

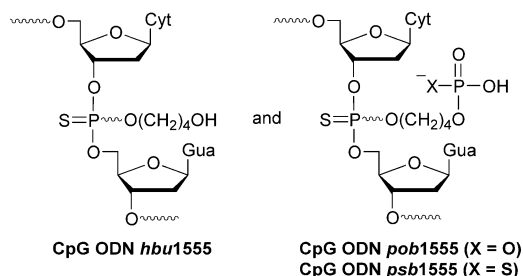
Solid-Phase Synthesis of Thermolytic DNA Oligonucleotides Functionalized with a Single 4-Hydroxy-1-butyl or 4-Phosphato-/Thiophosphato-1-butyl Thiophosphate Protecting Group[†]

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Several thermolytic CpG-containing DNA oligonucleotides analogous to **1** have been synthesized to serve as potential immunotherapeutic oligonucleotide prodrug formulations for the treatment of infectious diseases in animal models. Specifically, the CpG motif (GACGTT) of each DNA oligonucleotide has been functionalized with either the thermolabile 4-hydroxy-1-butyl or the 4-phosphato-/thiophosphato-1-butyl thiophosphate protecting group. This functionalization was achieved through incorporation of activated deoxyribonucleoside phosphoramidite **8b** into the oligonucleotide chain during solid-phase synthesis and, optionally, through subsequent phosphorylation effected by phosphoramidite **9**. Complete conversion of CpG ODNs *hbu1555*, *psb1555*, and *pob1555* to CpG ODN 1555 (homologous to **2**) occurred under elevated temperature conditions, thereby validating the function of these diastereomeric oligonucleotides as prodrugs in vitro. Noteworthy is the significant increase in solubility of CpG ODN *psb1555* and CpG *pob1555* in water when compared to that of neutral CpG ODN *fma1555* (homologous to **1**).

Introduction

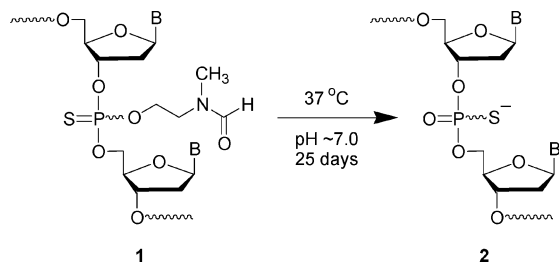
Oligonucleotide prodrugs have attracted considerable attention over the past decade in an effort to overcome problems associated with the cellular uptake of antisense oligonucleotides and the sensitivity of these biomolecules to ubiquitous nucleases present in biological media and physiological environments. A strategy to improve cellular permeation of negatively charged oligonucleotide drugs entails temporarily masking the phos-

phodiester groups of these biopolymers with acyloxymethyl,¹ 2-(*S*-acylthio)ethyl,² and 2-/4-(acyloxy)benzyl³ protecting groups or with groups derived from bis(hydroxymethyl)-1,3-dicarbonyl

(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’Aquila, 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. (g) Bologna, J.-C.; Vivès, E.; Imbach, J.-L.; Morvan, F. *Antisense & Nucleic Acid Drug Dev.* **2002**, *12*, 33–41. (h) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2471–2476.

[†] S.L.B. dedicates this paper to his doctoral mentor, Prof. Kelvin K. Ogilvie.
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SCHEME 1. Thermolytic Conversion of DNA Oligonucleotide Prodrugs to Bioactive Therapeutic Oligonucleotides^a



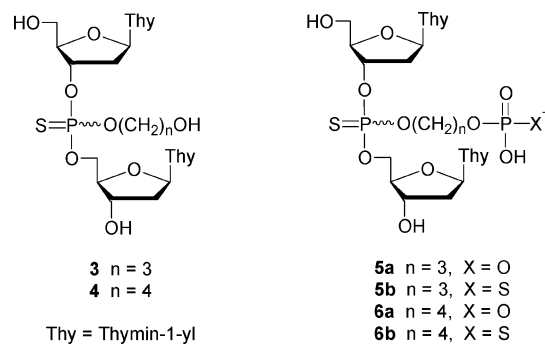
^a B = thymine-1-yl, cytosine-1-yl, adenine-9-yl, or guanine-9-yl.

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.^{5a,b} 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 are stable to hydrolytic nucleases. A distinctive feature of this class of modified oligonucleotides is that only an aqueous environment, maintained at a temperature of 37 °C or above, is necessary for converting oligonucleoside 2-(*N*-formyl-*N*-methyl)aminoethyl phosphorothioate triesters (**1**) to functionally bioactive oligonucleoside phosphorothioate diesters (**2**) within a reasonable period of time.^{5a,b}

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 phosphodiester in terms of the number of cells secreting cytokines, chemokines, and immunoglobulins is generated.^{5a,b} However, a delay in the induction of these immunostimulatory events is observed, consistent with the thermolytic conversion half-time of 2-(*N*-formyl-*N*-methyl)aminoethyl thiophosphate triesters ($t_{1/2} = 73$ h at 37 °C) to the biologically active phosphorothioate diesters. A noteworthy outcome of these findings relates to the co-administration of CpG ODN prodrugs of type **1** and conventional CpG ODN of type **2** in mice, which widened the timetable of therapeutic

treatment against specific viral infections.^{5a,b} These observations prompted us to design CpG ODN prodrugs exhibiting either a shorter or longer half-time of thiophosphate deprotection relative to that of CpG ODN **1** for the preparation of effective and long-acting immunotherapeutic oligonucleotide formulations against various infectious diseases in animal models. 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 of cellular uptake, which is reportedly commensurate to the relative lipophilicity of oligonucleotides.^{2f}

Given that CpG ODN *fma1555* [$d(\text{G}_{\text{PS}(\text{FMA})}\text{C}_{\text{PS}(\text{FMA})}\text{T}_{\text{PS}(\text{FMA})}^{-}\text{A}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}\text{A}_{\text{PS}(\text{FMA})}\text{C}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}\text{T}_{\text{PS}(\text{FMA})}^{-}\text{T}_{\text{PS}(\text{FMA})}^{-}\text{A}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}\text{C}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}\text{T})$, where PS(FMA) stands for the thermolytic 2-(*N*-formyl-*N*-methyl)aminoethyl phosphorothioate triester function] is functional as a prodrug in mice,^{5a,b} we propose to evaluate the biophysical implications of replacing the thermolytic thiophosphate protecting group of the CpG within the motif $\text{G}_{\text{PS}(\text{FMA})}\text{A}_{\text{PS}(\text{FMA})}\text{C}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}^{-}\text{T}_{\text{PS}(\text{FMA})}\text{T}_{\text{PS}(\text{FMA})}^{-}$ with thermolabile groups displaying slower or faster deprotection kinetics than those of the FMA group. These new heat-sensitive groups were designed to impart similar or better solubility properties in biological media than those in the FMA groups.⁶ The dinucleoside phosphorothioate derivatives **3–6** were prepared to serve as models for determining the thermolytic deprotection kinetics of each thiophosphate protecting group. With the exception of the 3-hydroxy-1-propyl group of dinucleotide **3**, the thiophosphate protecting groups of dinucleotides **4–6** are expected to follow an intramolecular cyclodeesterification pathway⁷ under thermolytic conditions at near-neutral pH.⁸



We now report the preparation of deoxyribonucleoside phosphoramidites **7a** and **8a–d**,⁹ which are required for the synthesis of all model dinucleotides and the solid-phase assembly of the DNA oligonucleotides shown in Table 1 of the Supporting Information (SI). The usefulness of the phosphitylating reagent, bis[*S*-(4,4'-dimethoxytrityl)-2-mercaptoethyl]-*N,N*-diisopropylphosphoramidite (**9**),¹⁰ in the preparation of, minimally, negatively charged CPG ODN prodrugs is also reported.

(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.

