Hydroxyalkylated phosphoramidate, phosphoramidothioate and phosphorodiamidothioate derivatives as thiophosphate protecting groups in the development of thermolytic DNA prodrugs†‡

Andrzej Grajkowski,[a] Jacek Cieślak,[a] Alexei Gapeev[b] and Serge L. Beaucage*[a]

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The hydroxyalkylated phosphoramidate **4a**, phosphoramidothioates **4b**, **4e–j**, and phosphorodiamidothioates **4c** and **4d** have been identified as a new class of heat-sensitive thiophosphate protecting groups in the development of thermolytic immunomodulatory DNA prodrugs. These alcohols are converted to their deoxyribonucleoside phosphoramidite derivatives **6a–j**, which are then used in the preparation of the thermosensitive dinucleoside phosphorothioates **7a–j**. The negatively charged thiophosphate protecting groups of **7a–b** and **7e–j** presumably undergo thermolytic cyclodeesterification at elevated temperature under essentially neutral conditions. The thiophosphate protecting groups of **7e** and **7f** show relatively rapid deprotection kinetics at 37 °C ($t_{\frac{1}{2}}$ = 20 and 42 h, respectively) and are therefore suitable for the protection of phosphodiester functions flanking the CpG motifs of immunomodulatory DNA sequences, whereas the thiophosphate protecting groups of **7g–j** with thermolytic deprotection half-lives in the range of 94–265 h at 37 °C are more appropriate for the thiophosphate protection of CpG motifs. Furthermore, the thermostability of the group protecting the thiophosphate function of **7a** ($t_{\frac{1}{2}}$ = 82 min at 90 °C) should offer adequate protection of the 5′- and/or 3′-terminal phosphodiester functions of DNA prodrugs against ubiquitous extracellular and intracellular exonucleases.

Introduction

The polyanionic nature of DNA or RNA sequences is known to negatively affect the cellular uptake of these biomolecules, thereby limiting their abilities to modulate the expression of therapeutically relevant proteins through either an antisense or an RNA interference pathway. An additional complication stemming from negatively charged DNA or RNA sequences, is their vulnerability to ubiquitous extracellular and intracellular nucleases that are present in biological environments. In order to mitigate these shortcomings, the concept of oligonucleotide prodrugs has been proposed[1] and has evolved over the years as a viable approach to facilitate cellular uptake of DNA sequences while imparting these with increased stability to extracellular and intracellular nucleases. The prodrug approach consists of converting the negatively charged phosphodiester groups of DNA sequences to neutral acylated or *S*-acylated phosphotriester functions,[2–4] which upon cellular entry, are hydrolyzed by intracellular esterases to their bioactive phosphodiester state. A modification of the original prodrug approach has been proposed by us[5] and consists of masking the phosphodiester functions of DNA sequences with thermosensitive phosphate/thiophosphate protecting groups. Such a modification eliminates the requirement for esterases activity; only an aqueous environment at elevated temperature (>30 °C) is necessary to thermolytically convert oligonucleoside phosphotriesters to bioactive oligonucleoside phosphodiesters. This approach to the preparation of DNA prodrugs allows the selection of phosphate/thiophosphate protecting groups with differential thermosensitivity properties in order to optimize cellular uptake of the prodrugs through better control of their lipophilic and hydrophilic attributes.

When applied in the context of immunomodulatory single-stranded DNA oligonucleoside phosphorothioates containing unmethylated CpG motifs (type K CpG ODNs),[6] oligonucleoside phosphorothioates with thermolytic 2-(*N*-formyl-*N*-methyl)-aminoethyl (FMA) thiophosphate protection induced an immunostimulatory response in mice similar to that generated from the administration of a conventional CpG ODN.[5] These findings prompted us to identify thermosensitive thiophosphate protecting groups, which by virtue of the range of their deprotection rates at 37 °C, should generate bioactive CpG ODNs in a time-dependent manner and produce an immunostimulatory response in animal models lasting longer than that obtained with conventional CpG ODNs.

We recently investigated thermolytic thiophosphate protecting groups derived from 2-(*N*-formyl-*N*-methyl)aminoethanol and 4-(methylthio)butan-1-ol;[7] the majority of these groups revealed deprotection rates that were either faster or

[a] *Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA. E-mail: serge.beaucage@fda.hhs.gov; Fax: +1 301-480-3256; Tel: +1 301-827-5162*
[b] *Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, Maryland, USA*
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considerably slower than that of the FMA group at 37 °C ($t_{\frac{1}{2}}$ = 72 h). The thermolytic groups with relatively faster deprotection kinetics may be used for the thiophosphate protection of immunomodulatory CpG DNA motifs and/or that of their flanking DNA sequences, whereas those with exceedingly slow deprotection rates offer DNA prodrugs with adequate protection of terminal phosphodiester functions against exonucleases that may be found in biological systems.[7]

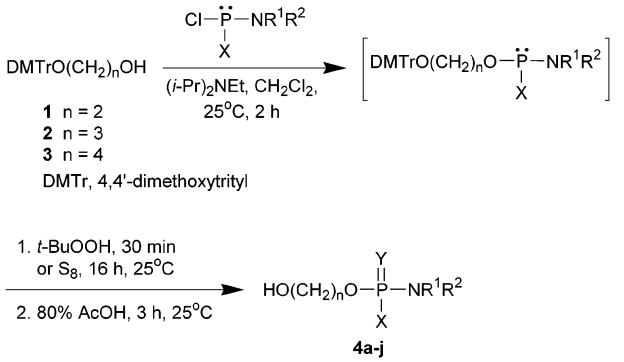
In order to better assess the biological consequences of sustained CpG ODN immunostimulation in animal models, it would be desirable to identify thiophosphate protecting groups with thermolytic deprotection half-lives in the range of 100–200 h at 37 °C. On the basis of our earlier work on the application of a 3-hydroxypropyl phosphoramidothioate function to the thermosensitive release of DNA sequences from controlled-pore glass under near neutral conditions,[8] we are now reporting the results of our investigations regarding the thermolytic properties of hydroxyalkylated phosphoramidate, phosphoramidothioate and phosphorodiamidothioate derivatives as thiophosphate protecting groups, as well as their potential application to the development of thermosensitive immunomodulatory CpG ODN prodrugs.

