{2}-Isobutyryl-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-[(*N*,*N*-diisopropylamino)(4-(2,4-dioxopent-1-yl) oxy-1-butyloxy)]phosphinyl-2'-deoxyguanosine (8d).** ^{31}P NMR (121 MHz, C_6H_6): δ 148.2, 147.9. APESI-HRMS: calcd for $C_{50}H_{66}N_5O_{11}P$ $(M + H)^+$ 957.4527, found 957.4523.

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 CH_2Cl_2 for 1 min. After washing the support extensively with CH_2Cl_2 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 1*H*-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 3*H*-1,2-benzodithiol-3-one 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 $NH_2NH_2•H_2O$ in pyridine/acetic acid (3:2 v/v) for 10 min at ~25 °C.

Following extensive washing of the CPG support with CH_2Cl_2 and MeCN, removal of the 5'-DMTr groups was effected by the use of 3% TCA in CH_2Cl_2 over a period of 1 min. After careful washing of the support with CH_2Cl_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 for $C_{23}H_{33}N_4O_{12}PS$ $(M)^+$ 620.16, found…?.. **Thymidylyl-(3'→5')-thymidine thiophosphate 4-hydroxy-1-butyl ester (4)**. ESI-MS: calcd for $C_{24}H_{35}N_4O_{12}PS$ $(M)^+$ 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_{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_R) for **4** and $T_{PS}T$ 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_{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$_2$NEt (1.0 g, mmol) in dry C_6H_6 (? 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 $CHCl_3$ (2 mL) and applied to the top of a chromatography column packed with silica gel (40 g) equilibrated in $CHCl_3$ The product was eluted from the column with $CHCl_3$ affording **17** (?g, ?mmol) as a colorless oil. Yield: 80%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.38 (m, 4H), 7.20 (m, 6H), 4.23 (dt, $J_{HP} = J = 6.4$ Hz, 2H), 3.95 (s, 1H), 3.38 (t, $J = 6.4$ Hz, 2H), 1.66 (m, 2H), 1.45 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 150.0 (d, $^2J_{CP}$ = 7.2 Hz), 130.0, 125.4, 119.8 (d, J_{CP} = 4.8 Hz), 69.2 (d, $^2J_{CP}$ = 6.0 Hz), 60.0, 28.2, 26.4 (d, J_{CP} = 6.0 Hz). ^{31}P NMR (121 MHz, DMSO-d_6): 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_6/D_2O (5:1 v/v, 0.5 mL) and heated for 4 h at 90 °C. ^{1}H 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 ^{1}H NMR spectrum of commercial THF.[12] Signals at 7.1-7.4 ppm correspond to those of diphenyl phosphate. ^{31}P NMR analysis of the reaction shows an almost complete conversion (>95%) of **17** (δ_P −12 ppm) to diphenyl phosphate (δ_P −13 ppm). Chemical shifts are upfield relative to an external standard of phosphoric acid in D_2O (δ_P 0 ppm).[12]