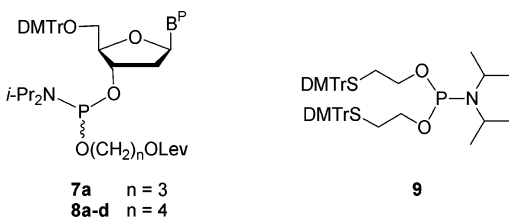
(4) (a) Ora, M.; Mäki, E.; Poijärvi, P.; Neuvonen, K.; Oivanen, M.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **2001**, 881–885. (b) Poijärvi, P.; Mäki, E.; Tomperi, J.; Ora, M.; Oivanen, M.; Lönnberg, H. *Helv. Chim. Acta* **2002**, *85*, 1869–1876. (c) Poijärvi, P.; Oivanen, M.; Lönnberg, H. *Lett.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

(5) (a) Grajkowski, A.; Pedras-Vasconcelos, J.; Wang, V.; Ausín, C.; Hess, S.; Verthelyi, D.; Beaucage, S. L. *Nucleic Acids Res.* **2005**, *33*, 3550–3560. (b) Grajkowski, A.; Pedras-Vasconcelos, J.; Ausín, C.; Verthelyi, D.; Beaucage, S. L. *Ann. N.Y. Acad. Sci.* **2005**, *1058*, 26–38. (c) The $\text{C}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}$ sequence within the 5'-proximal $\text{G}_{\text{PS}(\text{FMA})}\text{A}_{\text{PS}(\text{FMA})}^{-}\text{C}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}\text{T}_{\text{PS}(\text{FMA})}\text{T}$ motif is critical for bioactivity in vivo. Replacing $\text{C}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}$ of the motif with $\text{G}_{\text{PS}(\text{FMA})}\text{G}_{\text{PS}(\text{FMA})}$ (ODN *fma1555*) results in complete suppression of the immunostimulatory properties of CpG ODN *fma1555* in mice (see ref 5a).

(6) The maximum concentration of CpG ODN *fma1555* obtainable in water is ~1 mM.

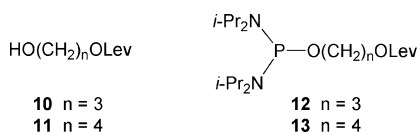
(7) (a) Wilk, A.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem.* **1997**, *62*, 6712–6713. (b) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Org. Chem.* **1999**, *64*, 7515–7522. (c) Wilk, A.; Grajkowski, A.; Srinivasachar, K.; Beaucage, S. L. *Antisense & Nucleic Acid Drug Dev.* **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. 1, pp 2.7.1–2.7.12.

Results and Discussion



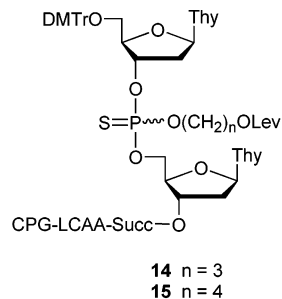
B^p = thymine-1-yl (**a**), N⁴-benzoylcytosine-1-yl (**b**), N⁶-benzoyladenine-9-yl (**c**), N²-isobutrylguanin-9-yl (**d**); DMTr = 4,4'-dimethoxytrityl; Lev, levulinyl

A prerequisite to the synthesis of dinucleoside phosphotriesters **3** and **4**, and that of the CpG DNA oligonucleotides, is the preparation of deoxyribonucleoside phosphoramidites **7a** and **8a,b**. Specifically, 1,3-propanediol or 1,4-butanediol was 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** or **11** with an equimolar amount of bis(*N,N*-diisopropylamino)chlorophosphine and excess *i*-Pr₂NEt in dry CH₂Cl₂ gave the corresponding phosphorodiamidite **12** or **13**, which was purified by silica gel chromatography and isolated in yields of ca. 70%.



Dry N-protected 5'-O-DMTr-2'-deoxyribonucleosides were reacted with phosphorodiamidite **12** or **13** and 1*H*-tetrazole in anhydrous MeCN over a period of 3 h. While the preparation of **7a** or **8a-c** was complete under these conditions, complete formation of **8d** was accomplished within 16 h. The crude deoxyribonucleoside phosphoramidites were purified by silica gel chromatography employing an eluent containing Et₃N to neutralize the inherent acidity of silica gel, which otherwise would cause hydrolysis and partial dedimethoxytritylation of the phosphoramidite monomers during purification. It is, however, critically important to remove residual Et₃N from the purified phosphoramidite monomers to prevent reduced coupling efficiency caused by neutralization of 1*H*-tetrazole while performing solid-phase oligonucleotide synthesis. Removal of the remaining Et₃N from purified **7a** or **8a-d** was achieved by dissolving the phosphoramidite in a minimum amount of dry C₆H₆ (~5 mL/g) and by adding the solution to a large volume

(20×) of cold hexane. The phosphoramidite precipitate that ensued was dissolved in C₆H₆ (~10 mL/g), and the resulting solution was freeze-dried under high vacuum over an extended period of time (16–24 h) to give triethylamine-free **7a** or **8a-d**.¹¹



CPG-LCAA-Succ: succinyl long chain alkylamine controlled-pore glass

The solid-phase-linked dinucleoside thiophosphate triesters **14** and **15** were prepared on a 0.2 μmol scale by adding a solution of phosphoramidite **7a** or **8a** and 1*H*-tetrazole in dry MeCN to a controlled-pore glass support (CPG) functionalized with thymidine. A sulfuration reaction effected by 0.05 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide¹² in MeCN completed the preparation of **14** or **15**. Sequential treatment of the solid-phase-linked dinucleotide **14** or **15** with (i) 0.5 M hydrazine hydrate in pyridine/acetic acid (3:2 v/v) to remove the levulinyl group, (ii) 3% trichloroacetic acid (TCA) in CH₂Cl₂ to cleave the 4,4'-dimethoxytrityl (DMTr) group, and (iii) pressurized MeNH₂ gas (~2.5 bar) for 3 min gave the hydroxyalkylated dinucleoside thiophosphate triester **3** or **4**. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis of the crude dinucleotide revealed that the purity of **3** or **4** was ~97% (data shown in Charts 1 and 2 of the SI). Parenthetically, a solution-phase approach to the preparation of the 4-hydroxy-1-butyl phosphate analogue of **4** was reported in the literature more than 25 years ago.¹³

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,⁸ it is anticipated that the 4-hydroxy-1-butyl thiophosphate protecting group of **4** would undergo intramolecular cyclodeesterification⁷ under elevated temperature conditions to liberate the dinucleoside phosphorothioate diester **T_{PS}T** with the concomitant formation of tetrahydrofuran (Scheme 2). It is also anticipated that the 3-hydroxy-1-propyl thiophosphate protecting group of **3** would not elicit significant thermolytic cyclodeesterification considering the unfavorable formation of trimethylene oxide (Scheme 2). As predicted, when RP-HPLC-purified dinucleotide **4** was heated in 1X phosphate-buffered saline (PBS, pH 7.2) at 90 °C, a clean and quantitative conversion to the parent dinucleoside phosphorothioate diester **T_{PS}T** was achieved

(8) (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. 1, 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.

(9) Even though only **7a**, **8a**, and **8b** are required for the present work, phosphoramidites **8c,d** were also prepared and characterized for use in experiments that are beyond the scope of this report.

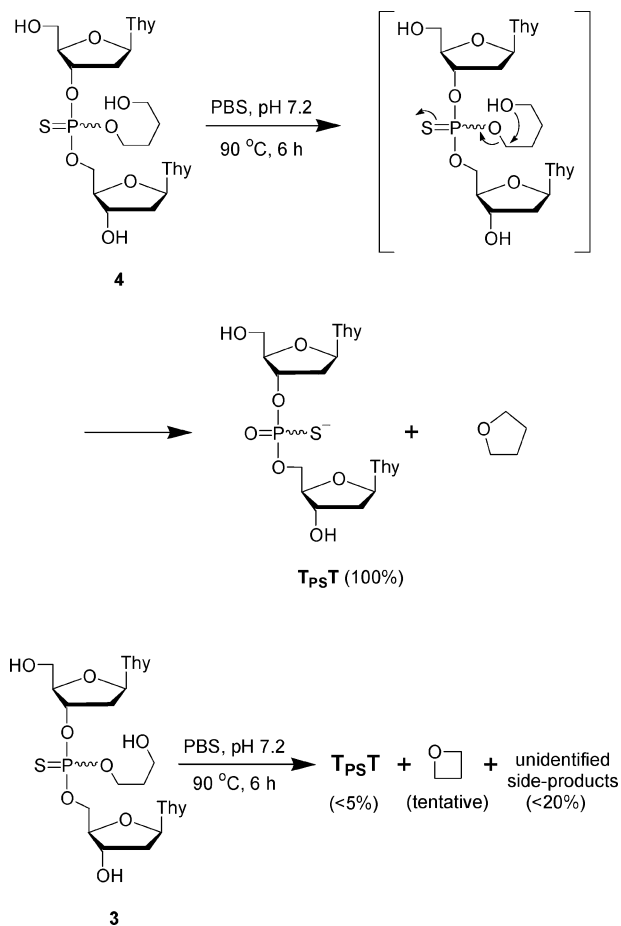
(10) Ausín, C.; Grajkowski, A.; Cieślak, J.; Beaucage, S. L. *Org. Lett.* **2005**, *7*, 4201–4204.