Results and discussion

The synthesis of hydroxyalkylated phosphoramidate, phosphoramidothioate and phosphorodiamidothioate derivatives (**4a–j**) began with the preparation the protected alcohols **1–3** (Scheme 1) according to published literature procedures.[9–11] The reaction of **1** with 2-cyanoethyl (*N*,*N*-diisopropyl)-phosphoramidochloridite (1.1 molar equiv.) and *N*,*N*-diisopropylethylamine (5 molar equiv.) in CH_2Cl_2 was followed by oxidation of the resulting phosphoramidite intermediate with either *tert*-butyl hydroperoxide or elemental sulfur to give, after removal of the 4,4-dimethoxytrityl (DMTr) group under acidic conditions and purification by chromatography on silica gel, the hydroxyalkylated phosphoramidate **4a** or the phosphoramidothioate **4b** in yields of 80% and 84%, respectively. The hydroxyalkylated phosphoramidothioates **4e** and **4h** were similarly prepared from the protected alcohols **2** and **3**, respectively, and were isolated in similar yields after silica gel chromatography. The replacement of 2-cyanoethyl (*N*,*N*-diisopropyl)phosphoramidochloridite with either 2-cyanoethyl (*N*,*N*-diethyl)phosphoramidochloridite[12] or 2-cyanoethyl (*N*-morpholinyl)phosphoramidochloridite[13] under the conditions used for the synthesis of **4b** afforded the hydroxyalkylated phosphoramidothioates, **4f**, **4g**, **4i** and **4j**, the yields of which were in the range of 79–83%. The hydroxyalkylated phosphorodiamidothioate derivatives **4c** and **4d** were obtained from the reaction of alcohol **1** with either bis(*N*,*N*-diisopropylamino)chlorophosphine or bis(*N*,*N*-diethylamino)chlorophosphine under the conditions employed for the preparation of **4b** (Scheme 1). The hydroxyalkylated phosphorodiamidothioate derivatives **4c** and **4d** were isolated after purification by chromatography on silica gel in yields comparable to those of **4a** and **4b**. The identity of the alcohols **4a–j** was confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy.

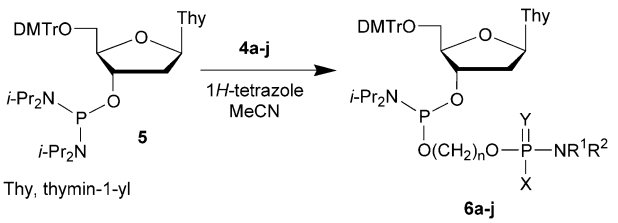
The deoxyribonucleoside phosphoramidites **6a–j** (Scheme 2) were prepared from the reaction of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-bis(*N*,*N*-diisopropylamino)phosphinyl-2′-deoxythymidine (**5**)[8] with equimolar amounts of any of the alcohols **4a–j** in dry MeCN and solid 1*H*-tetrazole, which was added to the solution in small portions over a period of 1 h at ambient temperature. The crude phosphoramidites **6a–j** were purified by chromatography on silica gel that was equilibrated in benzene containing Et_3N (10% v/v) to prevent premature activation of **6a–j** during purification. This solution was also used to elute **6a–j** from the chromatography column. Complete removal of residual Et_3N from purified phosphoramidites by lyophilization from dry benzene under high vacuum is critically important for optimal coupling of **6a–j** in the solid-phase synthesis of the dinucleoside phosphorothioate triesters **7a–j** (Scheme 3). The identity of phosphoramidites **6a–j** was determined by ^{31}P NMR spectroscopy and by high resolution mass spectrometry, which also confirmed the identity of **4a–j**.

The synthesis of **7a–j** was performed by the reaction of any of the phosphoramidites **6a–j** with the 5′-hydroxy function of deoxythymidine that is covalently attached to long chain alkylamine controlled-pore glass (CPG) through a 3′-*O*-hemisuccinate linker (TsuccCPG, Scheme 3). This reaction proceeded smoothly in the presence of 1*H*-tetrazole in dry MeCN to produce a phosphite triester intermediate,

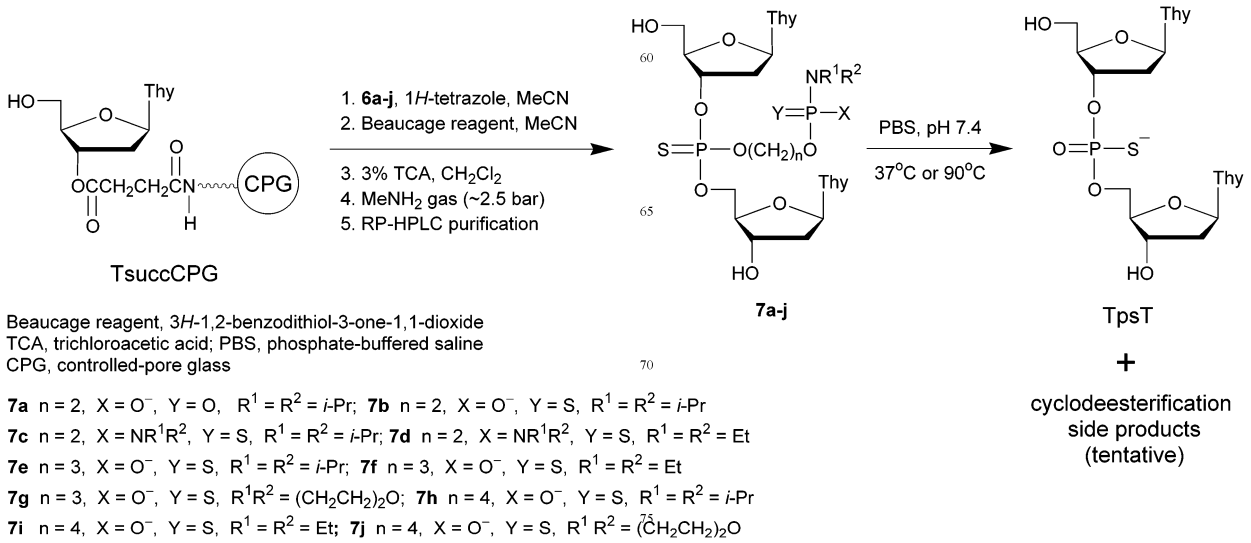


a n = 2, X = OCH_2CH_2CN, Y = O, $R^1 = R^2$ = *i*-Pr
b n = 2, X = OCH_2CH_2CN, Y = S, $R^1 = R^2$ = *i*-Pr
c n = 2, X = NR^1R^2, Y = S, $R^1 = R^2$ = *i*-Pr
d n = 2, X = NR^1R^2, Y = S, $R^1 = R^2$ = Et
e n = 3, X = OCH_2CH_2CN, Y = S, $R^1 = R^2$ = *i*-Pr
f n = 3, X = OCH_2CH_2CN, Y = S, $R^1 = R^2$ = Et
g n = 3, X = OCH_2CH_2CN, Y = S, $R^1 R^2 = (CH_2CH_2)_2O$
h n = 4, X = OCH_2CH_2CN, Y = S, $R^1 = R^2$ = *i*-Pr
i n = 4, X = OCH_2CH_2CN, Y = S, $R^1 = R^2$ = Et
j n = 4, X = OCH_2CH_2CN, Y = S, $R^1 R^2 = (CH_2CH_2)_2O$

Scheme 1



Scheme 2



Scheme 3

which was oxidized to the corresponding dinucleoside phosphorothioate derivative by treatment with 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in MeCN. Removal of the 5′-DMTr protecting group under acidic conditions was followed by the cleavage of the 2-cyanoethyl group from the phosphoramidate/phosphoramidothioate function and release of **7a–j** from CPG upon exposure to pressurized $MeNH_2$ gas (Scheme 3). Each of the dinucleoside phosphorothioate triesters **7a–j** was purified by reversed-phase (RP) HPLC prior to its thermolytic conversion to the dinucleoside phosphorothioate diester TpsT in phosphate-buffered saline (PBS, pH 7.4) at 37 °C or 90 °C.