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_2Cl_2 for 1 min. After carefully washing the support with CH_2Cl_2 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 1*H*-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 3*H*-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 $NH_2NH_2{\bullet}H_2O$ 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 CH_2Cl_2 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 1*H*-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 3*H*-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 CH_2Cl_2 over a period of 10 min. The support was then washed with CH_2Cl_2 and MeCN followed by a solution of 1.2 % (w/v) DTT in H_2O (1 mL) containing 5% (v/v) Et_3N over a period of 30 min. After extensive washing of the support with CH_2Cl_2, 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)**. ^{31}P NMR (121 MHz, D_2O): δ_P ? +MALDI-TOF MS: calcd for $C_{23}H_{34}N_4O_{14}P_2S_2$ $(M + 2H)^+$ 716.10, found ? . **Thymidylyl-(3'→5')-thymidine thiophosphate 4-thiophosphato-1-butyl ester (25)**. ^{31}P NMR (121 MHz, H_2O): δ 67.1, 66.9, 51.9. −MALDI-TOF MS: calcd for $C_{24}H_{35}N_4O_{14}P_2S_2$ $(M - H)^-$ 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 CH_2Cl_2, instead of 3*H*-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 H_2O containing 5% (v/v) Et_3N 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 (t_R) of diastereomeric **24** is 27.5 and 27.6 min. **Thymidylyl-(3'→5')-thymidine thiophosphate 4-phosphato-1-butyl ester (24).** ^{31}P NMR (121 MHz, H_2O): δ 68.0, 1.8. +MALDI-TOF MS: calcd for $C_{24}H_{36}N_4O_{15}P_2S$ $(M + 2H)^+$ 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 $T_{PS}T$ 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 $T_{PS}T$ 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 $T_{PS}T$ 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_3N (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_2Cl_2 and purified by silica gel chromatography. The product was eluted from the column using a gradient of CH_2Cl_2:MeOH (0 → 5%) affording 9.98 g of pure **28** (44.2 mmol, 88%). ^1H NMR (300 MHz, DMSO-d_6): δ ??. ^{13}C NMR (75 MHz, DMSO-d_6): δ ??. ^{31}P NMR (121 MHz, CH_2Cl_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_2Cl_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_2Cl_2 and purified by silica gel chromatography. The product was eluted from the column using a gradient of CH_2Cl_2:MeOH (0 → 5%) to give pure **29** (1.04 g, 2.07 mmol, 47%) as a pale yellow oil. ^1H NMR (300 MHz, DMSO-d_6): δ ??. ^{13}C NMR (75 MHz, DMSO-d_6): δ ??. ^{31}P NMR (121 MHz, CH_2Cl_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%). ^1H 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_2Cl_2 (80 mL). The solution was extracted with brine (20 mL). The organic layer was washed further with H_2O (20 mL) and then dried over anhydrous Na_2SO_4. The suspension was filtered and evaporated under reduced pressure. The residue was purified by silica gel chromatography using CH_2Cl_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): δ ??. ^{13}C (75 MHz, DMSO-d_6): δ ??. ^{31}P NMR (121 MHz, DMSO-d_6): δ 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_3N (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.[5] 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 $NH_2NH_2 \bullet H_2O$ 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_2Cl_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 1*H*-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 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN (? mL) for 2 min, whereas the support of the other column was treated 0.1 M ethyl(methyl)dioxirane in CH_2Cl_2 (? 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_2O (1 mL). The recovered yield of CpG ODN *hbu*1555 was ? 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_2Cl_2 for 10 min. Excess TCA was washed off the synthesis column with CH_2Cl_2 (3 mL) and MeCN (3 mL). The solid-phase bound oligonucleotides were then agitated for ? min in 1.2% (w/v) DTT/H_2O containing 5% (v/v) Et_3N. The synthesis columns were flushed with H_2O 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 *psb*1555 and CpG ODN *pob*1555 was determined by UV spectrophotometry at 260 nm upon reconstitution of the purified oligonucleotides in ddH_2O (1 mL). The recovered yields of CpG ODN ODN *psb*1555 and CpG ODN *pob*1555 were ? and ? OD_{260} units, respectively. Pure CpG ODN *psb*1555 and CpG ODN *pob*1555 were stored frozen at −20 °C.

Oligonucleotide characterization. RP-HPLC analysis of purified CpG ODN *hbu*1555, CpG ODN *psb*1555 and CpG ODN *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 *hbu*1555 had a retention time (t_R) of ? min, whereas CpG ODN *psb*1555 and CpG ODN *pob*1555 displayed t_Rs of ? min and ? 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 *hbu*1555 (+ESI-TOF MS): calcd for $C_{203}H_{289}N_{65}O_{89}P_{14}S_{14}$ $(M)^+$ 5946, found……… CpG ODN *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 *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.

References and Footnotes

(1) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Chem.* **1995**, *23*, 1-21.

(2) (a) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2471-2476. (b) Barber, I.; Rayner, B.; Imbach, J. -L. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 563-568. (c) Tosquellas, G.; Alvarez, K.; Dell'Aquilla, C.; Morvan, F.; Vasseur, J. -J.; Imbach, J. -L.; Rayner B. *Nucleic Acids Res.* **1998**, *26*, 2069-2074. (d) Bologna, J. -C.; Morvan, F.; Imbach, J. -L. *Eur. J. Org. Chem.* **1999**, 2353-2358. (e) Morvan, F.; Brès, J. -C.; Lefebvre, I.; Vasseur, J, -J.; Pompon, A.; Imbach, J. -L.; *Nucleosides Nucleotides & Nucleic Acids.* **2001**, *20*, 1159-1163. (f) Morvan, F.; Bologna, J. -C.; Vivès, E.; Imbach, J. -L.; *Nucleosides Nucleotides & Nucleic Acids.* **2001**, *20*, 1165-1168. (f) Bologna, J. -C.; Vivès, E.; Imbach, J. -L.; Morvan, F. *Antisense Nucl. Acid Drug Dev.* **2002**, *12*, 33-41. (g) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2471-2476.