(11) Pure deoxyribonucleoside phosphoramidites **7a** and **8a-d** were characterized by ³¹P NMR spectroscopy and by high-resolution mass spectrometry. ³¹P NMR spectra of **7a** and **8a-d** are presented in the SI.

(12) (a) Beaucage, S. L.; Iyer, R. P.; Egan, W.; Regan, J. B. *Ann. N.Y. 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. (d) Regan, J. B.; Phillips, L. R.; Beaucage, S. L. *Org. Prep. Proced. Int.* **1992**, *24*, 488–492.

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

SCHEME 2. Thermolytic Cyclodeesterification of the Hydroxyalkylated Thiophosphate Protecting Groups from Dinucleotides **3 and **4** under Near-Neutral Conditions^a**

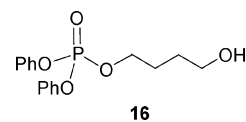


^a Keys: PBS, 1X phosphate-buffered saline.

within 6 h.¹⁴ A reference sample of **T_{Ps}T** exhibited a RP-HPLC retention time ($t_R = 20.5$ and 21.1 min) identical to that of **T_{Ps}T** generated from the thermolytic deprotection of **4**.¹⁴ 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 (see Table 2 of the SI). In accordance with our expectations, heating the 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 determined by RP-HPLC analysis of the reaction mixture. Several unidentified products were detected under these conditions, accounting for less than 20% of the total peak area (data shown in Chart 2 of the SI). Aside from these unidentified products, which were generated presumably through cleavage of the internucleotidic linkage, most of the dinucleoside triester **3** (ca. 80%) was left unreacted.

To confirm whether 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 ¹H NMR spectroscopy during the process. Specifically, the reaction of 1,4-butanediol with diphenyl chlorophosphate and *i*-Pr₂NEt in dry C₆H₆ afforded the 4-hydroxy-1-butyl ester of diphenylphosphate (**16**) in a yield of 77% after purification on silica gel. ¹H NMR analysis of pure **16** that was

heated in DMSO-*d*₆/D₂O (5:1 v/v) for 4 h at 90 °C showed signals at 1.7 and 3.5 ppm that were identical to those obtained from commercial THF in terms of chemical shifts and signal multiplicity (data shown in Chart 3 of the SI). Signals at 7.1–7.4 ppm corresponded to those of diphenyl phosphate. This assignment was further supported by ³¹P NMR analysis of the deprotection reaction, which clearly showed the conversion of **16** ($\delta_P -12$ ppm) to diphenyl phosphate ($\delta_P -13$ ppm) (data presented in Chart 4 of the SI).



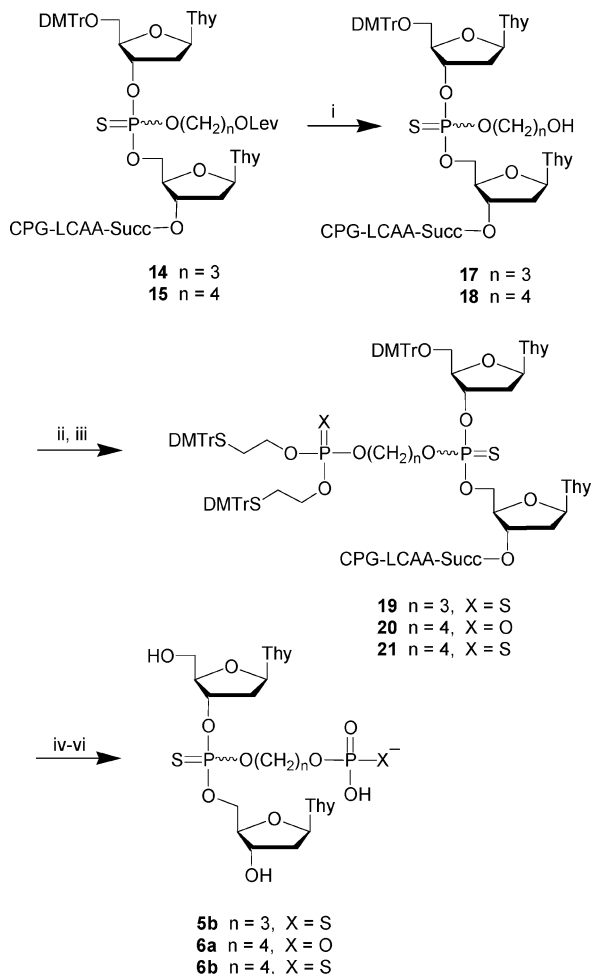
Given that the 3-hydroxy-1-propyl group for thiophosphate protection in **3** is relatively stable under thermolytic conditions, we rationalized that phosphorylation/thiophosphorylation of this protecting group would considerably enhance its thermolability. In order to test the validity of this rationale, hydrazinolysis of the levulinylated solid-phase-linked dinucleoside phosphorothioate triester **14** was carried out over a period of 10 min, without cleaving the dinucleotide from the support,¹⁵ to give **17** (Scheme 3). Phosphitylation of **17** using activated **9** in excess, followed by sulfuration with 0.1 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in MeCN, produced **19**. Removal of all of the DMTr groups was effected under acidic conditions (3% TCA in CH₂Cl₂) over a period of 15 min. Treatment of the detritylated solid-phase-linked dinucleotide with an aqueous solution of DL-dithiothreitol (DTT) and Et₃N was necessary to reduce the amount of side products produced during elimination of the 2-mercaptoethyl groups. Release of the thiophosphatoalkylated dinucleotide **5b** 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 **5b**, as less than 2% of unreacted dinucleotide **3** was detected. However, the chromatographic profile of RP-HPLC-purified **5b** hinted to its thermolability, given that **T_{Ps}T** (<10%) was also present in spite of the mildness of the conditions under which the analysis was performed.¹⁶ RP-HPLC-purified **5b** was characterized by ³¹P NMR spectroscopy and MALDI-TOF mass spectrometry (data shown in the SI). As anticipated, the 3-thiophosphato-1-propyl group was easily cleaved from **5b** in PBS (pH 7.2) to produce **T_{Ps}T**; the half-time of the thermolytic deprotection reaction was 156 min at 37 °C (see Table 2 of the SI). Such relatively rapid deprotection kinetics would be of limited value in the development of long-acting oligonucleotide prodrug formulations. Consequently, oligonucleotides functionalized with 3-thiophosphato-1-propyl groups for thiophosphate protection were not given further consideration as potential oligonucleotide prodrugs. Oligonucleotides functionalized with 3-phosphato-1-propyl groups for thiophosphate protection were also not considered for the development of thermolytic oligonucleotide prodrugs, as ubiquitous phosphatases might convert 3-phosphato-1-propyl groups

(15) (a) Iwai, S.; Ohtsuka, E. *Tetrahedron Lett.* **1988**, *29*, 5383–5386. (b) Iwai, S.; Ohtsuka, E. *Nucleic Acids Res.* **1988**, *16*, 9443–9456. (c) Ueno, Y.; Shibata, A.; Matsuda, A.; Kitade, Y. *Bioconjugate Chem.* **2003**, *14*, 684–689.

(16) RP-HPLC chromatogram of the dinucleotide is shown in Chart 13A of the SI.

(14) RP-HPLC chromatograms of the thermolytic conversion of **4** to **T_{Ps}T** are shown in Chart 1 of the SI.

SCHEME 3. Solid-Phase Synthesis of the Phosphato-/Thiophosphatoalkylated Dinucleoside Thiophosphate Triesters 5b and 6a–b^a



^a Reagents and conditions: (i) 0.5 M $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$ in $\text{C}_5\text{H}_5\text{N}/\text{AcOH}$ (3:2 v/v), 10 min; (ii) **9**, 0.45 M 1*H*-tetrazole/MeCN, 3 min; (iii) 0.1 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide/MeCN, 2 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.