The thermolytic conversion of dinucleoside phosphotriester models to TpsT rather than to TpT is preferred for assessing the deprotection rates of thermosensitive protecting groups because *S*-alkylation of the internucleoside thiophosphate function, if occurring during the thermolytic deprotection reaction, can be easily monitored by RP-HPLC. *S*-Alkylation of the internucleoside phosphorothioate diester by deprotection side products typically results in its hydrolytic desulfurization and formation of TpT along with other minor degradation products. Since the structural integrity of phosphorothioate diester groups is critically important for safeguarding the immunostimulatory properties of type K[6] and type D[6,14] CpG ODNs by providing resistance to the nucleolytic activities of extracellular and intracellular nucleases, *S*-alkylation and desulfurization of phosphorothioate diester functions must be avoided.

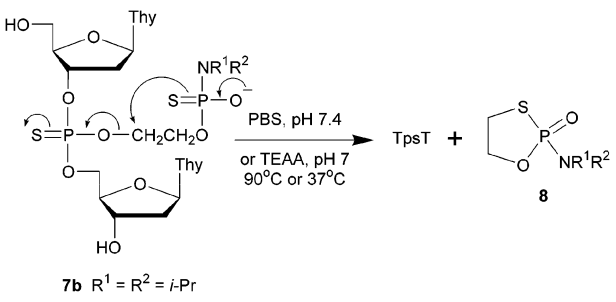
The thermolytic thiophosphate deprotection of RP-HPLC purified **7a** in PBS (pH 7.4) to give TpsT occurred with a half-life of 60 min at 90 °C (Table 1). The deprotection rate of the 2-[*O*-(*N*,*N*-diisopropylphosphoramido)]ethyl group would definitely be too slow at 37 °C for thiophosphate protection of the CpG motifs and adjacent flanking sequences of CpG ODN prodrugs. This group may nonetheless be useful in the protection of terminal 5′- and/or 3′-thiophosphate functions of oligonucleotide prodrugs. Conversely, the cleavage of the 2-[*O*-(*N*,*N*-diisopropylphosphoramidothioyl)]ethyl group from **7b** was too rapid under the same thermolytic conditions ($t_{\frac{1}{2}}$ = ~1 min at 90 °C or 2.5 h at 37 °C) to be of practical use in the development of CpG ODN prodrugs.

Table 1 Thermolytic deprotection rates of the dinucleoside phosphorothioate triester derivatives **7a–j** to TpsT

7a–j → TpsT		
Triester derivative	$t_{\frac{1}{2}}$ (90 °C) [min]	$t_{\frac{1}{2}}$ (37 °C) [h]
7a	60	ND[a]
7b	~1[b]	2.5
7c	20	NA[c]
7d	72	NA
7e	5	20
7f	10	42
7g	13	94
7h	30	135
7i	36	245
7j	49	265

[a] Not determined. [b] The deprotection rate is too rapid to be accurately determined. [c] Not applicable.

As shown in Scheme 4, the thermolytic deprotection of **7b** followed a cyclodeesterification pathway.[5,7,15] ^{31}P NMR analysis of the deprotection reaction conducted at 37 °C in 0.1 M triethylammonium acetate (TEAA, pH 7) revealed the formation of TpsT (δ_P 54 ppm) and that of **8** (δ_P 48 ppm) as a



Scheme 4

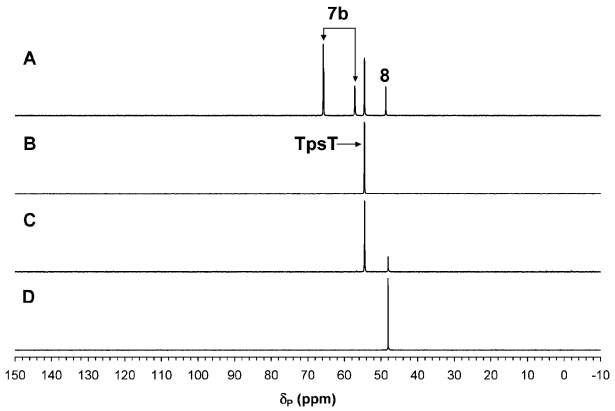


Fig. 1 ^{31}P NMR analysis of the thermolytic conversion of RP-HPLC purified **7b** to TpsT and **8** in 0.1 M TEAA (pH 7). (A) Spectrum of **7b** after being heated at 37 °C for 2.5 h. (B) Spectrum of the reference standard TpsT in 0.1 M TEAA (pH 7). (C) Spectrum of **7b** after being heated at 37 °C for 20 h. (D) Spectrum of synthetic **8** in 0.1 M TEAA (pH 7).

cyclodeesterification side product (Fig. 1). The identity of **8** was corroborated by its chemical synthesis, which was carried out by the oxidation of 2-(*N*,*N*-diisopropylamino)-1,3,2-oxathiaphospholane[16] with *tert*-butyl hydroperoxide, and through spiking experiments (data not shown). It is therefore likely that the thermolytic thiophosphate deprotection of **7e–7j** proceeds generally through a cyclodeesterification process with the concomitant formation of cyclic phosphoramidothioate side products.

In an effort to slow down the thiophosphate deprotection of **7b**, the 2-[*O*-bis(*N*,*N*-diisopropylphosphorodiamidothioyl)]-ethyl group for thiophosphate protection was then investigated. The uncharged bis(*N*,*N*-diisopropylphosphorodiamidothioyl) function of **7c** is significantly less nucleophilic than the negatively charged *N*,*N*-diisopropylphosphoramidothioyl functionality of **7b** and should therefore slow down thiophosphate deprotection rates. Indeed, the thiophosphate deprotection of **7c** proceeded with a half-life of 20 min at 90 °C (Table 1). RP-HPLC analysis of the deprotection reaction showed a clean conversion of **7c** to TpsT at 90 °C, but not at 37 °C; in addition to TpsT, new unidentified peaks with longer retention times were also produced. Replacement of the bis(*N*,*N*-diisopropylphosphorodiamidothioyl) function in **7c** with a bis(*N*,*N*-diethylphosphorodiamidothioyl) function gave **7d**. The thermolytic thiophosphate deprotection kinetics of **7d** should provide information on the steric effect of the diisopropyl groups on the rates of deprotection. Surprisingly, the rate of thermal deprotection of **7d** was slower ($t_{\frac{1}{2}}$ = 72 min) than that of **7c** by a factor greater than 3 at 90 °C (Table 1). These findings suggest that electronic rather than steric factors were predominant in the transition state of the thiophosphate deprotection reaction. RP-HPLC analysis of the thermal thiophosphate deprotection of **7d** also indicated that TpsT was exclusively formed at 90 °C only. On the basis of these results, the use of bis(*N*,*N*-dialkylphosphorodiamidothioyl)-ethyl groups as thiophosphate protecting groups for CpG ODN prodrugs must be avoided; the formation of unexpected side products at 37 °C may interfere with the interpretation of the immunostimulatory function of CpG ODN prodrugs *in vivo*. Further investigations are therefore necessary to characterize these side products and assess their immunostimulatory properties, if any, in animal models.