(3) (a) Iyer, R. P.; Yu, D.; Devlin, T.; Ho, N. -H.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1917-1922. (b) Iyer, R. P.; Ho, N. -H.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 871-876.

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(4) (a) Ora, M.; Mäki, E.; Poijärvi, P.; Neuvonen, K.; Oivanen, M.; Lönnberg, H. *J. Chem. Soc. Perkin Trans. 2* **2001**, 881-885. (b) Poijärvi, P.; Mäki, E.; Tomperi, J.; Ora, M.; Oivanen, M.; Lönnberg, H. *Helv. Chim. Acta* **2002**, *85*, 1869-1876. (c) Poijärvi, P.; Oivanen, M.; Lönnberg, H. *Lett. Org. Chem.* **2004**, *1*, 183-188. (d) Poijärvi, P.; Heinonen, P.; Virta, P.; Lönnberg, H. *Bioconjugate Chem.* **2005**, *16*, 1564-1571.

(5) Grajkowski, A.; Pedras-Vasconcelos, J.; Wang, V.; Ausín, C.; Hess, S.; Verthelyi, D.; Beaucage, S. L. *Nucleic Acids Res.* **2005**, *33*, 3550-3560.

(6) (a) Wilk, A.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem.* **1997**, *62*, 6712-6713. (b) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Org. Chem.* **1999**, *64*, 7515-7522. (c) Wilk, A.; Grajkowski, A.; Srinivasachar, K.; Beaucage, S. L. *Antis. Nucl. Acid Drug Dev.* **1999**, *9*, 361-366. (d) Wilk, A.; Grajkowski, A.; Chmielewski, M. K.; Phillips, L. R.; Beaucage, S. L. In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A., Eds.; John Wiley & Sons: New York, 2001; Vol. I, pp. 2.7.1-2.7.12.

(7) (a) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 2149-2156. (b) Grajkowski, A.; Wilk, A.; Chmielewski, M. K.; Phillips, L. R.; Beaucage, S. L. *Org. Lett.* **2001**, *3*, 1287-1290. (c) Wilk, A.; Chmielewski, M. K.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *Tetrahedron Lett.* **2001**, *42*, 5635-5639. (d) Wilk, A.; Chmielewski, M. K.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Org. Chem.* **2002**, *67*, 6430-6438. (e) Wilk, A.; Chmielewski, M. K.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A., Eds.; John Wiley & Sons: New York, 2002; Vol. I, pp. 3.9.1-3.9.16. (f) Cieślak, J.; Beaucage, S. L. *J. Org. Chem.* **2003**, *68*, 10123-10129. (g) Cieślak, J.; Grajkowski, A.; Livengood,V.; Beaucage, S. L. *J. Org. Chem.* **2004**, *69*, 2509-2515.

(8) Ausín, C.; Grajkowski, A.; Cieślak, J.; Beaucage, S. L. *Org. Lett.* **2005**, *7*, 4201-4204.

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(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.

(10) (a) Beaucage, S. L.; Iyer, R. P.; Egan, W.; Regan, J. B. *Ann. New York Acad. Sci.* **1990**, *616*, 483-485. (b) Iyer, R. P.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Am Chem. Soc.* **1990**, *112*, 1253-1254. (c) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693-4699. (c) Regan, J. B.; Phillips, L. R.; Beaucage, S. L. *Org. Prep. Proc. Int.* **1992**, *24*, 488-492.

(11) Ogilvie, K. K.; Beaucage, S. L. *Nucleic Acids Res.* **1979**, *7*, 805-824.

(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.

(14) Kataoka, M.; Hattori, A.; Okino, S.; Hyodo, M.; Asano, M.; Kawai, R.; Hayakawa, Y. *Org. Lett.* **2001**, *3*, 815-818.

(15) (a) Ruda, K; Lindberg, J.; Garegg, P. J.; Oscarson, S.; Konradsson, P. *Tetrahedron* **2000**, *56*, 3969-3975. (b) Noble, N. J.; Dubreuil, D.; Potter, B. V. L. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 471-476.

(16) Koga, M.; Moore, M. F.; Beaucage, S. L. *J. Org. Chem.* **1991**, *56*, 3757-3759.

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

(18) Murthy, N.; Campbell, J.; Fausto, N.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate. Chem.* **2003**, *14*, 412-419.

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