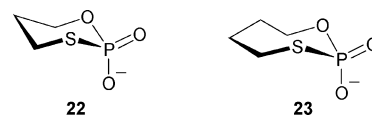
to thermolytically stable 3-hydroxy-1-propyl groups *in vivo* (vide infra).¹⁷

The solid-phase preparation of dinucleotides **20** and **21** was achieved in a manner identical to that of dinucleotide **19**, with the exception of using a solution of 0.1 M ethyl(methyl)dioxirane in CH_2Cl_2 ¹⁸ as an oxidant in the synthesis of **20**. When employing **9** as a phosphitylating reagent, the use of this oxidant instead of iodine or *tert*-butyl hydroperoxide produced higher yields of the phosphate monoesters.¹⁰ Conversion of **20** and **21** to **6a** and **6b**, respectively, proceeded under conditions identical to those described for the conversion of **19** to **5b**. Consistent with a slower thermolytic cyclodeesterification process, the formation of **T_{PS}T** ($\sim 2\%$) during the preparation of **6b** was significantly less than that produced during the preparation of **5b** (see Charts 5 and 6 of the SI). The formation of **T_{PS}T** was

not detected during the preparation of **6a**, which was reflective of the “harder” nucleophilic character of the phosphate monoesters relative to that of the thiophosphate monoesters in inducing cyclodeesterification reactions. RP-HPLC-purified **6a** and **6b** were characterized by ³¹P NMR spectroscopy and MALDI-TOF mass spectrometry (Experimental Section, see also Charts 5 and 7 of the SI). The purified dinucleotide **6a** was dissolved in PBS (pH 7.2) and heated at elevated temperatures to induce thermolytic cleavage of the 4-phosphato-1-butyl group and production of **T_{PS}T**. The thermolytic thiophosphate deprotection of **6a** proceeded slowly with a half-time of 305 min at 90 °C or 45 days at 37 °C (see Table 2 and Chart 12 of the SI). Such slow deprotection kinetics raise doubts on the usefulness of the 4-phosphato-1-butyl group in the development of thermolytic oligonucleotide prodrugs. However, the dinucleotide **6a** served as a substrate for human alkaline phosphatase *in vitro* as it was quantitatively dephosphorylated to its 4-hydroxybutylated derivative **4** within 2 h at 37 °C (data shown in Chart 8B of the SI). Thus, oligonucleotides functionalized with the 4-phosphato-1-butyl group for phosphate/thiophosphate protection may, theoretically, be dephosphorylated *in vivo* by intracellular phosphatases to the corresponding 4-hydroxybutylated oligonucleotide prodrugs and, then, may proceed through the thermolytic prodrug-to-drug conversion process. This potential phosphatase-dependent approach to the generation of thermolytic oligonucleotide prodrugs *in situ* is interesting and deserves further investigations.

The purified dinucleotide **6b** is, unlike **6a**, a poor substrate for human alkaline phosphatase, as dethiophosphorylation to dinucleotide **4** occurred to the extent of $\sim 15\%$ within 30 h at 37 °C (data shown in Chart 9B of the SI). However, given the “soft” nucleophilic character of thiophosphate monoesters, thermolytic cleavage of the 4-thiophosphato-1-butyl thiophosphate protecting group from dinucleotide **6b** occurred smoothly; the half-time of the deprotection reaction was 13 min at 90 °C or 30 h at 37 °C (see Table 2 and Chart 14 of the SI). As discussed above, the thermolytic thiophosphate deprotection of **5b** and that of dinucleotide **6b** proceeded, presumably via a cyclodeesterification mechanism leading to the generation of **T_{PS}T** and the concomitant formation of the respective oxathiaphosphorinane and oxathiaphosphpepane derivatives **22** and **23**.

To confirm the identity of cyclodeesterification product **23**



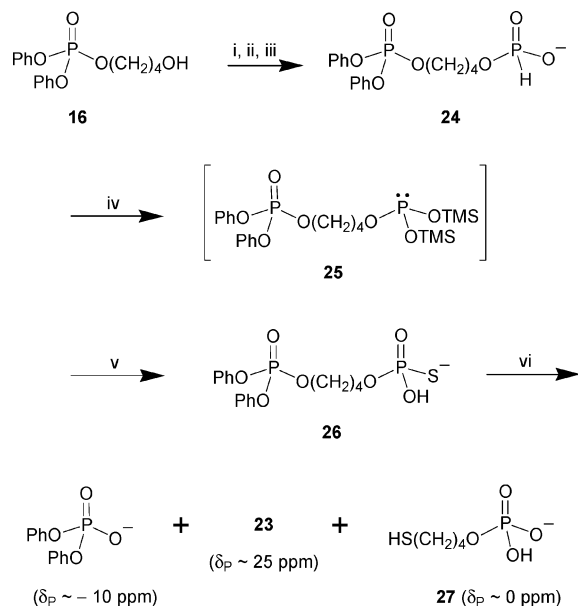
by means of spectroscopic methods, the structurally simpler thiophosphatoalkylated phosphate triester **26** (Scheme 4) was prepared. Typically, the reaction of *O,O*-diphenyl-*O*-(4-hydroxy-1-butyl)phosphate (**16**) with 2-chloro-1,3,2-benzodioxaphosphorin-4-one, as described for the preparation of deoxyribonucleoside 3'*H*-phosphonates,¹⁹ gave the *H*-phosphonate monoester **24** in a yield of 51% after silica gel chromatography. Condensation of **24** with trimethylsilyl chloride and Et_3N in dry pyridine²⁰ produced the bistrimethylsilyl phosphite triester derivative **25**, which, without workup, was treated with elemental sulfur to

(17) This statement is substantiated by the rapid and homologous conversion of dinucleotide **6a** to the hydroxybutylated dinucleotide **4** upon reaction with human alkaline phosphatase at 37 °C.

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

(19) Stawinski, J.; Strömberg, R. 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; pp 2.6.1–2.6.15 and references therein.

(20) Hata, T.; Sekine, M. *J. Am. Chem. Soc.* **1974**, *96*, 7363–7364.

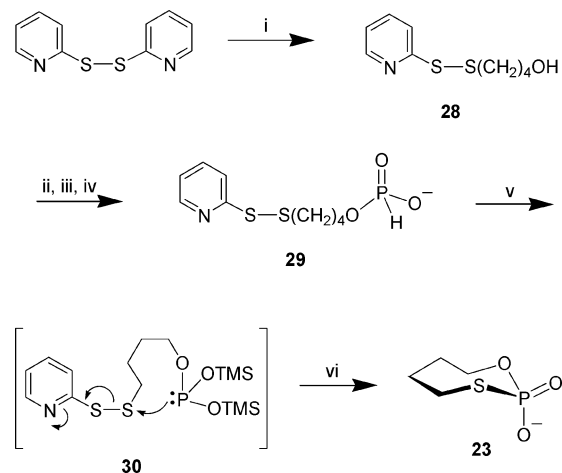
SCHEME 4. Preparation of the 4-Thiophosphato-1-butyl Phosphotriester **26 and Evaluation of Its Thermolytic Cyclodeesterification Side Products^a**


^a Reagents and conditions: (i) 2-chloro-1,3,2-benzodioxaphosphorin-4-one/dioxane, 1 h, 5→25 °C; (ii) 1 M triethylammonium bicarbonate (TEAB), pH 7.5; (iii) silica gel chromatography [CHCl₃/MeOH (9:1 v/v)]; (iv) Me₃SiCl/Et₃N, C₅H₅N, 4 h, 25 °C; (v) S₈, 16 h, 25 °C, RP-HPLC purification; (vi) DMSO-*d*₆/D₂O (5:1 v/v), 6 h, 90 °C.

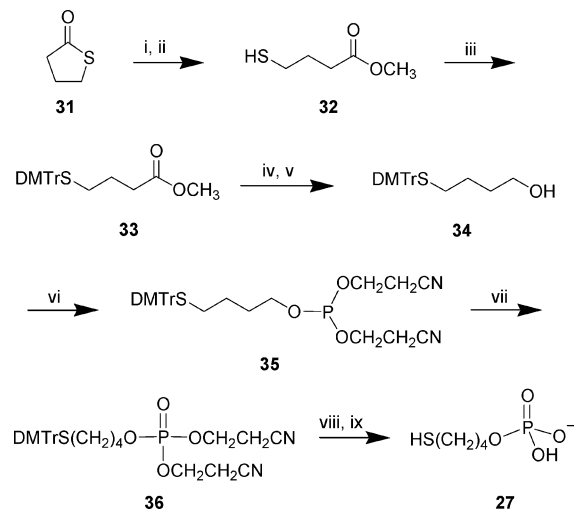
give **26** in a yield of ca. 90% on the basis of ³¹P NMR analysis of the reaction products. RP-HPLC-purified **26** was then heated to 90 °C in DMSO-*d*₆/D₂O (5:1 v/v) for 6 h to induce cyclodeesterification of the 4-thiophosphato-1-butyl phosphate protecting group. ³¹P NMR analysis of the thermolytic cyclodeesterification reaction revealed the formation of the oxathiaphosphepane derivative **23** ($\delta_P \sim 25$ ppm) along with that of diphenyl phosphate ($\delta_P \sim -10$ ppm). The formation of the most likely 4-mercaptobut-1-yl phosphate monoester (**27**, $\delta_P \sim 0$ ppm) was also detected, presumably as a result of the sensitivity of **23** to ring-opening hydrolysis under thermolytic conditions (data shown in Chart 10 of the SI).