An expansion of the five-membered cyclic transition state operating in the thermal thiophosphate deprotection of **7b** to a six-membered cyclic transition state decreased, as expected, the thermal thiophosphate deprotection of **7e**, which occurred with a half-life of 5 min at 90 °C or 20 h at 37 °C (Table 1). Because of the relatively rapid thermolytic cleavage of the negatively charged 3-[*O*-(*N*,*N*-diisopropylphosphoramidothioyl)]prop-1-yl group from **7e**, this functional group may find application in the thiophosphate protection of the DNA sequences flanking the CpG motif of CpG ODN prodrugs and provide increased aqueous solubility to lipophilic oligonucleotide prodrugs.[7] As discussed above, replacement of the 3-[*O*-(*N*,*N*-diisopropylphosphoramidothioyl)]prop-1-yl group in **7e** with a 3-[*O*-(*N*,*N*-diethylphosphoramidothioyl)]prop-1-yl group gave **7f**, which underwent thermolytic thiophosphate deprotection at a slower rate ($t_{\frac{1}{2}}$ = 10 min at 90 °C or 42 h at 37 °C) than **7e** under identical conditions (Table 1). These findings are consistent with the thermal thiophosphate deprotection rate of **7g**, which was found slower ($t_{\frac{1}{2}}$ = 13 min at 90 °C or 94 h at 37 °C) than that of **7f**. These results suggest that the inductive effect of the oxygen atom in the morpholine ring of **7g** had negatively affected the deprotection rate of the 3-[*O*-(*N*-morpholinophosphonothioyl)]prop-1-yl group and thus further support the significance of electronic effects in the thermolytic cyclodeesterification of these thiophosphate protecting groups. The negatively charged 3-[*O*-(*N*,*N*-diethylphosphoramidothioyl)]prop-1-yl and 3-[*O*-(*N*-morpholinophosphonothioyl)]prop-1-yl groups can be used for thiophosphate protection of the CpG motif of CpG ODN prodrugs.

An expansion of the six-membered cyclic transition state taking place in the thermal thiophosphate deprotection of **7e** was necessary to decrease its deprotection rate. Thus, replacement of the negatively charged 3-[*O*-(*N*,*N*-diisopropylphosphoramidothioyl)]prop-1-yl group in **7e** with the negatively charged 4-[*O*-(*N*,*N*-diisopropylphosphoramidothioyl)]but-1-yl group led to **7h**, which under thermolytic conditions produced TpsT with a half-life of 30 min at 90 °C or 135 h at 37 °C (Table 1). When the 4-[*O*-(*N*,*N*-diethylphosphoramidothioyl)]-but-1-yl or 4-[*O*-(*N*-morpholinophosphonothioyl)]but-1-yl group was used for thiophosphate protection of **7i** or **7j**, respectively, the thermolytic cleavage of these protecting groups to TpsT proceeded with respective half-lives of 36 min at 90 °C and 245 h at 37 °C or 49 min at 90 °C and 265 h at 37 °C (Table 1). These groups are also suitable for thiophosphate protection of the CpG motif of CpG ODN prodrugs.

Conclusion

In this report, many of the thiophosphate protecting groups listed in Table 1 have shown thermolytic deprotection half-lives in the range of 94 h to 265 h at 37 °C. These deprotection rates are complementary to those of thiophosphate protecting groups identified earlier,[7] the thermolytic deprotection half-lives

of which were in the range of 6 h to 40 h at 37 °C. Altogether, these heat-sensitive groups are likely to protect adequately the thiophosphate functions of CpG motifs and those of adjacent DNA sequences in immunomodulatory CpG ODN prodrugs. The judicious selection of uncharged and negatively charged phosphate/thiophosphate protecting groups, in proper ratios, should provide aqueous solubility to lipophilic oligonucleotide prodrugs and enhance their pharmacokinetics and pharmacodynamic properties *in vivo*. Phosphate/thiophosphate protecting groups with deprotection half-lives exceeding 300 h[7] at 37 °C should be useful for the protection of terminal 5′- and 3′-phosphodiester functions of CpG ODN prodrugs and increase the resistance of these biomolecules to pervasive nucleases. Furthermore, in the context of immunomostimulatory CpG ODN prodrugs, a library of CpG motifs modified with thermolytic phosphate/thiophosphate groups is being prepared to evaluate the correlation between extended immunostimulation and resistance to viral and/or bacterial infections in animal models.

As discussed earlier,[7] thermosensitive groups have also been applied to the protection of the 5′-hydroxyl[17] and exocyclic amino[18] functions of deoxyribonucleosides, thereby supporting the potentially general application of these groups as alcohols and amine protecting groups. Interestingly, the incorporation of thermolabile phosphate protecting groups into DNA oligonucleotide primers[19] prevents the premature extension of these primers at the initial set up stages of the polymerase chain reaction. A thermal activation step induces the cleavage of the heat-sensitive phosphate protecting groups and generates the corresponding unmodified DNA primers, which then enable a clean amplification of the desired DNA target sequences. These useful applications highlight the importance and versatility of thermolytic groups in the development of DNA prodrugs and DNA diagnostics.

Experimental

Methods and materials

Common chemicals and solvents in addition to bis(*N*,*N*-diethylamino)chlorophosphine, bis(*N*,*N*-diisopropylamino)chlorophosphine and 2-cyanoethyl (*N*,*N*-diisopropyl)-phosphoramidochloridite were purchased from commercial suppliers and were used as received. 2-[(4,4′-Dimethoxytrityl)-oxy]ethan-1-ol (**1**),[9] 3-[(4,4′-dimethoxytrityl)oxy]propan-1-ol (**2**),[10] 4-[(4,4′-dimethoxytrityl)oxy]butan-1-ol (**3**),[11] 2-cyanoethyl (*N*,*N*-diethyl)phosphoramidochloridite,[12] 2-cyanoethyl (*N*-morpholinyl)phosphoramidochloridite,[13] 2-(*N*,*N*-diisopropylamino)-1,3,2-oxathiaphospholane[16] and 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-bis(*N*,*N*-diisopropylamino)phosphinyl-2′-deoxythymidine (**5**)[8] were prepared as described in the literature. Silica gel 60 (EMD, 0.040–0.063 mm) was used for chromatographic purifications. All NMR experiments were carried out using a Bruker Avance DRX 300 spectrometer operating at fields of 300.13 MHz for ^{1}H, 75.47 MHz for ^{13}C, and 121.5 MHz for ^{31}P. ^{1}H, proton-decoupled ^{13}C, and proton-decoupled ^{31}P NMR spectra were recorded in deuterated solvent as indicated. High resolution mass spectra used to determine the elemental composition of compounds **6a–j** were obtained on a Bruker Daltonics Apex III FT-ICR mass spectrometer. Electrospray ionization in positive ion mode was used to generate $[M + H]^+$ and $[M + Na]^+$ ions out of 0.1 mg mL^{-1} test sample solutions in 2-propanol–water (1 : 1 v/v). Spectra were externally calibrated using a 1 mg mL^{-1} solution of CsI, which yielded a series of peaks in the mass range used for analysis (200–2000 *m*/*z*).

Procedures

General procedure for the preparation of hydroxyalkylated phosphoramidate, phosphoramidothioate and phosphorodiamidothioate derivatives (4a–j). To a stirred solution of any of the alcohols **1–3** (5 mmol) and dry *N*,*N*-diisopropylethylamine (25 mmol) in anhydrous CH_2Cl_2 (20 mL) was added, under an inert gas atmosphere, the appropriate phosphoramidochloridite (5.5 mmol). After 2 h at 25 °C, the reaction was complete (TLC); a 5.5 M solution of *tert*-butyl hydroperoxide (10 mmol) in decane or elemental sulfur (10 mmol) was added to the reaction mixture. The *tert*-butyl hydroperoxide oxidation reaction was allowed to proceed for 30 min, whereas the sulfurization reaction was left stirring for 16 h at 25 °C. The reaction mixture was then diluted to a final volume of 50 mL with CH_2Cl_2 and was washed with water (3 × 25 mL); the organic extracts were collected, dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. The material left was dissolved in 80% AcOH (30 mL); the solution was stirred for 3 h at 25 °C and was then evaporated to an oil under reduced pressure. The crude product was purified by chromatography on silica gel (~30 g) using a gradient of CH_3OH (0 → 5%) in $CHCl_3$. The alcohol derivatives **4a–j** were isolated in yields ranging from 79% to 86%.

O-*(1-Hydroxyethyl)*-O-*(2-cyanoethyl)*-N,N-*diisopropyl phosphoramidate* (**4a**). Yield: 1.10 g (80%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.83 (1H, br s, O***H***), 4.02 (2H, m, C***H***$_2$CH$_2$OH), 3.95–3.77 (2H, m, CH$_2$C***H***$_2$OH), 3.57 (2H, t, *J* = 5.8 Hz, C***H***$_2$CH$_2$CN), 3.44 (1H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 3.37 (1H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 2.87 (2H, t, *J* = 5.8 Hz, CH$_2$C***H***$_2$CN), 1.16 (12H, d, *J* = 6.8 Hz, (C***H***$_3$)$_2$CHN); δ_C(75 MHz; DMSO-d_6) 118.4 (CH$_2$CH$_2$***C***N), 66.7 (d, $^{2}J_{C-P}$ = 5.7 Hz, ***C***H$_2$CH$_2$OH), 60.1 (d, $^{3}J_{C-P}$ = 6.9 Hz, CH$_2$***C***H$_2$OH), 60.0 (d, $^{2}J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CN), 45.2 (d, $^{2}J_{C-P}$ = 4.6 Hz, (CH$_3$)$_2$***C***HN), 22.1 (d, $^{3}J_{C-P}$ = 14.9 Hz, (***C***H$_3$)$_2$CHN), 19.1 (d, $^{3}J_{C-P}$ = 8.0 Hz, CH$_2$***C***H$_2$CN); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 9.1.

O-*(1-Hydroxyethyl)*-O-*(2-cyanoethyl)*-N,N-*diisopropyl phosphoramidothioate* (**4b**). Yield: 1.25 g (84%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.83 (1H, br s, O***H***), 4.12–3.99 (2H, m, C***H***$_2$CH$_2$OH), 3.94 (1H, m, CH$_2$C***H***$_2$OH), 3.84 (1H, m, CH$_2$C***H***$_2$OH), 3.75 (1H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 3.68 (1H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 3.59 (2H, t, *J* = 5.2 Hz, C***H***$_2$CH$_2$CN), 2.89 (2H, t, *J* = 5.8 Hz, CH$_2$C***H***$_2$CN), 1.22 (12H, d, *J* = 6.8 Hz, (C***H***$_3$)$_2$CHN); δ_C(75 MHz; DMSO-d_6) 118.4 (CH$_2$CH$_2$***C***N), 67.7 (d, $^{2}J_{C-P}$ = 5.7 Hz, ***C***H$_2$CH$_2$OH), 60.9 (d, $^{2}J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CN), 59.9 (d, $^{3}J_{C-P}$ = 10.3 Hz, CH$_2$***C***H$_2$OH), 46.4 (d, $^{2}J_{C-P}$ = 4.6 Hz, (CH$_3$)$_2$***C***HN), 22.1 (***C***H$_3$)$_2$CH$_2$CN), 18.8 (d, $^{3}J_{C-P}$ = 9.2 Hz, CH$_2$***C***H$_2$CN); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 72.4.

O-*(1-Hydroxyethyl)-bis(*N,N-*diisopropyl) phosphorodiamidothioate (***4c***)*. Yield: 1.40 g (86%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.77 (1H, br s, O***H***), 3.91 (1H, t, *J* = 5.7 Hz, C***H***$_2$CH$_2$OH), 3.88 (1H, t, *J* = 5.7 Hz, C***H***$_2$CH$_2$OH), 3.64 (2H, m, CH$_2$C***H***$_2$OH), 3.61 (2H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 3.57 (2H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 1.25 (12H, d, *J* = 6.8 Hz, (C***H***$_3$)$_2$CHN), 1.21 (12H, d, *J* = 6.8 Hz, (C***H***$_3$)$_2$CHN); δ_C(75 MHz; DMSO-d_6) 65.1 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$OH), 59.9 (d, $^3J_{C-P}$ = 10.3 Hz, CH$_2$***C***H$_2$OH), 45.5 (d, $^3J_{C-P}$ = 5.7 Hz, (CH$_3$)$_2$***C***HN), 23.0 (***C***H$_3$)$_2$CHN), 21.2 (***C***H$_3$)$_2$CHN); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 71.8.