To validate the spectral characteristics of **23**, the compound was prepared using an alternate route (Scheme 5). Specifically, the reaction of 2,2'-dithiopyridine with 4-mercaptobutan-1-ol gave 4-(2-pyridyldithio)butan-1-ol (**28**) in a yield of 39%. Phosphonylation of purified **28** with 2-chloro-1,3,2-benzodioxaphosphorin-4-one was achieved under conditions similar to those described in the literature for a different dithioalcohol.²¹ Purification of the crude *H*-phosphonate derivative by silica gel chromatography afforded pure **29** in a yield of 61%. Silylation of **29** led to the silylated phosphite intermediate **30**, which underwent intramolecular cyclization to afford the oxathiaphosphepane **23** after hydrolytic workup. ³¹P NMR analysis of crude **23** in DMSO-*d*₆/D₂O (5:1 v/v) revealed a signal exhibiting a chemical shift (δ_P 24.5 ppm, Chart 10C of the SI) identical to that obtained from the cyclodeesterification of **26** (Chart 10B of the SI), which was unambiguously demonstrated through a spiking experiment (data shown in Chart 11 of the SI).

Due to the inherent instability of the oxathiaphosphepane **23**, its characterization was difficult. Indeed, ³¹P NMR analysis of

SCHEME 5. Alternate Synthesis of the Oxathiaphosphepane Derivative **23^a**


^a Reagents and conditions: (i) 4-mercaptobutan-1-ol, Et₃N, 3 h, 25 °C; (ii) 2-chloro-1,3,2-benzodioxaphosphorin-4-one, 1,2-dichloroethane, 16 h, 5 °C; (iii) 0.3 M TEAB, pH 7.5; (iv) silica gel chromatography [CH₂Cl₂/MeOH (5→12% v/v)]; (v) Me₃SiCl/Et₃N, C₅H₅N, 5 h, 25 °C; (vi) MeCN/H₂O (99:1 v/v), 30 min, 25 °C.

SCHEME 6. Preparation of the *O*-(4-Mercaptobut-1-yl) Phosphate Monoester **27^a**


^a Reagents and conditions: (i) CH₃ONa, MeOH, 16 h, reflux; (ii) Dowex 50WX4–100 hydrogen form; (iii) DMTrCl, pyridine, 14 h, 25 °C; (iv) LiAlH₄, THF, 2 h, 0 °C; (v) silica gel chromatography; (vi) *i*-Pr₂NP(OCH₂CH₂CN)₂, 1*H*-tetrazole, MeCN, 2 h, 25 °C; (vii) 0.2 M I₂ in THF/Py/H₂O, 5 min, 25 °C; (viii) concd NH₄OH/dioxane (5:1 v/v), 8 h, 55 °C; (ix) 0.9% TFA in CHCl₃/MeOH (15:2 v/v), 2 min, 25 °C.

crude **23** that was stored in DMSO-*d*₆/D₂O (5:1 v/v) at –20 °C for 4 days tentatively revealed the formation (ca. 50%) of 4-mercaptobut-1-yl phosphate monoester (**27**, Scheme 4 and Chart 11B of the SI), which exhibited a ³¹P NMR chemical shift (δ_P 0.47 ppm) identical to that of the thermolytic side product obtained from the cyclodeesterification of **26**.

To corroborate the identity of **27**, its synthesis was achieved as shown in Scheme 6. Commercial γ -thiobutyrolactone (**31**) was refluxed with an equimolar amount of sodium methoxide in absolute methanol to provide methyl-4-mercaptopropionate (**32**) in 80% yield. Reaction of crude **32** with 4,4-dimethoxytrityl chloride in dry pyridine gave the *S*-DMTr derivative **33**, which was subsequently reduced to the corresponding alcohol **34** by

(21) Lartia, R.; Asseline, U. *Tetrahedron Lett.* **2004**, *45*, 5949–5952.

treatment with LAH in anhydrous THF. The alcohol **34** was purified by silica gel chromatography and phosphitylated upon reaction with bis(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite and 1*H*-tetrazole in MeCN. After aqueous workup, the phosphite ester **35** was isolated by precipitation from hexane. The phosphite **35** was then converted to the phosphate triester **36** via oxidation with 0.2 M iodine in pyridine/THF/H₂O.²² The crude phosphate triester **36** was dissolved in a concd NH₄OH/dioxane (5:1 v/v) mixture and heated at 55 °C until β-elimination of the 2-cyanoethyl phosphate protecting groups was complete. Following the removal of excess NH₄OH, the crude phosphate monoester was precipitated from diethyl ether and, then, lyophilized from dioxane. The lyophilisate was dissolved in ~1% TFA in CHCl₃/MeOH to remove the *S*-(4,4'-dimethoxytrityl)group, and crude **27** was isolated by precipitation from diethyl ether. The ³¹P NMR spectrum of **27** obtained from **36** was identical to that of **27** produced from the cyclodeesterification of **26** (Scheme 4, Charts 10 and 11 of the SI). These results rigorously confirmed the thermolytic conversion of dinucleotide prodrug model **6b** to its phosphorothioate diester **T_{PS}T** through a cyclodeesterification pathway.

The information obtained collectively from the dinucleotide prodrug models in terms of prodrug-to-drug conversion under thermolytic conditions prompted us to initiate the solid-phase synthesis of oligonucleotide CpG ODN *hbu*1555, which was performed by employing deoxyribonucleoside phosphoramidites functionalized with the 2-(*N*-formyl-*N*-methyl)aminoethyl group for phosphorus protection and phosphoramidite **8b** under conditions identical to those used for the preparation of CpG ODN *fma*1555.^{5a} The average coupling efficiency of these phosphoramidites was 98–99%, as determined by colorimetric measurements of the dimethoxytrityl cation after each chain elongation step. Upon completion of the synthesis in the “tritylon” mode, the solid-phase-linked oligonucleotide was first subjected to hydrazinolysis to remove the levulinyl group from the thermolabile oligonucleotide.²³ Then, pressurized ammonia gas was used to deprotect the nucleobases and release the oligonucleotide from the support.²⁴ The crude 5'-DMTr oligonucleotide was purified by 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 pure CpG ODN *hbu*1555, which was characterized satisfactorily by ESI–TOF mass spectrometry (see Table 1 of the SI).

The solid-phase synthesis of oligonucleotide CpG ODN *pob*1555 and CpG ODN *psb*1555 was achieved differently than that described for CpG ODN *hbu*1555 to enable an efficient chromatographic separation of the modified oligonucleotides from their respective failures sequences. Although the solid-phase synthesis of CpG ODN *pob*1555 and CpG ODN *psb*1555 was accomplished initially, in a manner identical to that of CpG ODN *hbu*1555, it was stopped temporarily at the penultimate chain elongation cycle to perform hydrazinolysis of the levulinyl group protecting the single internucleotidic 4-hydroxybutyl thiophosphate triester function. The resulting solid-phase-linked 4-hydroxybutylated oligonucleotide was then reacted with phosphoramidite **9** in the presence of 1*H*-tetrazole. This coupling

reaction was repeated once more, and the newly formed phosphite triester was oxidized with either 3*H*-1,2-benzodithiol-3-one-1,1-dioxide or ethyl(methyl)dioxirane to produce the respective 4-thiophosphato- or 4-phosphato-1-butylated oligonucleotide. After execution of a standard capping reaction, complete 5'-*O*- and *S*-dedimethoxytritylation was achieved by acidification with 3% TCA in CH₂Cl₂, which was immediately followed by an aqueous treatment with DTT and Et₃N to mitigate the formation of side products. The final chain elongation cycle was then resumed to complete the oligonucleotide assembly. The terminal 5'-DMTr group was left intact to facilitate oligonucleotide purification.

Nucleobase deprotection and RP-HPLC purification of oligonucleotides CpG ODN *psb*1555 and CpG ODN *pob*1555 were effected under conditions identical to those described above for CpG ODN *hbu*1555. The recovery of CpG ODN *psb*1555 and CpG ODN *pob*1555 was lower than that of CpG ODN *hbu*1555, thus indicating that steric factors may presumably be responsible for the less than optimal solid-phase phosphorylation (~85%) of the 4-hydroxybutyl thiophosphate protecting group effected by phosphoramidite **9**. This limitation is currently being investigated, and a solution to this shortcoming will be communicated later.

Desalted CpG ODN *pob*1555 and CpG ODN *psb*1555 were characterized by ESI–TOF mass spectrometry (see Table 1 of the SI). Conversion of diastereomeric RP-HPLC-purified CpG ODN *hbu*1555, CpG ODN *psb*1555, and CpG ODN *pob*1555 to CpG ODN 1555^{5a,b} was accomplished by heating to 90 °C in PBS (pH 7.2) over a period of 6, 4, and 16 h, respectively. Analysis of each thermolytic deprotection reaction by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions revealed, as expected, a band exhibiting an electrophoretic mobility identical to that of diastereomeric CpG ODN 1555 (data shown in Chart 18 of the SI). The thermolytic conversion of CpG ODN *pob*1555 to CpG ODN 1555 occurred with some internucleotidic bond cleavage; two faint fast-moving bands were observed by PAGE analysis. These findings were expected given that 2'-deoxyadenosine dephosphorylates to the extent of less than 3% when heated in PBS (pH 7.2) over a period of 16 h at 90 °C (data shown in Chart 19 of the SI).

All analytical methods used confirmed the identity of each of the modified oligonucleotides and their abilities to function as prodrugs, *in vitro*, through clean thermolytic conversion to CpG ODN 1555. Noteworthy is the increased solubility of CpG ODN *pob*1555 and CpG ODN *psb*1555 relative to that of CpG ODN *fma*1555 in aqueous media. This attribute was assessed by independent reconstitution of equal amounts of either lyophilized CpG ODN *pob*1555 or CpG ODN *psb*1555 and CpG ODN *fma*1555 in a limited but equal volume of water. The concentration of each reconstituted oligonucleotide was determined by UV spectrophotometry. When compared with each other, the concentration of reconstituted CpG ODN *pob*1555 or CpG ODN *psb*1555 was either 49 or 25% higher than that of CpG ODN *fma*1555. These findings suggest that the incorporation of a phosphate/thiophosphate monoester into an otherwise uncharged oligonucleotide may be sufficient to enhance aqueous solubility without significantly altering cellular uptake.

The resistance of oligonucleotide prodrugs CpG ODN *hbu*1555, CpG ODN *pob*1555, and CpG ODN *psb*1555 to nucleases was evaluated by exposing each of the prodrugs to snake venom phosphodiesterase (SVP) and S1 nuclease over a

(22) (a) Letsinger, R. L.; Lunsford, W. B. *J. Am. Chem. Soc.* **1976**, *98*, 3655–3661. (b) Letsinger, R. L.; Finnan, J. L.; Heavner, G. A.; Lunsford, W. B. *J. Am. Chem. Soc.* **1975**, *97*, 3278–3279.

(23) Only hydrazinolysis was effective in the cleavage of the levulinyl group within an acceptable period of time at ~25 °C.

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

period of 24 h at 37 °C. RP-HPLC analysis of the hydrolysates showed that the stability of all three oligonucleotide prodrugs to SVP and S1 nuclease was similar to that of CpG ODN *fma1555*, which was used as a negative control. Less than 2 and 4% degradation products were detected from the respective SVP and S1 digests. Under similar conditions, unmodified DNA oligonucleotides were completely digested within 15 min.²⁵ These results provide assurance that these three oligonucleotide prodrugs will maintain structural integrity while being tested as immunotherapeutic formulations in animal models for extended periods of time.

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 thermolability relative to that of the previously studied 2-(*N*-formyl-*N*-methyl)aminoethyl thiophosphate protecting group. Although CpG ODN *hbu1555* and CpG ODN *psb1555* will not require intracellular enzymes for conversion to the bioactive CpG ODN 1555, the oligonucleotide prodrug CpG ODN *pob1555* shall benefit from intracellular phosphatase activity for faster conversion to CpG ODN 1555 through the thermolabile intermediate CpG ODN *hbu1555*. Such a two-stage conversion process to CpG ODN 1555 may provide an additional degree of control on prodrug-to-drug conversion. By virtue of incorporating a phosphate/thiophosphate monoester function into the oligonucleotide chain, the solubility of CpG ODN *psb1555* and CpG ODN *pob1555* in water has increased significantly relative to that of CpG ODN *fma1555*. These findings thus foster the chemical development of novel, minimally charged, oligonucleotide prodrugs.

Studies assessing cellular uptake and biological activity of diastereomeric CpG ODNs *hbu1555*, *psb1555*, and *pob1555* in animal models are ongoing, and the results of these studies will be reported elsewhere in due course. Other thermolytic oligonucleotide prodrugs are currently being synthesized in our laboratories to provide increasingly effective heat-sensitive therapeutic oligonucleotide formulations against infectious diseases and certain types of cancer in animals.

Experimental Section

3-Hydroxy-1-propyl Levulinate (10). To a stirred solution of 1,3-propanediol (7.61 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 ~25 °C. 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 CHCl₃–MeOH (96:4 v/v), and the solution was added to the top of a column packed with silica gel (~150 g), pre-equilibrated in CHCl₃–MeOH (96:4 v/v). The column was then eluted employing the equilibration solvent to give pure 3-hydroxy-1-propyl levulinate (**10**) in 49% yield (8.51 g, 48.8 mmol): ¹H 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); ¹³C 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 purification conditions. Following

silica gel chromatography, 4-hydroxy-1-butyl levulinate (**11**) was isolated as a pure oil (8.02 g, 42.6 mmol) in 43% yield: ¹H 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); ¹³C 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 (**10**, 0.87 g, 4.99 mmol) and *i*-Pr₂NEt (4.15 g, 34.6 mmol) in dry CH₂Cl₂ was added bis(*N,N*-diisopropylamino)chlorophosphine (1.35 g, 5.06 mmol). Progress of the reaction was monitored by ³¹P NMR spectroscopy, which showed complete conversion of bis(*N,N*-diisopropylamino)chlorophosphine (δ_P 135.5) to the phosphorodiamidite **12** (δ_P 122.0) within 2 h at ~25 °C. The suspension was filtered off, and the filtrate was evaporated to an oil under reduced pressure. The crude phosphorodiamidite was dissolved in a minimal volume of C₆H₆/Et₃N (9:1 v/v); the solution was added to the top of a chromatography column packed with silica gel (~30 g) that was equilibrated in C₆H₆/Et₃N (9:1 v/v). The column was eluted using the equilibration solvent, and fractions containing the product were identified by ³¹P NMR spectroscopy. Fractions containing the product were collected together and rotoevaporated under low pressure to give an oil: Yield 75% (1.52 g, 3.75 mmol); ¹H NMR (300 MHz, C₆D₆) 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); ¹³C NMR (75 MHz, C₆D₆) δ 204.4, 172.3, 61.9, 61.0 (d, ²*J*_{CP} = 21.5 Hz), 44.8, 44.6, 37.6, 31.1 (d, ³*J*_{CP} = 8.4 Hz), 29.1, 28.1, 24.7, 24.6, 24.1, 24.0; ³¹P NMR (121 MHz, C₆D₆) 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 employing 4-hydroxy-1-butyl levulinate (**11**, 15.0 mmol) under conditions identical to those used for the synthesis and purification of **12**: Yield 72% (4.52 g, 10.8 mmol); ¹H NMR (300 MHz, C₆D₆) 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); ¹³C NMR (75 MHz, C₆D₆) 204.5, 172.3, 64.5, 64.2 (d, ²*J*_{CP} = 21.5 Hz), 44.7, 44.6, 37.6, 29.1, 28.4 (d, ³*J*_{CP} = 8.4 Hz), 28.1, 26.0, 24.8, 24.7, 24.1, 24.0; ³¹P NMR (121 MHz, C₆D₆) δ 122.8.

General Procedure for the Preparation of Deoxyribonucleoside Phosphoramidites 7a and 8a–d. Suitably protected 2'-deoxyribonucleosides (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 ~25 °C for 3 h²⁶ and was then rotoevaporated to a foam under vacuum. The material was dissolved in a minimal volume of C₆H₆/Et₃N (9:1 v/v). The solution was added to the top of a chromatography column packed with silica gel (~30 g) that was equilibrated in C₆H₆/Et₃N (9:1 v/v). The column was eluted employing 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 C₆H₆ (~5 mL), and the solution was added to cold (–10 °C) hexanes (100 mL). The precipitate was isolated by decanting off the hexanes and was dissolved by adding dry C₆H₆ (10 mL). The resulting solution was frozen in a

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

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

dry ice–EtOH bath and was 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 $\text{C}_{45}\text{H}_{59}\text{N}_3\text{O}_{11}\text{P}$ ($\text{M} + \text{H}$) $^+$, 848.3887; found, 848.3883.