O-*(1-Hydroxyethyl)-bis(*N,N-*diethyl) phosphorodiamidothioate (***4d***)*. Yield: 1.15 g (85%). δ_H(300 MHz; DMSO-d_6) 4.74 (1H, br s, O***H***), 3.82 (1H, t, *J* = 5.4 Hz, C***H***$_2$CH$_2$OH), 3.79 (1H, t, *J* = 5.4 Hz, C***H***$_2$CH$_2$OH), 3.57 (2H, t, *J* = 5.4 Hz, CH$_2$C***H***$_2$OH), 3.12–2.94 (8H, m, CH$_3$C***H***$_2$N), 1.04 (12H, t, *J* = 7.1 Hz, C***H***$_3$CH$_2$N); δ_C(75 MHz; DMSO-d_6) 65.8 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$OH), 60.0 (d, $^3J_{C-P}$ = 10.3 Hz, CH$_2$***C***H$_2$OH), 39.4 (d, $^2J_{C-P}$ = 5.7 Hz, CH$_3$***C***H$_2$N), 39.2 (d, $^2J_{C-P}$ = 5.7 Hz, CH$_3$***C***H$_2$N), 13.7 (d, $^3J_{C-P}$ = 4.6 Hz, ***C***H$_3$C$_2$HN), 13.6 (d, $^3J_{C-P}$ = 4.6 Hz, ***C***H$_3$CH$_2$N); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 79.0.

O-*(1-Hydroxypropyl)*-O-*(2-cyanoethyl)*-N,N-*diisopropyl phosphoramidothioate (***4e***)*. Yield: 1.3 g (84%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.06–3.90 (4H, m, C***H***$_2$CH$_2$C***H***$_2$OH), 3.71 (1H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 3.69 (1H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 3.49 (2H, t, *J* = 6.3 Hz, C***H***$_2$CH$_2$CN), 2.88 (2H, t, *J* = 6.3 Hz, CH$_2$C***H***$_2$CN), 1.76 (2H, quint, *J* = 6.3 Hz, CH$_2$C***H***$_2$CH$_2$OH), 1.22 (12H, d, *J* = 6.8 Hz, (C***H***$_3$)$_2$CHN); δ_C(75 MHz; DMSO-d_6) 118.4 (CH$_2$CH$_2$***C***N), 63.5 (d, $^2J_{C-P}$ = 5.7 Hz, ***C***H$_2$CH$_2$CH$_2$OH), 60.9 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CN), 57.0 (CH$_2$CH$_2$***C***H$_2$OH), 46.4 (d, $^2J_{C-P}$ = 5.7 Hz, (CH$_3$)$_2$***C***HN), 32.8 (d, $^3J_{C-P}$ = 8.0 Hz, CH$_2$***C***H$_2$CH$_2$OH), 22.1 (***C***H$_3$)$_2$CHN), 18.8 (d, $^3J_{C-P}$ = 9.2 Hz, CH$_2$***C***H$_2$CN); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 72.0.

O-*(1-Hydroxypropyl)*-O-*(2-cyanoethyl)*-N,N-*diethyl phosphoramidothioate (***4f***)*. Yield: 1.15 g (81%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.08–3.85 (4H, m, C***H***$_2$CH$_2$C***H***$_2$OH), 3.48 (2H, t, *J* = 6.0 Hz, C***H***$_2$CH$_2$CN), 3.17 (2H, q, *J* = 7.1 Hz, CH$_3$C***H***$_2$N), 3.13 (2H, q, *J* = 7.1 Hz, CH$_3$C***H***$_2$N), 2.89 (2H, t, *J* = 6.0 Hz, CH$_2$C***H***$_2$CN), 1.75 (2H, quint, *J* = 6.3 Hz, CH$_2$C***H***$_2$CH$_2$OH), 1.06 (6H, t, *J* = 7.1 Hz, C***H***$_3$CH$_2$N); δ_C(75 MHz; DMSO-d_6) 118.4 (CH$_2$CH$_2$***C***N), 63.5 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CH$_2$OH), 60.8 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CN), 57.0 (CH$_2$CH$_2$***C***H$_2$OH), 39.6 (d, $^3J_{C-P}$ = 4.6 Hz, CH$_3$***C***H$_2$N), 32.7 (d, $^2J_{C-P}$ = 8.0 Hz, CH$_2$***C***H$_2$CH$_2$OH), 18.7 (d, $^2J_{C-P}$ = 9.2 Hz, CH$_2$***C***H$_2$CN), 14.0 (***C***H$_3$CH$_2$N); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 76.0.

O-*(1-Hydroxypropyl)*-O-*(2-cyanoethyl)*-N-*morpholino phosphonothioate (***4g***)*. Yield: 1.15 g (79%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.52 (1H, t, *J* = 5.2 Hz, O***H***), 4.07 (1H, t, *J* = 5.8 Hz, C***H***$_2$CH$_2$CH$_2$OH), 4.04 (1H, t, *J* = 5.8 Hz, C***H***$_2$CH$_2$CH$_2$OH), 4.03–3.95 (2H, m, CH$_2$CH$_2$C***H***$_2$OH), 3.55 (4H, t, *J* = 4.9 Hz, C***H***$_2$CH$_2$N), 3.49 (1H, t, *J* = 6.0 Hz, C***H***$_2$CH$_2$CN), 3.47 (1H, t, *J* = 6.0 Hz, C***H***$_2$CH$_2$CN), 3.17 (4H, m, CH$_2$C***H***$_2$N), 2.90 (1H, t, *J* = 6.0 Hz, CH$_2$C***H***$_2$CN), 1.76 (2H, quint, *J* = 6.3 Hz, CH$_2$C***H***$_2$CH$_2$OH); δ_C(75 MHz; DMSO-d_6) 118.4 (CH$_2$CH$_2$***C***N), 66.1 (d, $^2J_{C-P}$ = 5.7 Hz, ***C***H$_2$CH$_2$CH$_2$OH), 63.9 (d, $^3J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$N), 61.2 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CN), 56.9 (CH$_2$CH$_2$***C***H$_2$OH), 44.8 (CH$_2$***C***H$_2$N), 32.7 (d, $^3J_{C-P}$ = 8.0 Hz, CH$_2$***C***H$_2$CH$_2$OH), 18.7 (d, ^3J = 9.2 Hz, CH$_2$***C***H$_2$CN); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 74.4.