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 $\text{C}_{46}\text{H}_{61}\text{N}_3\text{O}_{11}\text{P}$ ($\text{M} + \text{H}$) $^+$, 862.4044; found, 862.4032.

N⁴-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 $\text{C}_{52}\text{H}_{64}\text{N}_4\text{O}_{11}\text{P}$ ($\text{M} + \text{H}$) $^+$, 951.4309; found, 951.4303.

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): ^{31}P NMR (121 MHz, C_6H_6) δ 148.0; APESI–HRMS calcd for $\text{C}_{53}\text{H}_{64}\text{N}_6\text{O}_{10}\text{P}$ ($\text{M} + \text{H}$) $^+$, 975.4422; found, 975.4418.

N²-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 $\text{C}_{50}\text{H}_{66}\text{N}_5\text{O}_{11}\text{P}$ ($\text{M} + \text{H}$) $^+$, 957.4527; found, 957.4519.

Preparation of the Dinucleoside Thiophosphate Triesters 3 and 4. A commercial synthesis column packed with succinyl long-chain alkylamine controlled-pore glass (LCAA-CPG) functionalized with 5'-O-DMTr-2'-deoxythymidine (0.2 μmol) was treated with 3% TCA in CH_2Cl_2 for 2 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** (30 μmol) and 0.45 M 1*H*-tetrazole in MeCN (300 μL) for 10 min. The synthesis column was rinsed out with MeCN, and the support was mixed with 0.05 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in MeCN (1 mL) for 2 min. The solid support was then washed with MeCN and treated with 0.5 M $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ in pyridine/acetic acid (3:2 v/v, 3 mL) for 10 min to remove the levulinyl group. Following extensive washing of the CPG support with CH_2Cl_2 and MeCN, removal of the 5'-DMTr group was effected under acidic conditions (3% TCA in CH_2Cl_2) over a period of 2 min. After carefully washing 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 about 97% as determined by RP-HPLC (data shown in Charts 1A and 2A of the SI). **Thymidylyl-(3'→5')-thymidine Thiophosphate 3-Hydroxy-1-propyl Ester (3)**: +MALDI–TOF MS calcd for $\text{C}_{23}\text{H}_{32}\text{N}_4\text{NaO}_{12}\text{P}_2\text{S}$ ($\text{M} + \text{Na}$) $^+$, 642.14; found, 642.64. **Thymidylyl-(3'→5')-thymidine Thiophosphate 4-Hydroxy-1-butyl Ester (4)**: +MALDI–TOF MS calcd for $\text{C}_{24}\text{H}_{35}\text{N}_4\text{O}_{12}\text{P}_2\text{S}$ (M) $^+$, 634.17; found, 634.72.

Kinetic Analysis of the Thermolytic Cleavage of the 4-Hydroxy-1-butyl Thiophosphate Protecting Group from 4. RP-HPLC-purified **4** (~10 OD₂₆₀) was dissolved in PBS (pH 7.2, 500 μL) and heated to 90 \pm 2 °C. Aliquots of the deprotection reaction were taken out, periodically, over a period of 6 h. Each aliquot was analyzed by RP-HPLC using a 5 μm Supelcosil LC-18S column (25 cm \times 4.6 mm) under the following conditions: starting from 0.1 M triethylammonium acetate (TEAA), pH 7.0, a linear gradient of 1% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min.

The thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group was also investigated at 37 \pm 2 °C using ~20 OD₂₆₀ of RP-HPLC-purified **4** in PBS (pH 7.2, 1 mL). Aliquots of the deprotection reaction were retained, periodically, over a period of 200 h and were analyzed by RP-HPLC under chromatographic

conditions identical to those used for the kinetic study performed at 90 °C. The RP-HPLC retention time (t_R) of **4** and its thermolytic thiophosphate deprotection half-time ($t_{1/2}$) at both 37 and 90 °C are reported in Table 2 of the SI.

Kinetic Analysis of the Thermolytic Cleavage of the 3-Hydroxy-1-propyl Thiophosphate Protecting Group from 3. This analysis was achieved at 90 \pm 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. Under these conditions, unreacted dinucleoside phosphotriester **3** and unidentified side products accounted for ~80% and less than 20% of the total peak area of the chromatogram, respectively.

Preparation of the Thiophosphatoalkylated Dinucleoside Thiophosphate Triesters 5b and 6b. A commercial synthesis column packed with succinyl LCAA-CPG, functionalized with 5'-O-DMTr-2'-deoxythymidine (0.2 μmol), was treated with 3% TCA in CH_2Cl_2 for 2 min. After carefully washing the support with CH_2Cl_2 and MeCN, the coupling reaction was carried out by agitating the support in a solution composed of phosphoramidite **7a** or **8a** (30 μmol) and 0.45 M 1*H*-tetrazole in MeCN (300 μL) for 10 min. The synthesis column was flushed 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 3 min. Unused oxidant was washed out of the support with MeCN, affording the dinucleoside phosphotriesters **14** or **15**. The levulinyl group was then cleaved from **14** or **15** by treatment with 0.5 M $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ in pyridine/acetic acid (3:2 v/v, 3 mL) for 10 min at ~25 °C. Following extensive washing of the CPG support **17** or **18** with CH_2Cl_2 and MeCN, a coupling reaction was achieved using phosphoramidite **9** (27 mg, 30 μmol) activated with 0.45 M 1*H*-tetrazole in MeCN (300 μL) over a period of 3 min. The synthesis column was rinsed out with MeCN, and the support was 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 away from the support with MeCN to give the dinucleotides **19** or **21**. Removal of all of the DMTr groups was effected by slowly flushing 3% TCA in CH_2Cl_2 through the synthesis column over a period of 15 min. The support was then washed with CH_2Cl_2 and MeCN followed by treatment with a solution of 1.2% (w/v) DTT in H_2O (1 mL) containing 5% (v/v) Et_3N over a period of 60 min. After extensive washing of the support with MeCN, the synthesis column was exposed to pressurized methylamine gas (~2.5 bar) for 3 min to release the crude thiophosphatoalkylated dinucleoside thiophosphate triesters **5b** or **6b**. Each dinucleotide was purified by RP-HPLC under chromatographic conditions identical to those described for the thermolytic cleavage of the 4-hydroxy-1-butyl thiophosphate protecting group from **4**. The RP-HPLC retention time of diastereomeric **5b** or **6b** under these conditions is reported in Table 2 of the SI. **Thymidylyl-(3'→5')-thymidine Thiophosphate 3-Thiophosphato-1-propyl Ester (5b)**: ^{31}P NMR (121 MHz, D_2O) δ 67.9, 67.7, 57.4, 57.3, 49.7, 11.8; +MALDI–TOF MS calcd for $\text{C}_{23}\text{H}_{34}\text{N}_4\text{O}_{14}\text{P}_2\text{S}_2$ ($\text{M} + 2\text{H}$) $^+$, 716.10; found, 716.10. **Thymidylyl-(3'→5')-thymidine Thiophosphate 4-Thiophosphato-1-butyl Ester (6b)**: ^{31}P NMR (121 MHz, H_2O) δ 67.1, 66.9, 51.9; –MALDI–TOF MS calcd for $\text{C}_{24}\text{H}_{35}\text{N}_4\text{O}_{14}\text{P}_2\text{S}_2$ ($\text{M} - \text{H}$) $^-$, 729.63; found, 729.66.

Preparation of the Phosphatoalkylated Dinucleoside Thiophosphate Triester 6a. Dinucleotide **6a** was prepared in a manner identical to that described for the preparation of dinucleotides **5b** and **6b** with the following exception: upon phosphorylation of **18** 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 in MeCN, was used over a period of 1 min to give the dinucleotide **20**. The RP-HPLC purification of dinucleotide **6a** was done under conditions identical to those described for the purification of **5b** and **6b**. The RP-HPLC retention time of **6a** under such chromatographic conditions is reported in Table 2 of the SI. **Thymidylyl-(3'→5')**

thymidine Thiophosphate 4-Phosphato-1-butyl Ester (6a): ³¹P NMR (121 MHz, H₂O) δ 68.0, 1.8; +MALDI-TOF MS calcd for C₂₄H₃₅N₄O₁₅P₂SNa (M + H + Na)⁺, 736.12; found, 736.39

Kinetic Analysis of the Thermolytic Cleavage of the Thio-phosphatoalkyl and Phosphatoalkyl Thiophosphate Protecting Groups from 5b, 6b, and 6a. This analysis was carried out at 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 of the deprotection reaction were taken out periodically over a period of 200 h in the case of 5b and 6b or until the deprotection reaction was 50% complete in the case of 6a. The thermolytic thiophosphate deprotection half-times of dinucleotides 5b, 6b, and 6a are reported in Table 2 of the SI.

Enzymatic Dephosphorylation of Dinucleotide 6a Catalyzed by Human Alkaline Phosphatase. To a solution of RP-HPLC-purified dinucleotide 6a (3 OD₂₆₀) 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 under conditions identical to those used for the purification of dinucleotides 5b and 6a,b. The analysis revealed complete dephosphorylation of 6a (t_R = 27.8 min) to dinucleotide 4 (t_R = 30.5 min). When an identical experiment was carried out with the dinucleotide 6b, less than 5 and 15% dethiophosphorylation to dinucleotide 4 was detected after an incubation time of 2 and 30 h, respectively.

Solid-Phase Oligonucleotide Synthesis. Solid-phase synthesis of the modified oligonucleotide CpG ODN *hbu1555* was performed on a 1 μmol scale and under conditions identical to those described earlier for the preparation of CpG ODN *fma1555*^{5a} and CpG ODN 1555. An additional glass bottle containing the phosphoramidite 8b as a 0.1 M solution in dry MeCN was connected to the DNA/RNA synthesizer. The phosphoramidite 8b was numerically labeled in the programmed alphabetical DNA sequence and recognized as such during execution of the automated DNA chain extension.

Upon completion of the DNA chain assembly, the 5'-DMTr solid-phase-linked oligonucleotide was treated with 0.5 M NH₂NH₂·H₂O in pyridine/AcOH (3:2 v/v) by pushing the solution (3 mL) through the synthesis column, over a period of 10 min, with a 3 mL syringe. The excess reagent was discarded, and the column was rinsed out, consecutively, with CH₂Cl₂ and MeCN (10 mL each). Residual solvent was eliminated from the support by placing the synthesis column under reduced pressure for ~3 min.

Solid-phase synthesis of oligonucleotides CpG ODN *pob1555* and CpG ODN *psb1555* was achieved initially under conditions identical to those used for the preparation of CpG ODN *hbu1555* but was interrupted at the second to last chain elongation cycle, leaving the terminal 5'-DMTr group intact. Hydrazinolysis of the levulinyl group was carried out identically as described for CpG ODN *hbu1555*. A solution of activated phosphoramidite 9 (0.2 M in 0.45 M 1*H*-tetrazole/MeCN, 0.3 mL) was then syringed back and forth through each of the synthesis columns for 3 min. The synthesis columns were purged with dry MeCN (10 mL), and the phosphorylation step was performed again. 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 (1 mL) for 2 min, whereas the support of the other column was treated with 0.1 M ethyl(methyl)dioxirane in CH₂Cl₂ (1 mL) for 1 min. The oxidant was then flushed out of the columns with MeCN (10 mL), and an equal volume (0.5 mL) of commercial Cap A and Cap B reagents was syringed back and forth through each of the synthesis columns for 3 min. The capping reagents were expelled out of each column with MeCN (10 mL). The DMTr groups were then cleaved by pushing 3% TCA in CH₂-Cl₂ (15 mL) through each column over a period of 15 min to achieve complete *S*-dedimethoxytritylation. Residual acid was washed out of each column with CH₂Cl₂ (10 mL) and MeCN (3 mL). A solution of 1.2% (w/v) DTT/H₂O containing 5% (v/v) Et₃N (3 mL) was syringed through the columns over a period of 60 min. The synthesis columns were rinsed out with H₂O (10 mL) and then with MeCN

(10 mL). The final chain elongation cycle was completed using the DNA/RNA synthesizer, leaving on the terminal 5'-DMTr group. The synthesis columns were then placed under reduced pressure for ~3 min to remove residual solvent.

Oligonucleotide Deprotection and Purification. The solid-phase-linked 5'-DMTr oligonucleotides corresponding to CpG ODN *hbu1555*, CpG ODN *psb1555*, and CpG ODN *pob1555* were deprotected under conditions identical to those reported for the deprotection of CpG ODN *fma1555*.^{5a} Specifically, the columns were placed into a stainless steel pressure vessel and exposed to pressurized ammonia (10 bar at 25 °C) for 12 h. 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 TEAA, pH 7.0, (1 mL). Purification of each oligonucleotide was accomplished by RP-HPLC in the "trityl-on" mode and then in the "trityl-off" mode, as described for the purification of CpG ODN *fma1555*.^{5a} The product peaks were collected, and the eluates were evaporated using a stream of air without heating. The concentration of CpG ODN *hbu1555*, CpG ODN *psb1555*, and CpG ODN *pob1555* was determined by UV spectrophotometry at 260 nm upon reconstitution of the purified oligonucleotides in ddH₂O (1 mL). The recovered yields of CpG ODN *hbu1555*, CpG ODN *psb1555*, and CpG ODN *pob1555* were 71, 55, and 59 OD₂₆₀ units, respectively. The pure oligonucleotides were stored frozen at -20 °C.

Oligonucleotide Characterization. RP-HPLC analysis of purified CpG ODN *hbu1555*, CpG ODN *psb1555*, and CpG ODN *pob1555* (7 OD₂₆₀ each) was achieved using an analytical 5 μm Supelcosil LC-18S column (4.6 mm × 25 cm) under 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 and then held isocratically for 20 min. RP-HPLC profiles of the purified oligonucleotides are shown in Charts 15–17 of the SI. RP-HPLC retention times of the purified oligonucleotides are listed in Table 1 of the SI. Samples of the purified oligonucleotides were characterized by ESI-TOF MS operating in the positive-ion mode. The results of these analyses are presented in Table 1 of the SI.

Functional characterization of CpG ODN *hbu1555*, CpG ODN *psb1555*, and CpG ODN *pob1555* consisted of converting these oligonucleotides to CpG ODN 1555^{5a} upon heating at 90 °C in 1X PBS (pH 7.2) for 6, 4, and 16 h, respectively. RP-HPLC and PAGE analysis of the thermolytically deprotected oligonucleotides revealed complete conversion to CpG ODN 1555 (data shown in Charts 15–18 of the SI).

Determination of Relative Oligonucleotide Solubility in Water. In duplicate experiments, lyophilized CpG ODN *pob1555* or CpG ODN *psb1555* (20 OD₂₆₀) and CpG ODN *fma1555* (20 OD₂₆₀) were suspended in H₂O (50 μL). Each suspension was vigorously agitated using a vortexer, at full speed, for 30 s. Each suspension was sedimented by microcentrifugation at 16 000g for 30 s. The concentration of each oligonucleotide in 5 μL of each supernatant was determined by UV spectrophotometry at 260 nm and averaged 1.00 OD for CpG ODN *fma1555*, 1.49 OD for CpG ODN *pob1555*, and 1.25 OD for CpG ODN *psb1555*. Thus, the concentration of CpG ODN *pob1555* or that of CpG ODN *psb1555* is 49%, or 25% higher than that of CpG ODN *fma1555* in water.

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Supporting Information Available: Experimental Section including materials and methods; synthesis and characterization of

16, **23**, **24**, **26–29**, and **32–36**; generation of THF during thermal cyclodeesterification of **16**; stability of CpG ODN *hbu1555*, CpG ODN *psb1555*, and CpG ODN *pob1555* to snake venom phosphodiesterase and S1 nuclease; ¹H NMR spectra of **10–13** and **26**; ¹³C NMR spectra of **10–13**, **26**, **29**, **35**, and **36**; ³¹P NMR spectra of **5b**, **6a–b**, **7a**, **8a–d**, **12**, **13**, **23**, **24**, **26–27**, **29**, **35**, and **36**; ¹H and ³¹P NMR analysis of the thermolytic cyclodeesterification of **16**; RP-HPLC analysis of the thermolytic thiophosphate deprotection of **3**, **4**, **5b**, **6a**, and **6b**; RP-HPLC analysis of the digestion of **6a**

and **6b** catalyzed by human alkaline phosphatase; RP-HPLC analysis of RP-HPLC-purified CpG ODN *hbu1555*, CpG ODN *psb1555*, CpG ODN *pob1555*, and their respective thermolytic conversion to CpG ODN 1555; polyacrylamide gel analysis of the thermolytic conversion of CpG ODN *hbu1555*, CpG ODN *psb1555*, and CpG ODN *pob1555* to CpG ODN 1555; RP-HPLC analysis of heat-induced depurination of 2'-deoxyadenosine. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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