O-*(1-Hydroxybutyl)*-O-*(2-cyanoethyl)*-N,N-*diisopropyl phosphoramidothioate (***4h***)*. Yield: 1.35 g (83%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.44 (1H, t, *J* = 5.2 Hz, O***H***), 4.10–3.83 (4H, m, C***H***$_2$CH$_2$CH$_2$C***H***$_2$OH), 3.72 (1H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 3.69 (1H, hept, *J* = 6.8 Hz, (CH$_3$)$_2$C***H***N), 3.42 (1H, t, *J* = 6.3 Hz, C***H***$_2$CH$_2$CN), 3.40 (1H, t, *J* = 6.3 Hz, C***H***$_2$CH$_2$CN), 2.90 (2H, m, CH$_2$C***H***$_2$CN), 1.65 (2H, m, CH$_2$C***H***$_2$CH$_2$CH$_2$OH), 1.48 (2H, m, CH$_2$CH$_2$C***H***$_2$CH$_2$OH), 1.22 (12H, d, *J* = 6.8, (C***H***$_3$)$_2$CHN); δ_C(75 MHz; DMSO-d_6) 118.4 (CH$_2$CH$_2$***C***N), 66.1 (d, $^2J_{C-P}$ = 5.7 Hz, ***C***H$_2$CH$_2$CH$_2$CH$_2$OH), 60.9 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CN), 60.1 (CH$_2$CH$_2$CH$_2$***C***H$_2$OH), 46.4 (d, $^2J_{C-P}$ = 5.7 Hz, (CH$_3$)$_2$***C***HN), 28.7 (CH$_2$CH$_2$***C***H$_2$CH$_2$OH), 26.2 (d, $^3J_{C-P}$ = 9.2 Hz, CH$_2$***C***H$_2$CH$_2$CH$_2$OH), 22.1 (***C***H$_3$)$_2$CHN), 18.8 (d, $^3J_{C-P}$ = 9.2 Hz, CH$_2$***C***H$_2$CN); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 72.0.

O-*(1-Hydroxybutyl)*-O-*(2-cyanoethyl)*-N,N-*diethyl phosphoramidothioate (***4i***)*. Yield: 1.20 g (81%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.42 (1H, t, *J* = 5.2 Hz, O***H***), 4.08–3.79 (4H, m, C***H***$_2$CH$_2$CH$_2$C***H***$_2$OH), 3.42 (1H, t, *J* = 6.3 Hz, C***H***$_2$CH$_2$CN), 3.40 (1H, t, *J* = 6.3 Hz, C***H***$_2$CH$_2$CN), 3.18 (2H, q, *J* = 7.1 Hz, CH$_3$C***H***$_2$N), 3.13 (2H, q, *J* = 7.1 Hz, CH$_3$C***H***$_2$N), 2.88 (2H, t, *J* = 5.7 Hz, CH$_2$C***H***$_2$CN), 1.65 (2H, m, CH$_2$C***H***$_2$CH$_2$CH$_2$OH), 1.47 (2H, m, CH$_2$CH$_2$C***H***$_2$CH$_2$OH), 1.06 (6H, t, *J* = 7.1 Hz, C***H***$_3$CH$_2$N); δ_C(75 MHz; DMSO-d_6) 118.3 (CH$_2$CH$_2$***C***N), 66.1 (d, $^2J_{C-P}$ = 5.7 Hz, ***C***H$_2$CH$_2$CH$_2$CH$_2$OH), 60.8 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CN), 60.1 (CH$_2$CH$_2$CH$_2$***C***H$_2$OH), 39.6 (d, $^2J_{C-P}$ = 4.6 Hz, CH$_3$***C***H$_2$N), 28.6 (CH$_2$CH$_2$***C***H$_2$CH$_2$OH), 26.2 (d, $^3J_{C-P}$ = 9.2 Hz, CH$_2$***C***H$_2$CH$_2$CH$_2$OH), 18.7 (d, $^3J_{C-P}$ = 9.2 Hz, CH$_2$***C***H$_2$CN), 14.0 (***C***H$_3$CH$_2$N); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 76.0.

O-*(1-Hydroxybutyl)*-O-*(2-cyanoethyl)*-N-*morpholino phosphonothioate (***4j***)*. Yield: 1.25 g (80%). δ_H(300 MHz; DMSO-d_6; Me_4Si) 4.43 (1H, t, *J* = 5.2 Hz, O***H***), 4.07 (1H, t, *J* = 5.7 Hz, C***H***$_2$CH$_2$CH$_2$CH$_2$OH), 4.04 (1H, t, *J* = 5.7 Hz, C***H***$_2$CH$_2$CH$_2$CH$_2$OH), 3.93 (2H, m, CH$_2$CH$_2$CH$_2$C***H***$_2$OH), 3.55 (4H, t, *J* = 4.9 Hz, C***H***$_2$CH$_2$N), 3.42 (1H, t, *J* = 6.3 Hz, OC***H***$_2$CH$_2$CN), 3.40 (1H, t, *J* = 6.3 Hz, C***H***$_2$CH$_2$CN), 3.17 (4H, m, CH$_2$C***H***$_2$N), 2.90 (2H, t, *J* = 6.3 Hz, CH$_2$C***H***$_2$CN), 1.65 (2H, m, CH$_2$C***H***$_2$CH$_2$CH$_2$OH), 1.47 (2H, m, CH$_2$CH$_2$C***H***$_2$CH$_2$OH); δ_C(75 MHz; DMSO-d_6) 118.3 (CH$_2$CH$_2$***C***N), 66.6 (d, $^3J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$N), 66.1 (d, $^2J_{C-P}$ =5.7 Hz, ***C***H$_2$CH$_2$CH$_2$OH), 61.2 (d, $^2J_{C-P}$ = 4.6 Hz, ***C***H$_2$CH$_2$CN), 60.1 (CH$_2$CH$_2$CH$_2$***C***H$_2$OH), 44.8 (CH$_2$***C***H$_2$N), 28.5 (CH$_2$CH$_2$***C***H$_2$CH$_2$OH), 26.2 (d, $^3J_{C-P}$ = 8.0 Hz, CH$_2$***C***H$_2$CH$_2$OH), 18.7 (d, $^3J_{C-P}$ = 9.2 Hz, CH$_2$***C***H$_2$CN); δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 74.4.

General procedure for the synthesis of the deoxyribonucleoside phosphoramidites 6a–j. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-bis(*N*,*N*-diisopropylamino)phosphinyl-2′-deoxythymidine (**5**, 1 mmol, Scheme 2) and any of the alcohols **4a–j** (1 mmol) were placed in a flame-dried round-bottom flask to which was added, by syringe, anhydrous MeCN (10 mL) under an inert gas atmosphere. Solid 1*H*-tetrazole was added to the solution by portions (4 × 0.25 mmol) over a period of 1 h at 25 °C. The reaction mixture was left stirring for an additional 2 h and was then concentrated to a syrup under reduced pressure. The crude phosphoramidite was purified by chromatography on silica gel (25 g), which was equilibrated in C_6H_6–Et_3N (9 : 1 v/v). The equilibration solvent was also used as the eluent and fractions (10 mL) containing the product were identified by ^{31}P NMR spectroscopy. These fractions were pooled together and evaporated to dryness under reduced pressure. The purified phosphoramidite was dissolved in dry benzene (5 mL); the resulting solution was frozen in a dry ice–acetone bath and was then lyophilized under high vacuum to give the phosphoramidite **6a–j** as a white powder. Phosphoramidites were isolated in yields ranging from 80% to 85%.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-[(2-cyanoethyl)-N,N-diisopropylphosphoramidyl]ethoxy]-(N,N-diisopropylamino)-phosphinyl-2′-deoxythymidine (**6a**). Yield: 790 mg (83%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 147.8, 147.4, 7.4. +ESI-MS: Calcd for $C_{48}H_{67}N_5O_{11}P_2$ $[M + Na]^+$ 974.4205, found 974.4201.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-[(2-cyanoethyl)-N,N-diisopropylphosphoramidothioyl]ethoxy]-(N,N-diisopropylamino)-phosphinyl-2′-deoxythymidine (**6b**). Yield: 794 mg (82%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 147.7, 147.4, 147.2, 71.4, 71.3, 71.2, 71.1. +ESI-MS: Calcd for $C_{48}H_{67}N_5O_{10}P_2S$ $[M + H]^+$ 968.4157, found 968.4163.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-[bis(N,N-diisopropyl)-phosphorodiamidothioyl]ethoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**6c**). Yield: 848 mg (85%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 146.9, 146.8, 70.7. +ESI-MS: Calcd for $C_{51}H_{77}N_5O_9P_2S$ $[M + Na]^+$ 1020.4809, found 1020.4772.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-[bis(N,N-diethyl)phosphorodiamidothioyl]ethoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**6d**). Yield: 801 mg (85%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 147.2, 146.9, 78.3, 78.1. +ESI-MS: Calcd for $C_{47}H_{69}N_5O_9P_2S$ $[M + Na]^+$ 964.4183, found 964.4182.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[3-[(2-cyanoethyl)-N,N-diisopropylphosphoramidothioyl]prop-1-oxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**6e**). Yield: 786 mg (80%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 146.3, 146.2, 146.1, 145.9, 70.3, 70.2. +ESI-MS: Calcd for $C_{49}H_{69}N_5O_{10}P_2S$ $[M + Na]^+$ 1004.4133, found 1004.4108.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[3-[(2-cyanoethyl)-N,N-diethylphosphoramidothioyl]prop-1-oxy]-(N,N-diisopropylamino)-phosphinyl-2′-deoxythymidine (**6f**). Yield: 782 mg (82%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 146.7, 146.5, 75.2. +ESI-MS: Calcd for $C_{47}H_{65}N_5O_{10}P_2S$ $[M + H]^+$ 954.4006, found 954.3991.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[3-[(2-cyanoethyl)-N-morpholinophosphonothioyl]prop-1-oxy]-(N,N-diisopropylamino)-phosphinyl-2′-deoxythymidine (**6g**). Yield: 794 mg (82%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 146.8, 146.7, 146.5, 146.4, 73.5. +ESI-MS: Calcd for $C_{47}H_{63}N_5O_{11}P_2S$ $[M + H]^+$ 968.3793, found 968.3807.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[4-[(2-cyanoethyl)-N,N-diisopropylphosphoramidothioyl]but-1-oxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**6h**). Yield: 797 mg (80%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 146.3, 146.0, 145.9, 70.3. +ESI-MS: Calcd for $C_{50}H_{71}N_5O_{10}P_2S$ $[M + H]^+$ 996.4470, found 996.4448.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[4-[(2-cyanoethyl)-N,N-diethylphosphoramidothioyl]but-1-oxy]-(N,N-diisopropylamino)-phosphinyl-2′-deoxythymidine (**6i**). Yield: 784 mg (81%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 146.3, 146.0, 145.9, 74.7. +ESI-MS: Calcd for $C_{48}H_{67}N_5O_{10}P_2S$ $[M + H]^+$ 968.4157, found 968.4160.

5′-O-(4,4′-Dimethoxytrityl)-3′-O-[4-[(2-cyanoethyl)-N-morpholinophosphonothioyl]but-1-oxy]-(N,N-diisopropylamino)-phosphinyl-2′-deoxythymidine (**6j**). Yield: 805 mg (82%). δ_P(121 MHz, C_6D_6; extern. H_3PO_4/D_2O) 146.1, 145.9, 73.0. +ESI-MS: Calcd for $C_{48}H_{65}N_5O_{11}P_2S$ $[M + Na]^+$ 1004.3769, found 1004.3740.

General procedure for the manual solid-phase synthesis of the thermosensitive dinucleoside phosphorothioate triester derivatives 7a–j. A solution of 3% TCA in CH_2Cl_2 (3 mL) was slowly pushed by syringe through a commercial DNA synthesis column packed with 5′-DMTrTsuccCPG (1 μmol) until complete disappearance of any orange color (~2 min). Excess acid was washed away from TsuccCPG (Scheme 3) with CH_2Cl_2 (10 mL) and then with dry MeCN (10 mL). A premixed solution of any of the 5′-DMTrT-3′-phosphoramidites **6a–j** (30 μmol) and 0.45 M 1*H*-tetrazole in MeCN (0.3 mL) was then added by syringe to TsuccCPG; the suspension was manually agitated for 5 min prior to expelling the excess reagents from the synthesis column with MeCN (2 × 10 mL). The CPG support was treated with 0.05 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide[20] in MeCN (1 mL) for 2 min. The excess oxidant was washed off the synthesis column with MeCN (2 × 10 mL). Removal of the terminal 5′-DMTr group was effected by agitating the CPG support immersed in a solution of 3% TCA in CH_2Cl_2 (2 mL) over a period of 2 min. After carefully washing the support with MeCN (2 × 10 mL), the dinucleoside thiophosphate triester **7a–j** was released from the CPG support upon exposure (30 min) to pressurized (~2.5 bar) methylamine gas.[21] Crude **7a–j** was purified by RP-HPLC prior to determining its thermolytic thiophosphate deprotection kinetics (*vide infra*).

Thermolytic deprotection of the dinucleoside phosphorothioate triesters 7a–j. RP-HPLC-purified **7a–j** (~200 nmol) were dissolved in PBS (1X, pH 7.4, 500 μL) and were heated to the desired temperature (37 °C or 90 °C). Aliquots (50 μL)

were taken out at predetermined time points for analysis by RP-HPLC. The analyses were performed using a 5 μm Supelcosil LC-18S column (4.6 mm × 25 cm) under the following conditions: starting from 0.1 M triethylammonium acetate (pH 7.0), a linear gradient of 1% MeCN/min was pumped at a flow rate of 1 mL/min for 40 min. Thermolytic thiophosphate deprotection rates of **7a–j** are listed in Table 1.

*2-(*N,N*-Diisopropylamino)-2-oxo-1,3,2-oxathiaphospholane (***8***).* To a stirred solution of 2-(*N*,*N*-diisopropylamino)-1,3,2-oxathiaphospholane[16] (0.2 g, 1.0 mmol) in CH_2Cl_2 (5 mL) was added 5.5 M *tert*-butyl hydroperoxide in decane (0.36 mL, 2.0 mmol). The oxidation reaction was allowed to proceed for 30 min at 25 °C. The reaction mixture was concentrated to an oil under reduced pressure. The crude product was purified by chromatography on silica gel (~10 g) using a gradient of CH_3OH (0 → 3%) in $CHCl_3$, affording **8** as a white crystalline material in a yield of 85% (0.19 g, 0.85 mmol).

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